PhD Thesis

Systematic analysis of the *in vitro* effects of exogenous opioids on innate and adaptive immune function

Jason Boland

Immunobiology Research Group
Department of Oncology
University of Sheffield

November 2011

Acknowledgements

I dedicate this thesis to my parents, my mum and my late dad, who always loved and supported me and gave me the education and ability to complete this thesis. I thank Elaine for her unwavering support and encouragement during the course of this study.

I am indebted to my primary supervisor, Professor Graham Pockley, who has guided and given me expert supervision throughout the course of my research and in all aspects of my PhD. He has also given me the use of his laboratory, equipment and expertise, without which this would not have been possible.

I am also grateful to my secondary supervisor, Professor Sam H. Ahmedzai, for his guidance with my research and the writing of my thesis.

Professor Penella Woll and Dr Bill Noble, who have given me advice and mentorship throughout the course of this study.

I am extremely grateful to Gemma Foulds, research technician, who taught me the laboratory techniques and gave me assistance in some of my more challenging experiments. I would also like to thank Dan Flower and Gareth Penman for their support and motivation throughout the course of this study. I am grateful to Sue Newton and Kay Hopkinson in the Medical School Core Flow Cytometry Facility who taught me the use and application of flow cytometry.

I would also like to acknowledge the financial support of the Sheffield Palliative and Oncology Research Group and the Association of Palliative Medicine via its Twycross Prize for funding aspects of this project.

Summary

Background: Opioids are used to treat moderate to severe pain, including that associated with surgery, cancer and infection. Findings from a range of species and methodologies have demonstrated that whereas some opioids are immunoneutral (e.g. buprenorphine), others can be immunosuppressive (e.g. morphine) or immunostimulatory (e.g. tramadol). This study compared the effects of commonly used opioids on the potential to mount protective immunity against bacterial infections (neutrophil/monocyte phagocytosis and oxidative burst responses) and cancer (NK cell cytotoxicity/activation), as well as T cell activation and cytokine production.

Methodology: Peripheral blood was obtained from healthy volunteers and the influence of clinically relevant concentrations of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on neutrophil and monocyte phagocytosis of *E.coli* and oxidative burst responses to fMLP, PMA and *E.coli* were determined by flow cytometry. Their effects on NK cell cytotoxicity in IL-2 stimulated, platelet-free peripheral blood mononuclear cells (PBMCs) and NK and T cell activation in IL-2 and anti-CD3/28 mAb stimulated PBMCs were also assessed. Cytokine production by anti-CD3/28 mAb and IL-2 stimulated PBMCs was determined using a cytometric bead array technique.

Results: The only consistent, statistically significant effect was that methadone, oxycodone and diamorphine inhibited the production of IL-6 in IL-2 stimulated PBMCs, which could impair the acute phase response. There was however marked variability between individuals, both in their baseline and the effect of opioids, this was especially evident for morphine, tramadol, fentanyl and buprenorphine in the phagocytic and oxidative burst response to *E.coli*.

Conclusion: These findings suggest that opioid choice could influence the susceptibility to bacterial or fungal infection, but have little effect on anti-cancer or anti-viral protection. Clinical studies aimed at assessing the *in vivo* effects of opioid administration on immune function in relevant patient groups are required in order to assess the clinical significance of opioid choice.

Table of Contents

List of Figures

List of Tables

Abbreviations

μg	microgram (10^{-6}g)
^{3}H	^{3}Hydrogen (tritiated hydrogen)
5-HT	5-hydroxytryptamine (serotonin)
^{51}Cr	^{51}Chromium
ABC	ATP-binding cassette
ACTH	adrenocorticotropic hormone
AP-1	activator protein-1
APC(s)	antigen-presenting cell(s)
AUCC	area under the cytotoxic curve
C3b	complement component 3b
cAMP	cyclic adenosine monophosphate
CBA	Cytometric Bead Array
CD	cluster of differentiation
CD3-ε	CD3-epsilon
cGMP	cyclic guanosine monophosphate
C_{max}	maximum plasma concentration
CNS	central nervous system
COMT	catechol-O-methytransferase
Con A	concanavalin A
CRP	C-reactive protein
CTAP	αPhe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr amide
CTOP	αPhe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide
CYP	cytochrome P450
DAMGO	[D-Ala^{2}, N-MePhe^{4}, Gly-ol]-enkephalin
DC(s)	dendritic cell(s)
DNA	deoxyribonucleic acid
DOR	δ (delta) opioid receptor
DPDPE	[D-Pen^{2,5}]-enkephalin
DSLET	D-Ser^{2}-Leu-Enkephalin-Thr^{6}
E.coli	*Escherichia coli*
E:T	effector:target (cell ratio)
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
FBS	fetal bovine serum
Fc	Fragment, crystallisable
FITC	fluorescein isothiocyanate
fMLP	N-Formyl-Methionyl-Leucyl-Phenylalanine
FSc	forward light scatter (flow cytometry parameter)
GCH1	GTP cyclohydrolase 1
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
HLA	human leukocyte antigen
hMDMs	human monocyte-derived macrophages
HPA	hypothalamic pituitary adrenal
ICAM-1	intercellular adhesion molecule-1
IFN-	interferon-

Ig	immunoglobulin
IL-	interleukin-
IP-	inducible protein-
IRAK	IL-1 receptor–associated kinases
IRF	Interferon regulatory factor
JAK	Janus kinase
JNK	c-Jun terminal kinases
kg	kilogram (10^3g)
KOR	κ (kappa) opioid receptor
Lck	lymphocyte-specific protein tyrosine kinase
LPS	lipopolysaccharide
LTR	lung tumour retention
M	moles (concentration)
mAb(s)	monoclonal antibody(ies)
MAPK	mitogen-activated protein kinase
MFI	median fluorescent intensity
mg	milligram (10^{-3}g)
MHC	major histocompatibility complex
MOR	μ (mu) opioid receptor
mRNA	messenger ribonucleic acid
MTG	MitoTracker Green
N-CAM	Neural Cell Adhesion Molecule
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram (10^{-9}g)
NK (cell)	natural killer (cell)
NMDA	N-methyl D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
OPRM1	opioid receptor μ 1 (μ opioid receptor gene)
ORL1	opioid receptor-like 1 (nociceptin/orphanin FQ receptor)
PAG	peri-aqueductal grey
PBMC(s)	peripheral blood mononuclear cell(s)
PBS	phosphate buffered saline
PCA	patient controlled analgesia
PCR	polymerase chain reaction
pg	picogram (10^{-12}g)
PHA	phytohaemagglutinin
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute
SEM	standard error of the mean
sIL-2R	soluble IL-2 receptors
SNP	single-nucleotide polymorphism
SNS	sympathetic nervous system
SPSS	Statistical package for the Social Sciences
SRBC(s)	sheep red blood cell(s)
STAT	signal transducer and activator of transcription
TCR	T cell receptor
T_{reg} (cell)	regulatory T (cell)

CHAPTER 1

INTRODUCTION

1.1 Background

Opioids are potent analgesic drugs that are used in the clinical management of moderate to severe pain, including pain associated with surgery, cancer and infections. Opioids are also used for other indications, such as the control of diarrhoea, breathlessness (dyspnoea) and cough. Morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine are among the most commonly used opioids (Klepstad et al., 2011, Pergolizzi et al., 2008). By acting on the same receptors that are utilised by the ubiquitous endogenous opioid system, opioids prescribed for pain, diarrhoea, dyspnoea or cough also affect virtually every organ system in the body (Table 1.1). Opioid receptors are also present on activated immune cells and opioids have the potential to affect the immune system (Borner et al., 2008). As this effect is not immediately evident and with few well designed clinical studies on the subject, opioid-induced immune modulation is often not considered in the clinical setting (Brack et al., 2011, Sacerdote, 2008).

Table 1.1: Effects of opioids on different organ systems.

System	Effects
Gastrointestinal	Constipation, xerostomia, nausea and vomiting, delayed gastric emptying, gastro-oesophageal reflux, constriction of the sphincter of Oddi
Neurological	Analgesia, delirium, hallucinations, sedation, myoclonus, hyperalgesia, seizures, headaches, euphoria, dysphoria, dependency
Cardiovascular	Bradycardia, hypotension
Pulmonary	Respiratory depression, decreased cough reflex, non-cardiogenic pulmonary oedema
Urological	Urine retention, decreased urine production
Endocrinological	Hypogonadism/sexual dysfunction, osteoporosis
Immunological	Possible effects on neutrophil, macrophage, natural killer cell, T cell and cytokine function

The immune system has a crucial role in controlling and potentially eradicating disease in patients with cancer and infection (viral, bacterial, fungal and parasitic), thus anything that impairs its functional capacity might alter the clinical course of patients with cancer or infections. Previous *in vitro* and *in vivo* studies have shown that clinically used opioids have different effects on the immune system. Although previous studies

have used different methodologies and assessed different cell types, in general they have reported that some opioids have no effects on immune function (buprenorphine), whereas others are immunosuppressive (morphine, fentanyl), or even immunostimulatory (tramadol) (Budd, 2006, Gaspani et al., 2002, Shavit et al., 2004). The choice of prescribed opioids in clinical practice might therefore be critical in patients with cancer or infection who are dependent on an intact immune system to help control their disease. Patients with cancer may be prescribed opioids at any stage of their illness, for pain associated with potentially curative surgery or radiotherapy, through to the final stages of their illness. If patients have curative treatment, they may also need opioids to control symptoms caused by the treatment and it is vital that such medications do not increase the risk of any residual disease becoming clinically significant. Despite numerous studies evaluating the effect of some opioids on certain aspects of immune function, the effects of a range of opioids on multiple immune parameters have yet to be systematically assessed. This is the focus of this programme of work.

Although components of the adaptive immune system, such as T and B cells, are vital in the protection against cancer and infections, the first line of immune defence is provided by components of the innate immune system (Friedman et al., 2003, Li and Xu, 2008). Innate immune cells of the monocyte-macrophage lineage and neutrophils are involved in the direct control of bacterial and fungal infections, whereas natural killer (NK) cells are involved in anti-viral and anti-tumour immunity. All immune cells develop by sequential differentiation of haematopoietic pluripotent progenitor cells (Figure 1-1).

As well as consisting of immune cells as detailed in Table 1.2, immune protection also involves anatomical barriers such as the skin and mucous membranes, defences such as increased body temperature and lowering pH (in inflammatory tissue) as well as a variety of soluble factors such as lysozymes, interferons and complement (Galley et al., 2000).

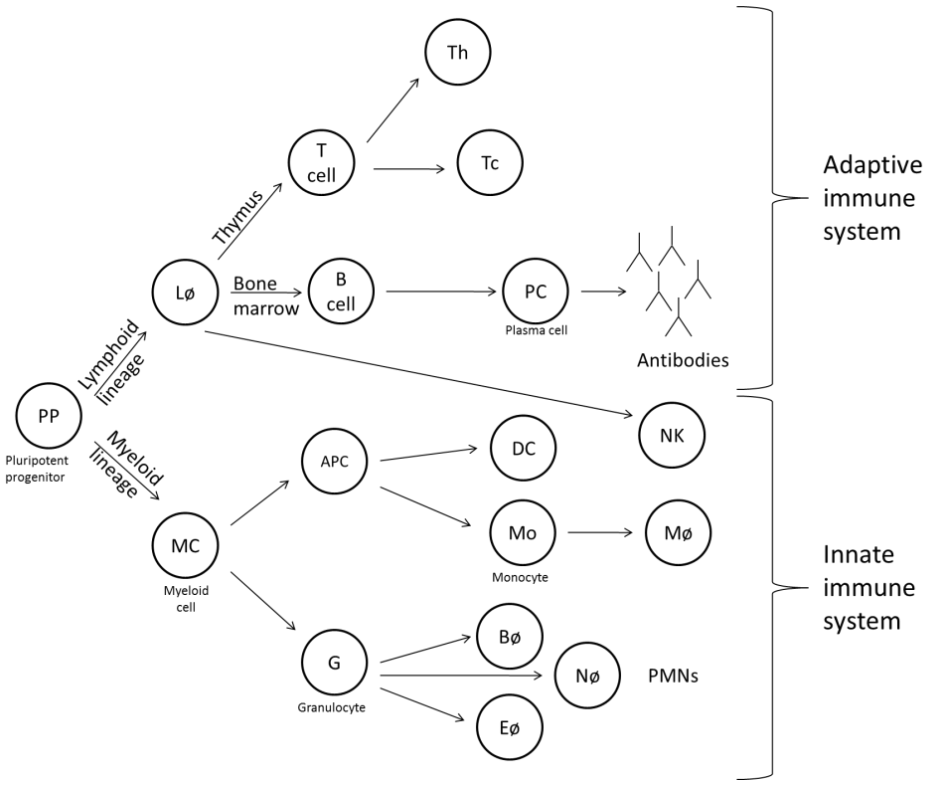

Figure 1-1: Development of immune cells.
All haematopoietic cells, including immune cells, are derived by differentiation from pluripotent progenitor cells. These initially develop into lymphocytes (Lø) which then further differentiate into T or B cells, before becoming a particular lymphocyte subset (i.e. $CD4^+$ T helper cells (T_H) or $CD8^+$ cytotoxic T cells (Tc), these constitute the adaptive immune system. Natural killer (NK) cells are derived from the lymphoid lineage and form part of the innate immune system. The other line of differentiation for pluripotent progenitor cells is to become myeloid cells, which can differentiate into antigen-presenting cells (APCs) which can become dendritic cells (DCs) or peripheral blood monocytes (Mo) or tissue-resident macrophages (Mø). Myeloid cells can also become granulocytes (or polymorphonuclear cells; PMNs), giving rise to basophils (Bø), eosinophils (Eø) and neutrophils (Nø), all of which are part of the innate immune system. Adapted from Cotran et al., 2010.

1.2 The innate immune system

The evolutionarily older innate immune system provides natural defence against pathogens and cancer without the need for previous exposure to a foreign antigen. The innate immune system is not only the sole arm of immunity in more primitive animals (pre-Gnathostomes), but also forms a very important part of the immune response in organisms that possess an adaptive immune system. Even in more evolved animals, cells of the innate immune system (monocytes/macrophages, neutrophils, NK cells) provide an instant immune response by eradicating bacteria, fungi and virally-infected cells as well as providing some defence against cancerous cells. However, in these more evolved animals, adaptive immune cells can stimulate or suppress the innate immune cells, providing greater flexibility of the immune response.

Table 1.2: Role and activation pattern of the main immune cells.

Cell	Role	Activators	Mechanism of activity	Immune test of activity	Arm
Dendritic cell	Antigen presentation	Multiple, including: bacterial products and cytokines	Presentation of antigenic peptides in the context of MHC class I and II molecules and the delivery of essential co-stimulatory molecules	Up-regulation of surface molecules (CD80, CD86, ICAM-1), the release of cytokines and T cell activation	Innate
Natural killer cell	Anti-tumour Anti-viral	Multiple, including: the lack of MHC class I expression	Release of cytotoxic molecules (granzymes, perforin)	Activation antigen expression (CD69, CD94) Cytotoxicity	Innate
Neutrophil	Anti-bacterial /fungal	opsonisation	Phagocytosis and oxidative burst	Phagocytosis and oxidative burst	Innate
Monocyte-Macrophage lineage	Anti-bacterial /fungal	Opsonisation Antigen presentation	Phagocytosis and oxidative burst	Phagocytosis and oxidative burst	Innate
$CD4^+$ T cell	Immune coordination/ regulation	Antigenic peptides presented by MHC class II plus essential co-stimulatory molecules	Regulating the activity of other immune cells	CD69 and CD25 expression Induction of antigen-specific immune responses	Adaptive
$CD8^+$ T cell	Cytotoxicity	Antigenic peptides presented by MHC class I	Induction of apoptosis by i) release of cytotoxins (perforin, granulysin, granzymes) ii) direct cell-cell contact, by up regulating surface Fas ligand	CD69 and CD25 expression Cytotoxicity	Adaptive
B cell	Antibody production	Antigens binding to surface immunoglobulin with help from $CD4^+$ T cells	Antibody production	Antibody secretion	Adaptive

A characteristic feature of innate immune cells is that they deliver the same immune response irrespective of previous encounters with pathogens. This contrasts with the amplified responses that are generated on subsequent encounters with immunological challenges that are characteristic of adaptive immune responses. Innate immune cells also present antigen to naïve and memory cluster of differentiation (CD) 4^+ and $CD8^+$ T cell populations, via dendritic cells (DCs) and monocytes/macrophages respectively.

1.2.1 Immune surveillance and anti-tumour immunity

The monitoring function of the immune system to recognise and react against aberrant cells, including infected and cancerous cells, arising within the body is known as 'immunosurveillance'. Cancer immunosurveillance reduces the potential for the primary cancer cells to develop and prevents the establishment of subsequent micrometastases. The initial eradication of newly developed tumour cells is principally carried out by NK cells. Strictly speaking, it is only this "un-primed" phase of immune-tumour interaction that is immunosurveillance. Once the tumour has become established, a number of complex immune-tumour interactions take place, for which the terms 'immunoediting' and 'immunosculpting' have been coined to describe this dynamic relationship (Reiman et al., 2007). These processes not only involve cells of the innate immune system, but also cytokines and T cell subsets (Reiman et al., 2007). As the tumour evolves, this may also involve down-regulation of major histocompatibility complex (MHC; human leukocyte antigen [HLA] in humans) class I and II antigens (Kim et al., 2007), which decreases the ability of adaptive immune cells to recognise the tumour.

Decreased or absent MHC class I expression is frequently associated with an invasive and metastatic tumour phenotype (Bubenik, 2003, Garrido and Algarra, 2001). Although this renders cells invisible to cytotoxic attack by $CD8^+$ cytotoxic T cells (which require MHC class I molecules to be expressed on their target cells), the loss of this expression renders tumour cells to be more susceptible to the cytotoxic effects of NK cells which target and kill MHC class I negative ('missing self') cells (Joncker et al., 2009, Ljunggren and Karre, 1990). This is particularly important in the context of anti-tumour immunity, as 60-90% of tumours can be MHC class I negative (Bubenik, 2003, Garrido and Algarra, 2001).

Cancer cells differ from most pathogens that interact with the immune system, as they evolve from normal host cells after developing gene mutations. This ultimately leads to unregulated proliferation, a reduced sensitivity to apoptotic signals and cytotoxicity by

immune cells, and an alteration in the presentation of surface antigens which would initially promote the immune response (Pawelec, 2004). These changes eventually allow some malignant cells to escape from immune recognition. The ability of immune surveillance for cancer cells varies for the different stages of tumour development. During the initial stages, malignant tissue is only exposed to immune cells after angiogenesis and the emission of danger signals (Fuchs and Matzinger, 1996). Once this occurs there is a selection pressure on the malignant cells by the immune system and the malignant cells continue to generate additional escape mechanisms, including tumour-derived soluble factors, which can result in an immune-resistant tumour.

The importance of the immune system in the protection against cancer has been demonstrated in a number of studies. A large retrospective cohort study of kidney transplant recipients compared the incidence of cancer when immunosuppression was reduced or stopped with that of patients on full immunosuppression (van Leeuwen et al., 2010). This showed that for some types of cancer (non-Hodgkin's lymphoma, lip cancer and melanoma) the incidence was higher when patients were on immunosuppression, whereas the incidence of leukaemia, lung, kidney and urinary tract cancers remained the same. Thyroid cancer was elevated in patients on dialysis (van Leeuwen et al., 2010). Chronic renal failure and dialysis are also likely to be immunosuppressive, albeit potentially affecting different immune cells. Although there are many confounding factors, it is likely that the immune system is involved in the elimination of some cancers more than others and this may be reflected in the suppression of certain types of immune cell, necessary for the control of a particular cancer at a particular time.

It has been demonstrated *in vitro* that activated NK cells function as effector cells against tumour target cells (Ames et al., 1987, Grimm et al., 1982). *In vivo*, NK cells penetrating into a solid tumour from the circulation are only a small portion of the total leukocyte population infiltrating the tumour (Menon et al., 2004). The observation that HLA class I antigen down regulation is associated with an improved survival of patients with breast carcinoma (Madjd et al., 2005), non-small cell lung carcinoma (Ramnath et al., 2006), uveal melanoma (Jager et al., 2002), and colon cancer (Menon et al., 2002) demonstrates the importance of NK cells in tumour surveillance and their potential therapeutic value. Although NK cell infiltration into malignant tumours has been related to a favourable outcome (Ishigami et al., 2000, Villegas et al., 2002), these studies used CD57 to identify NK cells which is a non-specific marker and thus it is impossible to be certain if this effect is purely due to NK cells. Using more specific NK and T cell

labelling it has been shown that tumours showing loss of MHC class I are infiltrated more by $CD8^+$ T cells (Sandel et al., 2005). It may be that the $CD8^+$ cytotoxic T cells have already killed the tumour cells that they have activity against; leaving the MHC class I negative tumour cells remaining, which have maintained the ability to attract $CD8^+$ cytotoxic T cells. This finding indicates that NK cells may be more important in preventing metastatic spread rather than the growth of the primary tumour.

It has been reported that patients with colorectal carcinoma who have a high NK cell cytotoxicity against K562 target cells (a human erythroleukaemic cell line) had less disease progression and a better survival compared to those with a low NK cell cytotoxicity (Liljefors et al., 2003). There was also a correlation between the patients' serum interleukin (IL)-2 concentrations and NK cell cytotoxicity, against K562 cells, again indicating that NK cell activity is important in the control of tumours, at least when tested *in vitro* (Liljefors et al., 2003).

NK cell cytotoxicity in the blood of 42 patients with colon cancer was measured before and one year after surgery and compared to control patients without cancer (Nüssler et al., 2007). Patients without metastasis at diagnosis had an increased NK cell cytotoxicity before surgery compared to control patients. In contrast, patients with metastatic disease at presentation had a decreased NK cell cytotoxicity compared to controls. One year after surgery, patients who remained free of metastasis had sustained NK cell cytotoxicity, whereas patients who developed metastasis had markedly decreased NK cell cytotoxicity. Retrospectively, the patients who developed metastasis already had reduced NK cell cytotoxicity prior to the "curative" colorectal surgery. Overall, metastatic spread was associated with decreased NK cell cytotoxicity (Nüssler et al., 2007).

Other studies have shown that a high NK cell cytotoxicity at diagnosis correlates with a good prognosis in patients with a variety of cancers, whereas low NK cell cytotoxicity may indicate early relapse (Gonzalez et al., 1998, Nakamura et al., 2000, Schantz and Ordonez, 1991, Taketomi et al., 1998).

The relationship between the suppression of NK cell cytotoxicity and the promotion of tumour metastasis has been studied in relation to alcohol (Ben-Eliyahu et al., 1996). Fischer 344 male rats were administered intraperitoneal ethanol and 1 hr later MADB106 cells (a NK cell-sensitive, rat mammary adenocarcinoma cell line which metastasises to the lungs) were injected into their tail vein. Three weeks later, the

lungs were removed and metastases counted or tumour retention measured using radioisotope uptake. There was a subgroup of rats which had selective *in vivo* depletion of NK cells with clone 3.2.3 monoclonal antibody (mAb) which recognises rat NK cells (Chambers et al., 1989). There was a marked increase in tumour load in the NK cell depleted rats and an intermediate tumour load in the ethanol treated rats, compared to control. NK depletion or ethanol had no effect when experiments were carried out using C4047 cells, an NK insensitive lung tumour cell line. The effect of ethanol on lung metastasis was present only if the MADB106 tumour cells were injected when ethanol levels were high, i.e. 1 hr after the intraperitoneal delivery of ethanol (Ben-Eliyahu et al., 1996). This suggests that even a transitory decrease in NK cell function may have a long term detrimental effect on cancer control.

1.2.1.1 Regulation of natural killer cell activation

Insight into the regulation of NK cell activity was provided when it was found that IL-2, interferon (IFN)-γ and more recently IL-15 augmented NK cell cytotoxicity (Handa et al., 1983, Henney et al., 1981, Zhang et al., 2011). Activated T cells produce IL-2, which is an important physiological activator of NK cells *in vivo*. Patients with advanced colorectal cancer (Hjelm Skog et al., 2001) and advanced non-small cell lung cancer (Orditura et al., 2000) had higher IL-2 serum levels than controls. IL-12 is also important, as in a rat tumour model, prophylactic IL-12 reduced post-operative metastasis, an effect which was mediated by increased prevalence of NK cells, rather than an increase in individual NK cell cytotoxicity (Schwartz et al., 2008).

In vivo, the overall level of NK cell activation results from a complex interaction of multiple stimulatory and inhibitory signals. There are many different stimulatory signals of different relative potencies, including both cytokines and direct cell-cell contact signals, i.e. the NKG2D NK cell receptor (Saito et al., 2011). These stimulatory signals would be balanced against the potential array of inhibitory signals including inhibitory NK cell receptors such as killer immunoglobulin-like receptor (Al Omar et al., 2011) to modulate the overall activation status of the NK cell (Figure 1-2).

One of the most potent inhibitors of NK cell activation is MHC class I, so much so that it is often considered that NK cells will only kill MHC class I negative (‘missing self’) cells (Ljunggren and Karre, 1990). Although tumour cells expressing MHC class I are less susceptible to NK cells, if there is otherwise an overwhelming balance of stimulatory signals to the NK cells then they will kill MHC class I expressing tumour cells.

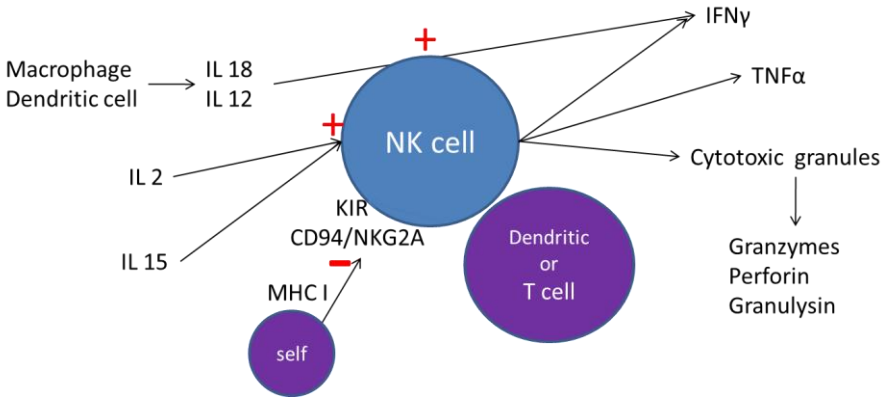

Figure 1-2: NK cell interactions and effector mechanisms.
NK cell activation results from a complex interaction of multiple stimulatory and inhibitory signals. One of the major inhibitory signals is from MHC class I via the heterodimeric CD94-NKG2 NK receptor family and the killer immunoglobulin-like receptor (KIR) receptors. There are a variety of cytokines which can activate NK cells including IL-12 and IL-18 (from macrophages and dendritic cells) which can induce NK cells to produce IFN-γ. IL-2 and IL-15 are also key activating cytokines. NK cells can also have direct cell-cell interactions with dendritic or T cells. NK cells principally have their effect by the production of cytotoxic granules (granzymes, granulysin and perforins) and by the production of cytokines (IFN-γ and TNF-α).

Although typically under the influence of $CD4^+$ T cells, a subgroup of murine NK cells have been shown to have a reciprocal control over adaptive immune responses by producing IL-10, these have been termed regulatory NK cells (Yoshida et al., 2010). Some murine NK cells have also been shown to have antigen specific properties and have the capacity to develop immunological memory (Paust et al., 2010).

1.2.2 Innate anti-infective immunity

As well as protecting against cancer, the innate immune system is also the principal effector arm against invading pathogenic organisms. Neutrophils and the monocyte-macrophage lineage are the main immune cells which ingest and kill extracellular bacteria and fungi. The regulation of these functions is described in the following sections.

1.2.2.1 Regulation of neutrophil function

Neutrophils, along with monocytes, are the principal innate immune effector cells that are involved in the control of bacterial and fungal infection and removal of foreign material by phagocytosis and subsequent oxidative burst reaction. Unactivated neutrophils roll along microvascular walls with low affinity interactions with endothelial cells. During inflammation, chemotactic factors and pro-inflammatory cytokines recruit neutrophils, causing activation of neutrophil β_2-integrins and high-affinity binding to endothelial cells (Zarbock and Ley, 2008). They then migrate along a chemotactic

gradient towards the site of inflammation, before penetrating the endothelial layer at the site of inflammation, where they congregate and adhere to extracellular matrix components such as laminin and fibronectin.

After the chemoattractants have guided the neutrophils to the site of inflammation, they can then phagocytose the bacteria, fungi or foreign material. The attachment of these to the cell surface of the neutrophil induces receptor clustering and activation resulting in intracellular scaffolding (actin-myosin contractile system) and signalling changes, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which initiate the formation of the phagosome and its internalisation. Phagocytosis is enhanced by opsonisation of the foreign material with immunoglobulin G (IgG) or complement component 3b (C3b), as neutrophils express immunoglobulin fragment crystallisable (Fc) and complement C3b receptors (Schutze et al., 1991).

Neutrophils are regulated by many factors including platelets, cytokines, bioactive lipids, neuroendocrine hormones, histamine, and adenosine, and the relative balance of these determines the level of neutrophil activation. Some cytokines enhance neutrophil function, whereas others are inhibitory. IL-1, IL-6 and Tumour Necrosis Factor (TNF)-α are potent neutrophil priming agents, which contribute to the activation of nicotinamide adenine dinucleotide phosphate oxidase. IL-8 is a potent chemoattractant which synergises with IFN-γ and TNF-α, to amplify neutrophil cytotoxicity against bacteria, protozoa and fungi. IFN-γ also amplifies antibody-dependent cytotoxicity of neutrophils. Neutrophils can also produce IL-1, IL-6, IL-8, and TNF-α which have autocrine and paracrine effects (Mantovani et al., 2011). IL-4 and IL-10 are anti-inflammatory and inhibit IL-8, TNF-α and IL-1 release, blocking neutrophil activation. Glucocorticoids, prostaglandins, adrenaline and β-endorphin inhibit the oxidative burst activity of neutrophils (Peterson et al., 1987, Trabold et al., 2007).

1.2.2.2 Regulation of monocyte function

Monocytes circulate in the blood and have a substantial splenic reserve (Swirski et al., 2009). Once migrated out of the circulation, monocytes differentiate into different types of macrophages or DCs in specific tissues. They are involved in the killing of bacteria and fungi and the removal of foreign material by phagocytosis and oxidative burst. They also interact with and modulate the adaptive immune response by the production of cytokines, either being stimulatory by secreting the pro-inflammatory TNF-α and IL-12 or by inhibiting $CD4^+$ T cell function by IL-10 production (Said et al., 2010).

The binding of pathogens to monocytes induces cell surface receptor clustering and activation and intracellular scaffolding and signalling changes, including NF-κB, which initiates the process of phagosome formation and subsequent internalisation. Opsonins such as C3b and IgG coat the bacteria or fungi to be phagocytosed increasing the ability of monocytes to internalise the organism into a phagosome before the subsequent oxidative burst reaction (Schutze et al., 1991).

The activation cascade in monocytes includes adenylyl cyclase and phospholipase C (PLC) stimulation by dissociated G-protein receptor subunits, which increases the second messengers cyclic adenosine monophosphate (cAMP), inositol trisphosphate and Ca^{2+}. These activate intracellular pathways affecting cell metabolism, motility, gene expression and cell division.

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, including *Escherichia coli* (*E.coli*), binds to Toll-like receptor (TLR) 4, in a complex with MD2. This then activates the MyD88-dependent pathway which recruits the IL-1 receptor–associated kinases (IRAK) and leads to the activation of NF-κB and mitogen-activated protein kinase (MAPK). The MAPK pathway activates the extracellular signal-regulated kinases (ERK) and c-Jun terminal kinases (JNK) pathways, as well as the early response transcription factor, activator protein-1 (AP-1), which is composed of proteins belonging to the c-Jun and c-Fos families (Aderem and Ulevitch, 2000). TLR4 also stimulates a Toll/Interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF) dependent pathway that activates interferon regulatory factor (IRF) 3 and NF-κB. Both these pathways lead to the production of pro-inflammatory cytokines and activation of immune responses (Kawai and Akira, 2010).

1.3 The adaptive immune system

Unlike the innate immune system, the phylogenetically more advanced adaptive immune system allows for a more coordinated and specific responses to pathogens and tumours. However, both immune arms are essential, as an effective adaptive immune response requires priming and takes time to initially develop. Plus, as the adaptive immune system evolved on the background of the innate immune system, some of its function is in the more specific control and modulation of innate immunity. The adaptive immune responses exhibit specificity, memory and amplification.

The principal mediators of adaptive immunity are T and B cells. T cells are so called as they mature in the thymus and are categorised into $CD4^+$ T cells ('T helper cells') and $CD8^+$ T cells ('cytotoxic T cells') (Liew, 2002). Several subtypes of $CD4^+$ T cells coordinate the immune response, including T_H1, T_H2, T_H9, T_H17, T_H22 and immunoregulatory T (T_{reg}) cells, described in section 2.8). $CD4^+$ T cells become activated when antigenic peptides that are associated with MHC class II expressed on antigen-presenting cells (APCs) are presented to them and recognised by appropriately-specific T cell receptors (TCRs). $CD8^+$ T cells are involved in the selected killing of tumour cells, virally infected cells and transplanted tissue to which the adaptive immune response has been primed by binding to cells that express antigenic determinants in the context of cell surface MHC class I via their TCR. MHC class I is ubiquitously expressed on the surface of nucleated cells, other than some virally-infected cells and tumour cells. $CD8^+$ T cells kill their target cells by the release of cytotoxins such as perforin (Ambach et al., 2001), granulysin (Stenger et al., 1998) and serine proteases such as granzymes (Bots and Medema, 2006). Granzymes also have a pro-inflammatory role by propagating immune signals (Cullen et al., 2010).

B cells are so called as they were originally identified as being matured in the bursa of Fabricius in birds. No such structure exists in mammals, in most of which immature B cells are derived in the bone marrow. When activated by a specific antigen, and with co-stimulation from $CD4^+$ T cells, B cells mature into plasma cells. The main function of plasma cells is to produce antibodies as the humoral immune response. However, it has recently been shown that B cells might have more diverse roles which include the regulation of $CD4^+$ T cell responses (Lund and Randall, 2010), with these so called regulatory B cells producing cytokines, such as IL-10, to suppress T cell activity (Amu et al., 2010).

Cytokines are a family of cell signalling protein based molecules that are primarily secreted from both innate and adaptive immune cells (principally $CD4^+$ T cells and macrophages) which regulate both humoral and cellular immune responses. There are many cytokines, each with specific cell surface receptor(s), initiating intracellular signalling cascades on binding to their receptor, resulting in alterations in transcription factors and subsequent gene transcription. There are several classification methods for cytokines, including both structural and functional, with the latter being most useful clinically and experimentally. There are two main groups of cytokines with this classification. The pro-inflammatory type 1 T_H cell cytokines (IL-2, IFN-γ and TNF-α, TNF-β), which stimulate cellular immune responses and can inhibit humoral immunity,

and the anti-inflammatory type 2 T_H cell cytokines (IL-3, IL-4, IL-5, IL-9, IL-10 and IL-13), which suppress cellular immune responses and promote humoral immunity (Figure 1-3) (Cutolo et al., 1998, Klein et al., 2004, Logani et al., 2011, Serreze et al., 2001, Teixeira et al., 2005, Yates et al., 2000).

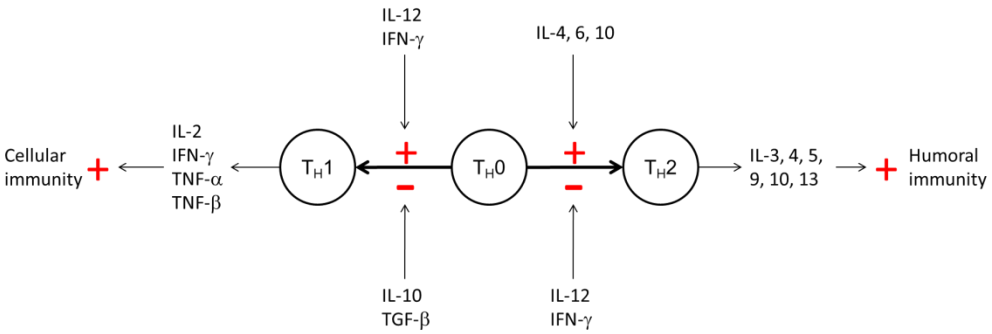

Figure 1-3: Effects of cytokines produced by T_{H1} and T_{H2} cells.
Naïve $CD4^+$ T helper cells (T_H0) under cytokine influence either differentiate into T_H1 or T_H2 cells. IL-12 and IFN-γ promote the differentiation towards T_H1 cells and thereby enhance the cellular immune response. IL-4, IL-6 and IL-10 promote differentiation towards T_H2 cells and enhance antibody (humoral) responses. The cytokines produced by the predominant arm of this response feedback to further enhance differentiation along that arm and inhibit the other arm. The T_H1 response drives cellular immune activation. The T_H2 response drives humoral immunity (Cutolo et al., 1998, Klein et al., 2004, Logani et al., 2011, Serreze et al., 2001, Teixeira et al., 2005, Yates et al., 2000).

1.3.1 Regulation of T cell activation

The primary function of the immune system is to protect the organism against invading pathogens. This need for the immune system to react to foreign antigens has to be balanced against reactivity against self. Thus, the primary aim of the healthy immune system is to be able to destroy non-self, while not damaging self. If the immune regulatory balance is too far towards immune activation then the host may be at increased risk of autoimmune disease as well as producing an excessive immune response to foreign antigens causing tissue damage, as in hypersensitivity reactions. However, if it is too much towards suppression of the immune response then this may increase the host's susceptibility to infectious diseases and cancer. There are many inbuilt mechanisms, both central and peripheral, to modulate this balance, primarily to induce and maintain immune tolerance to self.

For T cells, central tolerance is established in the thymus, but as some T cells 'escape' this clonal deletion process (auto-reactive T cells) there are mechanisms in the periphery to control this. Populations of T_{reg} cells are considered to be the major mediator of this by controlling the presence and activity of self-reactive T cells in the

periphery (Shalev et al., 2011). T_{reg} cells also control excessive immune responses to foreign antigens, preventing potential damage to the host (Shalev et al., 2011).

Cytokines are important in the fine tuning of the immune response and the profile of cytokines to which T cells are exposed dictates whether they are activated or inhibited, and defines their differentiation and functional properties (Wan and Flavell, 2009). In the case of the $CD8^+$ cytotoxic T cells this would result in either increased or decreased target cell killing.

T cell activation is induced by cell surface receptors that modulate signal transduction pathways. This involves the TCR which recognises antigenic peptides in the context of MHC and co-stimulatory signals from professional APCs such as DCs. The TCR is a heterodimer, with 95-98% of T cells having an α and β chain, and 2-5% having γ and δ chains (so called γ/δ T cells, which can recognise antigens directly) (Morita et al., 2000). The TCR complex consists of the TCR, CD3 and ζ-chains. Once the antigenic peptide/MHC complex on the APC binds to the TCR complex, the fate of the T cell depends on the delivery of essential co-stimulatory signals (Acuto and Michel, 2003). In T_H cells, CD4 interacts with MHC class II and in $CD8^+$ cytotoxic T cells, CD8 interacts with MHC class I.

The recognition of MHC class I and class II increases specificity and binding affinity, as well as recruits cell signalling molecules, such as lymphocyte-specific protein tyrosine kinase (Lck). CD28 is a co-stimulatory receptor constitutively expressed on T cells, which binds B7.1 (CD80) and B7.2 (CD86) on APCs. Triggering of both the TCR and CD28 potently activates the T cell (Acuto and Michel, 2003). Essential co-stimulatory signals can only be delivered by professional APCs and only these cells can activate naïve T cells. Previously activated T cells are not dependent on co-stimulatory signals and so can be activated by non-professional APCs such as monocytes and macrophages. The interaction of naïve T cells with professional APCs in the absence of co-stimulatory signals, which can be achieved using ‘immature’ DCs and/or by blocking co-stimulation leads to T cell non-responsiveness or anergy (Smith-Garvin et al., 2009).

T cell activation via the TCR induces receptor clustering, which activates tyrosine kinases on the intracellular surface of the plasma membrane. These phosphorylate tyrosine residues in the receptors which allow binding of additional kinases and other signalling molecules, including PLC-γ1, triggering intracellular signalling. Activated

PLC-γ1 cleaves membrane phosphatidylinositol bisphosphate into two signalling molecules, the diffusible inositol trisphosphate, and diacylglycerol, which remains bound to the intracellular surface of the plasma membrane. Inositol trisphosphate opens Ca^{2+} channels in the endoplasmic reticulum, allowing Ca^{2+} to enter the cytosol, which in turn opens plasma membrane Ca^{2+} channels, allowing further Ca^{2+} influx. Diacylglycerol binds protein kinase C (PKC) and anchors it to the inside of the cell membrane where it is activated by the Ca^{2+} influx. PKC is a serine/threonine kinase with several roles in cell activation, including via the NF-κB and MAPK/ERK signal transduction pathways. Raised Ca^{2+} levels also activate calmodulin, which stimulates other Ca^{2+}-dependent enzymes within the cell. Receptor phosphorylation triggers signal transducers and activators of transcription (STATs) to form homodimers or heterodimers, which can translocate to the nucleus where they bind to regulatory sites in the gene promoter region, inducing gene transcription (Smith-Garvin et al., 2009). These signalling pathways are common to many intracellular signalling pathways in immune cells and eventually converge in the cell nucleus to modify patterns of gene transcription.

Although the innate and adaptive immune systems are typically considered separately, there is a great deal of interconnectivity between them. For example, neutrophils and monocytes (innate immune cells) preferentially recognise bacteria that are opsonised (coated) with antibodies that have been produced by B cells (adaptive immune cells) which in turn have received 'help' from $CD4^+$ T cells (adaptive immune cells) (Figure 1-4). Cytokines are also produced by activated innate and adaptive immune cells and can exert a plethora of effects on all immune cells.

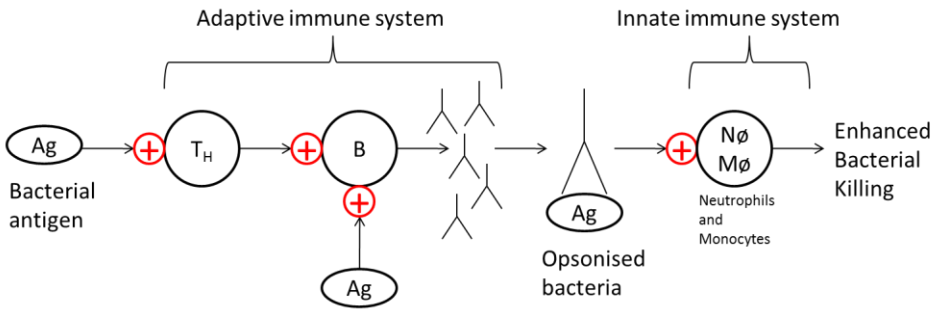

Figure 1-4: Interaction of adaptive and innate immune systems.
$CD4^+$ T helper cells (T_H) recognise an antigen (Ag) and provide "help" to B cells (B) which produce antibodies. These antibodies bind to invading pathogens (e.g. bacteria) enhancing their recognition by neutrophils (Nø) and monocytes (Mø) and subsequent eradication of the bacteria by phagocytosis and oxidative burst.

Optimal functioning of both the innate and the adaptive immune systems in patients with infections or cancer is critical, and anything which influences this might have significant effects on their clinical status. Many factors are known to inhibit immune function, including pain, surgery and general anaesthesia (Kawasaki et al., 2007, Page, 2003). It is also possible that certain opioids could further inhibit immune function and aggravate the problem of reduced immunosurveillance (Budd, 2006, Sacerdote, 2008). The following sections explore the actions of opioids and the differences between them.

1.4 Molecular actions of opioids

Although prescribed opioids principally work by modulating the effects of the endogenous opioid system, they also have a range of non-opioid effects (discussed in section 1.5). Endogenous opioids bind to specific opioid receptors (Table 1.3) and these interactions help to maintain the homeostasis of many of the body's organs. It is the modulation of these receptors, as well as non-opioid effects, by prescribed opioids which is responsible for their desired and unwanted effects (see Table 1.1).

Table 1.3: Endogenous opioid agonists and their opioid receptor binding patterns.

Endogenous opioid	Receptor
β-Endorphin	μ (and δ)
Dynorphin A	κ (and μ)
Enkephalins	δ (and μ)
Endomorphins	μ
Morphine	μ
Nociceptin/orphanin FQ	ORL1

The principal endogenous opioid peptides and the type(s) of opioid receptor to which they bind with the highest affinity. In brackets are the receptors to which these peptides bind with lower affinity. The endorphins, dynorphins, enkephalin and endomorphins are the classical endogenous opioids; opioid receptor-like-1 (ORL1), the nociceptin/orphanin FQ peptide receptor, has sequence homology with the classical opioid receptors, but has a different signalling system. Although typically used exogenously, endogenous morphine produced by immune cells, has been described (Glattard et al., 2010, Stefano et al., 2008a, Zhu et al., 2005).

1.4.1 Opioid receptors

The opioid receptor is a seven-transmembrane domain GTP-binding protein (G protein)-coupled receptor (GPCR). GPCRs are a superfamily of diverse receptors which evolved over 800 million years ago and are present in fungi, invertebrates and vertebrates (Peroutka and Howell, 1994). There are several groups of GPCR, with the

largest group being the type 1, rhodopsin-like, family of receptors, which includes the opioid receptors. Numerous other receptors are also classified into this group, including those for acetylcholine, cannabinoids, catecholamines, chemokines (i.e. IL-8), histamine and serotonin.

There are three classical opioid receptors: μ, κ and δ (MOR, KOR and DOR), and the non-classical opioid receptor-like 1 (ORL1), which are principally responsible for the effects of both endogenous and prescribed opioids. Of these, the MOR is generally considered the most important opioid receptor for mediating the effects of prescribed opioids. The more recently discovered ORL1 receptor has sequence homology with the classical opioid receptors, but it has a different signalling system, with the nociceptin/orphanin FQ peptide as its endogenous ligand (Chiou et al., 2007).

All of these receptors have subtypes which are thought to be splice variants, as each receptor only has one gene (Dietis et al., 2011). For example, numerous splice variants of the MOR have been identified, with at least 14 human MOR isoforms (Pan, 2005), with various affinities for morphine and its derivatives, which suggests that different morphine concentrations may activate specific receptor subtypes. For example, the μ3 receptor binds morphine, but is not activated by endogenous opioid peptides. Interestingly, it only has 6 transmembrane domains (Makman et al., 1995, Stefano et al., 1993).

Opioid receptors can form homodimers (i.e. two MORs) or heterodimers (i.e. MOR and DOR) and can also form heterodimers with other GPCRs on immune cells, including chemokine receptors (Suzuki et al., 2002). Dimerisation enhances ligand binding combinations and modulates receptor signalling transduction, it can also influence receptor internalisation and degradation properties (Faras-Melainis et al., 2009, Rozenfeld and Devi, 2007), and is in part responsible for the interindividual variability in response to opioids (Chakrabarti et al., 2010).

1.4.2 Opioid receptor signalling

Opioids bind to different points of the binding cleft which is made up of the transmembrane and extracellular domains of the seven-transmembrane opioid receptor. The specificity of ligand-receptor binding at different sites on the receptor is dependent on the type of opioid. Opioid alkaloids bind within the transmembrane portion of the receptor, whereas large peptidyl ligands bind to the extracellular loops (Watson et al., 1996).

The intracellular domains (principally the second and third intracellular loops) of the opioid receptor are coupled to a heterotrimeric G-protein complex, which is inhibitory under normal circumstances (G_i). The binding of opioid agonists to the binding cleft induces a conformational change in this GPCR and this initiates a downstream chain of secondary messenger signalling events. Initially, guanosine diphosphate (GDP) dissociates from the α-subunit of the G-protein, and is replaced by guanosine triphosphate (GTP). The Gα and the G$\beta\gamma$ subunits are then liberated and can now interact with downstream transduction pathways. These include the opening of G-protein activating inwardly rectifying potassium (GIRK) channels (Henry et al., 1995), which causes hyperpolarisation of the cell, making it more refractory to depolarisation and decreases the presynpatic release of vesicles, through de-activation of Ca^{2+} channels (Ikeda et al., 2000). These vesicles contain excitatory transmitters, such as, acetylcholine, glutamate, calcitonin gene related peptide and substance P (Yaksh, 1997). By activating G-proteins, opioids also have inhibitory effects at voltage-gated Ca^{2+} channels (Yaksh, 1997).

Opioid receptor activation also results in intracellular effects which are mediated by the secondary messengers cAMP, cyclic guanosine monophosphate (cGMP), PLC and PKC (Kelly et al., 2008, Rubovitch et al., 2003, Stefano et al., 2008b). Opioids inhibit adenylyl cyclase decreasing cAMP formation. Opioid receptor activation, via certain G-proteins, stimulates PLC, increasing the release of Ca^{2+} from intracellular stores, which activates several kinases, including PKC and MAPK (Law et al., 2000). These kinases have many functions, one of which is to phosphorylate opioid receptors and cause receptor desensitisation, reducing opioid responsiveness (Law et al., 2000). Opioids also increase nitric oxide (NO) and cGMP (Stefano et al., 2008b) (Figure 1-5).

The action of morphine on immune cells may be due to an effect via the μ3 receptor, with is linked to a nitric oxide synthase (NOS) molecule, producing NO when morphine activates this receptor (Cadet et al., 2003). The N-methyl D-aspartate (NMDA) receptor is also linked to NOS and the opioid receptor interacts with the NMDA receptor (Law et al., 2000). Furthermore, it has been shown that human monocytes are inactivated morphologically (going from amoeboid to round) when NO levels increase and had a rebound enhancement of activation and chemotaxis when NO levels subsequently decreased (Magazine et al., 2000).

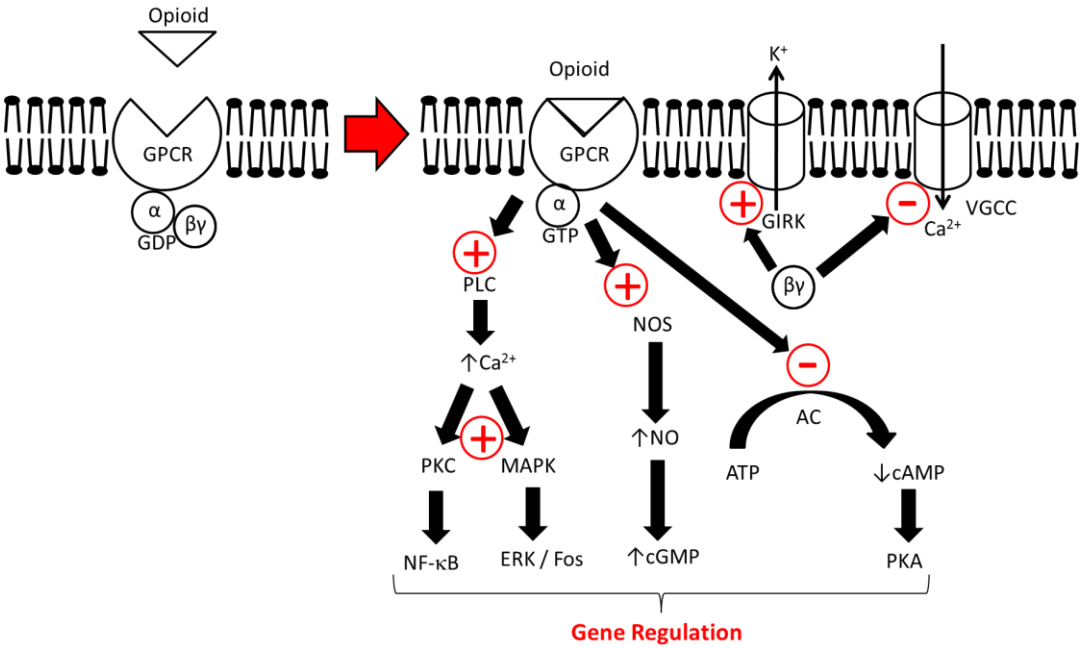

Figure 1-5: Intracellular opioid signalling cascades.
In the resting state the opioid receptor (GPCR) is bound to Gα and Gβγ subunits and guanosine diphosphate (GDP). On opioid binding, GDP dissociates and is replaced by guanosine triphosphate (GTP), the Gα and the Gβγ subunits then interact with downstream pathways. These include the opening of G-protein activating inwardly rectifying potassium (GIRK) channels and inhibition of voltage gated calcium channels (VGCC). Phospholipase C (PLC) is activated, increasing intracellular calcium and activating protein kinase C (PKC) and mitogen-activated protein kinase (MAPK). These then activate NF-κB and extracellular signal-regulated kinases (ERK) and c-Fos which regulate gene transcription. Nitric oxide synthase (NOS) is activated, increasing nitric oxide (NO) and cyclic guanosine monophosphate (cGMP). During acute opioid exposure, adenylyl cyclase (AC) is inhibited and cyclic adenosine monophosphate (cAMP) levels are decreased. This decrease in cAMP inhibits protein kinase A (PKA) which is involved in sugar and lipid metabolism, as well as gene transcription via cAMP response element-binding (CREB).

The duration of activation of the opioid receptor and its ability to signal is dependent on the dissociation of the G-protein subunits, which requires GTP binding. This is negatively regulated by GTP hydrolases, which convert GTP to GDP, causing re-association of the G-protein to its inactive form. This process is also under the control of regulators of G-protein signalling proteins which activate GTPase, terminating receptor activation (Tso and Wong, 2003).

During prolonged opioid exposure, the response of the receptor and its downstream mechanisms becomes attenuated, a process which has been termed ‘tolerance’. This phenomenon is seen with many other drugs and the evidence for its occurrence and multiple underlying mechanisms are becoming clearer. These result from diverse cellular and intercellular events including: reduced internalisation consequent on ligand binding; receptor down-regulation; impaired ability of the opioid receptor to activate G-proteins with a subsequent inhibition of downstream effects, such as in the secondary messengers, protein kinase-A (PKA) and PKC. The latter are responsible for key

events such as activating NMDA receptors, increasing intracellular Ca^{2+} influx which activates NOS, promoting NO as well as superoxide formation. These changes can occur quickly as in the case of receptor desensitisation or can take longer when gene transcription is affected, such as in the up-regulation of adenylyl cyclase (Law et al., 2000, Nestler, 2004). Opioid receptor desensitisation and down-regulation require receptor phosphorylation by kinases, such as G-protein receptor kinases, PKA, PKC, calcium-calmodulin dependent protein kinase-II and tyrosine kinase (Borgland, 2001).

There is a constant background recycling of opioid receptors as they are endocytosed and then either returned to the cell surface or degraded in lysosomes (Finn and Whistler, 2001, Keith et al., 1998). Endocytosis is a clathrin-mediated process. The activated receptor is phosphorylated by G-protein receptor kinases and then binds to β-arrestin and dynamin, which prevents further coupling of the receptor and the G-protein (Ahn et al., 2002, Luttrell and Lefkowitz, 2002). Small phosphorylase-rich endosomes are formed (Ferguson, 2001), in which the receptor is dephosphorylated and returned resensitised to the cell surface (von Zastrow et al., 2003), available for further ligand binding and activation.

Endocytosis is affected by the activation of opioid receptors by certain agonists. Once endocytosed, the receptor cannot undergo further activation, but is ready for further activation if returned to the cell membrane. The level of receptor activation versus the endocytosis is variable between opioids (Martini and Whistler, 2007). Opioids which do not induce opioid receptor endocytosis, such as morphine, are more likely to induce tolerance compared with those that do induce opioid receptor internalisation, such as methadone (Borgland, 2001, Lopez-Gimenez and Milligan, 2010, Whistler et al., 1999). However, over time it is likely that all opioids induce some degree of tolerance.

In Jurkat immune cell lines and neural cells, the MORs localise to the membrane and undergo internalisation after agonist binding (Trafton et al., 2000). In neurons, the MOR agonist [D-Ala^2, N-$MePhe^4$, Gly-ol]-enkephalin (DAMGO) causes rapid receptor internalisation, whereas morphine causes little internalisation (Trafton et al., 2000). In T cells internalisation in response to DAMGO is slower (Borner et al., 2008).

Morphine, at nanomolar concentrations, has been shown to increase NF-κB activation and TNF-α production in macrophages, which was blocked by the non-selective opioid receptor antagonist, naloxone. At micromolar concentrations, morphine suppressed NF-κB, which was not inhibited by naloxone. This suggests that only the effect of low

concentrations of morphine on NF-κB is mediated through opioid receptors (Roy et al., 1998b). Morphine has also been shown to inhibit NF-κB activity in LPS stimulated neutrophils and monocytes, and decrease the gene transcription of pro-inflammatory cytokines. This was dependent on NO and blocked by naloxone (Welters et al., 2000a).

In summary, opioids by binding to the seven-transmembrane G-protein coupled opioid receptor induce a host of intracellular effects, by modulating a range of intracellular secondary messengers and transcription factors. Most previous studies have used morphine as the test opioid, however there are many differences between opioids which, along with their non-opioid effects, are explored in the next section.

1.5 Opioid pharmacology

Most of the commonly used opioids principally elicit their effects by binding to the MOR. However, they can have very different physicochemical properties and pharmacological actions on other opioid and non-opioid receptor systems which may also be responsible for the differential effects between opioids (Keiser et al., 2009). These effects are outlined under the individual opioids below, and include effects on the monoaminergic system (serotonin and noradrenaline), muscarinic receptors, NMDA receptor and even the innate immune TLR's.

Our understanding of which receptors bind these ligands has previously been investigated at the molecular level using binding assays, but is now being explored by *in silico* techniques using detailed computer modelling approaches. *In silico* techniques, alongside biochemical approaches to define these profiles experimentally, has vastly expanded our understanding of the diversity of receptor binding affinities of the different opioids to a range of receptors (Keiser et al., 2009).

Although opioids have some similarities, namely binding to the opioid receptor, the disparate structure and binding profile of opioids results in differences in their effect profile, including antinociceptive and immunosuppressive effects (Keiser et al., 2009, Sacerdote et al., 1997).

1.5.1 Morphine

Morphine is a naturally occurring opiate alkaloid which primarily elicits its analgesic effects by agonistic activity at the MOR (Figure 1-6); it is also a low affinity agonist at the DOR and KOR (Kristensen et al., 1995). It is one of several opiates that are derived from the opium poppy, *Papaver somniferum* (Vallejo et al., 2011) and has been shown to be produced endogenously in humans (Glattard et al., 2010, Stefano et al., 2008a). Morphine is the most commonly used "strong" opioid for pain control and is administered orally and parenterally. As it is hydrophilic it does not readily cross the blood-brain barrier to gain access to the central nervous system (CNS) in which it produces its analgesic effects. It thus has the potential to cause significant systemic toxicity (Clark et al., 2004) on other organ systems (Table 1.1), as greater systemic concentrations are required in order to enable sufficient morphine to cross the blood-brain barrier and induce adequate analgesic effects (Figure 1-14). Morphine does not cause internalisation of the MOR and tolerance may develop to its effects (Lopez-Gimenez and Milligan, 2010).

Morphine is principally glucuronidated in the liver by UDP-glucuronosyl transferase-2B7 to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (Christrup, 1997), the latter having analgesic properties (Smith et al., 2009). *In vivo* it is not only important to consider the effects of morphine on the immune system, but also those of its active metabolites M6G and M3G. It has been suggested that M6G binds to a MOR subtype which is different to that of morphine (Rossi et al., 1996). Although M6G has a better efficacy to side effect profile than morphine (Kilpatrick and Smith, 2005) it has been shown to induce immunosuppression in the rat (Carrigan and Lysle, 2001). *In vivo, in vitro* and *in silico* techniques have shown that morphine and M3G are TLR4 agonists which might not only impact upon hyperalgesic states of microglial origin, but also potentially directly influence innate immunity (Hutchinson et al., 2010, Lewis et al., 2010).

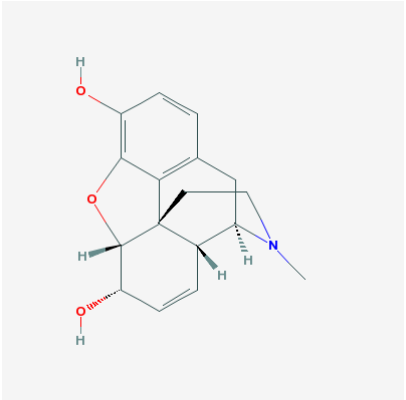

Figure 1-6: Schematic diagram showing the molecular structure of morphine.

Morphine has a molecular weight of 285.3 g/mol and is a moderately potent MOR agonist

Image from Pub Chem (NIH)

1.5.2 Tramadol

Tramadol is a synthetic opioid (Figure 1-7) which has a dual mechanism of analgesic action as it binds with low affinity to MORs and activates central monoaminergic pathways by inhibiting uptake of serotonin and noradrenaline, increasing their extracellular concentration (Grond and Sablotzki, 2004). Compared with morphine, tramadol has 6000 times less affinity for the MOR, a 600-fold lower affinity for the DOR and a 75-fold lower affinity for the KOR (Raffa, 1996), but has the ability to activate other GPCRs, such as the 5-hydroxytryptamine (5-HT) receptor (Driessen and Reimann, 1992) and the noradrenaline receptor (Driessen et al., 1993). The formulation of tramadol is as a racemic mixture in which the (+) enantiomer of tramadol binds to opioid receptors and inhibits serotonin uptake, and the (−) enantiomer of tramadol inhibits the uptake of noradrenaline (Frink et al., 1996, Raffa et al., 1993). Tramadol has lipophilic properties (Alhashemi and Kaki, 2003), which enables it to have good gastrointestinal absorption and penetration into the CNS. It is demethylated by CYP2D6 in the liver to O-desmethyltramadol, an active metabolite.

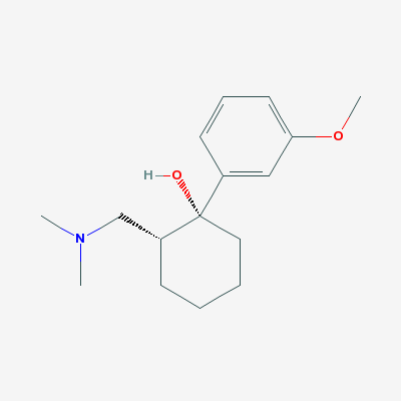

Figure 1-7: Schematic diagram showing the molecular structure of tramadol.

Tramadol (±) cis-2-[(dimethylamino)methyl] -1-(3-methoxyphenyl) cyclohexanol hydrochloride has a molecular weight of 263.4 g/mol (299.8 for tramadol hydrochloride) and it is a weak MOR agonist and an inhibitor of norepinephrine and serotonin reuptake.

Image from Pub Chem (NIH)

1.5.3 Fentanyl

Fentanyl is a very potent, lipophilic, synthetic MOR agonist. It is approximately 100 times the potency of morphine and due to its lipophilicity it can be administered in transdermal and mucosally absorbed preparations (Figure 1-8). Due to its rapid penetration into the CNS, fentanyl causes less systemic, especially gastrointestinal tract, toxicity (Figure 1-14), it also causes less somnolence compared with morphine (Clark et al., 2004). Fentanyl has very low affinities for the KOR and DOR (Maguire et al., 1992) and may interact with serotonin receptors as the analgesia produced by fentanyl is attenuated by 5-HT_{1A} receptor antagonists (Clarke and Ward, 2000). Fentanyl is principally metabolised by N-dealkylation to the inactive metabolites norfentanyl and hydroxyfentanyl by cytochrome P450 (CYP) 3A4 in the small intestine

and liver (Feierman and Lasker, 1996). *In vitro* and *in silico* techniques have shown that fentanyl is a TLR4 receptor agonist and might have a direct influence on innate immune function (Hutchinson et al., 2010).

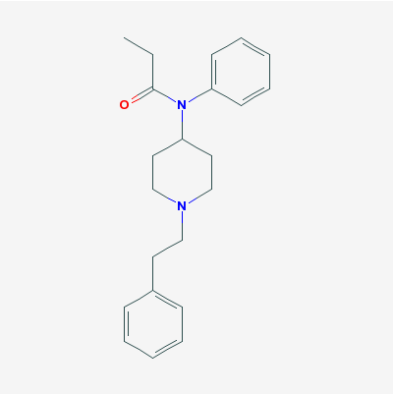

Figure 1-8: Schematic diagram showing the molecular structure of fentanyl.

Fentanyl, N-Phenyl-N-(1-(2-phenylethyl)-4-piperidinyl) propanamide, has a molecular weight of 336.5 g/mol and is a very potent, lipophilic, MOR agonist

Image from Pub Chem (NIH)

1.5.4 Buprenorphine

Buprenorphine is a synthetic opioid derived from the naturally occurring opiate thebaine (Figure 1-9). It is a potent MOR agonist (it is a partial agonist at very high concentrations, exhibiting full agonist properties at concentrations used clinically), from which it has a slow dissociation. It has partial antagonistic activity at the KOR and is a low affinity antagonist at the DOR (Evans and Easthope, 2003). It is also a low affinity agonist at the non-classical ORL1 receptor (Huang et al., 2001). Buprenorphine is associated with less dependence and tolerance compared with other commonly used opioids and has a role not only in chronic pain, but also in opioid dependent patients (Negus and Woods, 1995). It has been shown to have less of an effect on the respiratory drive centres compared to fentanyl (Dahan et al., 2005, Dahan et al., 2006).

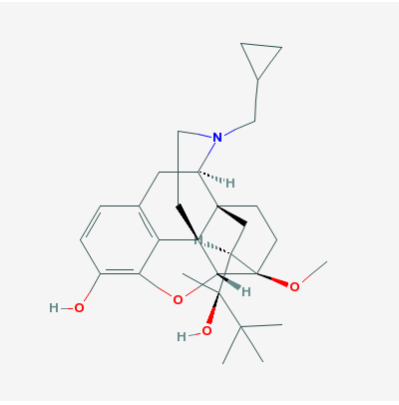

Figure 1-9: Schematic diagram showing the molecular structure of buprenorphine.

Buprenorphine (17-(cyclopropylmethyl)-α-(1,1-dimethyl ethyl)-4, 5-epoxy-18, 19-dihydro-3-hydroxy-6-methoxy-α-methyl-6, 14-ethenomorphinan-7-methanol) has a molecular weight of 467.3 g/mol. It is a potent MOR agonist, a low affinity ORL1 agonist and a KOR and DOR antagonist. It is weakly acidic with limited solubility in water.

Image from Pub Chem (NIH)

Buprenorphine is metabolised by N-dealkylation to norbuprenorphine, which other than being a potent full agonist at the DOR, has similar receptor binding profiles to the

parent molecule (Huang et al., 2001). There are complex interactions between opioid receptors, including dimerisations which can be between the same or different types of opioid receptors (van Rijn et al., 2010), leading to different receptor internalisation and degradation properties. This alters the intracellular actions of opioids when different subtypes of opioid receptor are activated (He et al., 2011, Schramm and Honda, 2010) as potentially occurs with the wide binding properties of buprenorphine. *In vitro* and *in silico* techniques have shown that buprenorphine is a TLR4 receptor agonist and might directly affect immune cells (Hutchinson et al., 2010).

1.5.5 Methadone

Methadone is a synthetic, lipophilic MOR agonist which is used as both an analgesic and in patients with drug dependency (Figure 1-10). It is a potent MOR agonist, NMDA antagonist (Ebert et al., 1995), muscarinic M3 antagonist (Keiser et al., 2007) and inhibits serotonin re-uptake (Codd et al., 1995). Methadone is thought to cause less tolerance compared with morphine, and this might be due to its ability to bind to the NMDA receptor (Ebert et al., 1995) and/or its ability to induce receptor endocytosis (Martini and Whistler, 2007). In a Xenopus oocyte model, the biological activity of methadone was due to its MOR agonist properties and its ability to inhibit NMDA receptors (Callahan et al., 2004).

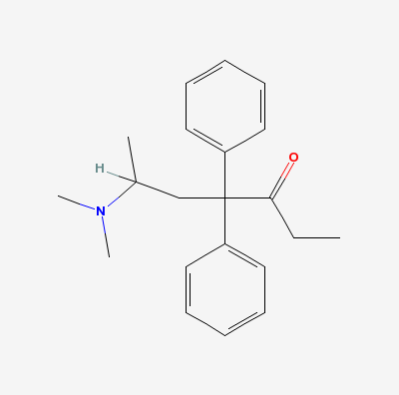

Figure 1-10: Schematic diagram showing the molecular structure of methadone.

Methadone, 6-(dimethylamino)-4,4-diphenyl-3-hepatanone hydrochloride, has a molecular weight of 309.4 g/mol (345.9 for methadone hydrochloride) and is a potent MOR agonist having antagonistic actions at the NMDA receptor and also inducing MOR endocytosis.

Image from Pub Chem (NIH)

Methadone has a long half-life and large distribution volume in humans. It is *N*-demethylated by several isoforms of the cytochrome P450 enzyme system, especially the CYP2B6 and CYP3A4 isotypes (Clark, 2008), to the inactive metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyraline (EMDP) (Oda and Kharasch, 2001). These enzymes are highly polymorphic (Ferrari et al., 2004, Zanger et al., 2007) and have the potential to cause a great deal of interindividual variation when methadone is used *in vivo*. Methadone has

two enantiomeric forms, (R and S isomers), each with differing pharmacological properties. However, the racemic mixture is the form which is commonly used in clinical and laboratory studies (Inturrisi, 2005). R-methadone has a 10-fold higher affinity for μ1 and μ2 receptors than S-methadone, it also binds with low affinity to the δ and κ receptors (Kristensen et al., 1995). *In vitro* and *in silico* studies have shown that both enantiomers of methadone are TLR4 receptor agonists potentially having a direct influence on innate immune cells (Hutchinson et al., 2010).

1.5.6 Oxycodone

Oxycodone is a semi-synthetic opioid (Figure 1-11), derived from thebaine. It is a MOR, KOR and weak DOR agonist (Ordonez Gallego et al., 2007). Having KOR agonist activity, it may have a more sex-specific and oestrogen-dependent effect on pain (Chakrabarti et al., 2010, Lawson et al., 2010). As it has activity at several opioid receptor subtypes, oxycodone could potentially have different activation and internalisation properties on opioid receptor heterodimers (He et al., 2011, Schramm and Honda, 2010). *In vitro* and *in silico* techniques have shown that oxycodone is a TLR4 receptor agonist and it might have a direct effect on innate immune cell function (Hutchinson et al., 2010). Compared with morphine it has a lower incidence of CNS adverse effects, it is twice as potent orally, and has a higher and more predictable oral bioavailability (Lenz et al., 2009). There are however gender differences in the serum concentrations of oxycodone (Andreassen et al., 2011). Oxycodone is principally metabolised by hepatic O-demethylation by CYP2D6 and CYP3A4 to oxymorphone, also a potent opioid analgesic (Andreassen et al., 2011, Samer et al., 2010). Oxycodone is also N-dealkylated to noroxycodone during first-pass metabolism.

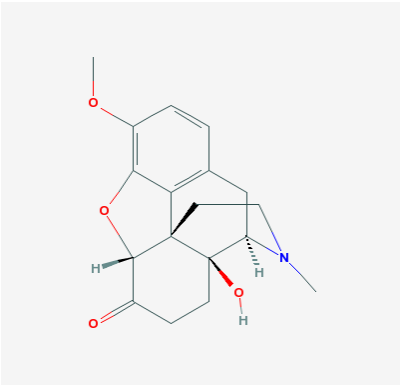

Figure 1-11: Schematic diagram showing the molecular structure of oxycodone.

Oxycodone, 14-hydroxydihydrocodeinone, has a molecular weight of 315.4 g/mol (351.8 for oxycodone hydrochloride) and is a MOR, KOR and weak DOR agonist.

Image from Pub Chem (NIH)

1.5.7 Diamorphine

Diamorphine (heroin; diacetylmorphine) is a 3,6-diacetyl ester synthesised from morphine, which greatly enhances its water and lipid solubility compared to the parent

compound (Figure 1-12). The half-life of diamorphine *in vivo* is 3 min (Inturrisi et al., 1984, Rentsch et al., 2001), as it is quickly hydrolysed back to morphine by sequential deacetylation of two ester bonds (Salmon et al., 1999). Initially, it is rapidly deacetylated to 6-monoacetylmorphine, which then undergoes a slower subsequent deacetylation to morphine (Rossi et al., 1996). Once hydrolysed to morphine it then behaves the same as morphine, but being so soluble and lipophilic, diamorphine readily penetrates into the CNS, the main site for analgesic effect, before deacetylation occurs. *In vivo*, this may also influence its immune effects during acute administration, depending on the body compartment of the immune cell under consideration. For example, diamorphine is likely to remain in the peripheral circulation for less time compared with morphine or oxycodone. Although morphine contributes significantly to diamorphine's pharmacological effects (Cone et al., 1993, Inturrisi et al., 1984), it has been reported that diamorphine directly binds to a MOR variant, different to that of morphine (Rossi et al., 1996). Diacetylmorphine been shown to degrade to 6-monoacetylmorphine and morphine in cell culture (Hutchinson and Somogyi, 2002) and thus it could have an effect *in vitro* either directly or after conversion to morphine (Rossi et al., 1996).

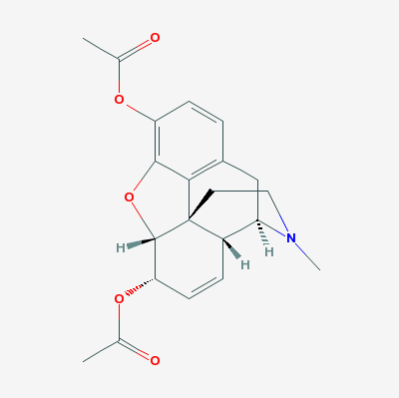

Figure 1-12: Schematic diagram showing the molecular structure of diamorphine.

Diamorphine, 3,6-diacetyl-morphine, has a molecular weight of 369.4 g/mol and is deacetylated to morphine, a potent, lipophilic, MOR agonist.

Image from Pub Chem (NIH)

1.5.8 Codeine

Codeine is a weak opiate which has about one tenth of the potency of morphine (Figure 1-13). Like morphine, it is a naturally occurring opiate alkaloid which is derived from the opium poppy, *Papaver somniferum*. Codeine is a pro-drug of morphine which is principally converted to its active form by CYP2D6 enzymes in the liver. Leukocytes have also been shown to express CYP2D6 (Zhu et al., 2005) and can convert codeine into morphine *in vitro*. Eight percent of Caucasians are slow metabolisers for this enzyme and do not significantly convert codeine to morphine, and do not derive any analgesic benefits from the drug. Similarly this group would be less likely to have any

clinical immune effects from codeine, as it and its 6-glucuronide metabolite only bind very weakly to the MOR and have even less affinity for the other opioid receptors (Mignat et al., 1995). However, codeine has been shown to have effects outwith those due to its conversion to morphine and these might be mediated by its metabolite codeine-6-glucuronide (Lotsch et al., 2006). About 2% of Caucasians are extensive metabolisers for CYP2D6 enzymes and these people get enhanced morphine-derived effects from codeine (Kirchheiner et al., 2007).

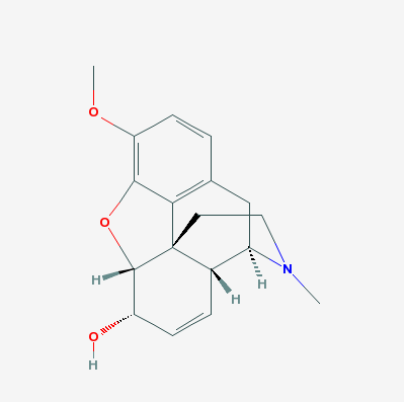

Figure 1-13: Schematic diagram showing the molecular structure of codeine.

Codeine, 3-methylmorphine, has a molecular weight of 299.4 g/mol and is pro-drug of morphine. It is a weak MOR agonist (after conversion to morphine).

Image from Pub Chem (NIH)

1.5.9 Summary

These commonly prescribed opioids are structurally very different which causes differences in their pharmacokinetics, pharmacodynamics and receptor binding profiles (Table 1.4). As our knowledge of the effects of opioids expands, these structural dissimilarities cause a different effect profile of these drugs, not only in analgesic potency and propensity to induce tolerance, but also in the likelihood to cause different toxicities. The differences between the constipating effect of some opioids has been extensively studied. There are both animal (Meert and Vermeirsch, 2005) and clinical (Clark et al., 2004, Tassinari et al., 2008) studies that show the more lipid-soluble opioids such as fentanyl and buprenorphine are less likely to cause constipation, compared with water-soluble opioids such as morphine and oxycodone, while maintaining the same degree of analgesic effect. This is probably caused by their reduced time in the systemic circulation as well as the lower peripheral concentration needed for a CNS concentration to induce an equivalent analgesic effect (Figure 1-14).

Other toxicities which have been studied, show that buprenorphine causes less respiratory depression compared to fentanyl (Dahan et al., 2005, Dahan et al., 2006) and fentanyl causes less somnolence compared with morphine (Clark et al., 2004). It is likely that this heterogeneous group of drugs will have differing effects on immunity.

Although there has been many studies investigating the effect of some opioids on certain immune cells in various models and even a proposal of how opioid structure might affect immune activity (Sacerdote et al., 1997), this has never been systematically studied.

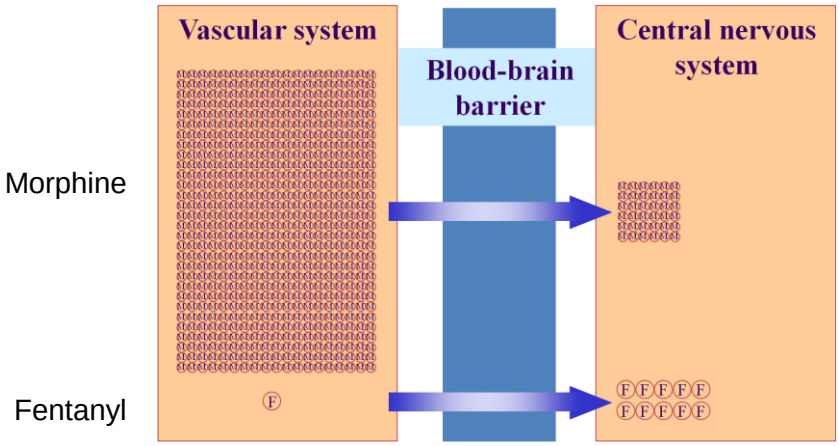

Figure 1-14 Schematic showing the effect of lipophilicity on opioid distribution.
Hydrophilic opioids such as morphine (M) need much greater concentrations in the systemic circulation (vascular system) to cross the blood-brain barrier and cause a similar analgesic effect in the central nervous system when compared with lipophilic opioids like fentanyl (F). This is likely to contribute to the differences in their peripheral effect profile. Adapted from Twycross, 1997.

Table 1.4: Principal pharmacological properties of commonly-used opioids.

Opioid	Pharmacology
Morphine	MOR agonist, TLR4 receptor agonist, hydrophilic
Tramadol	Weak MOR agonist, serotonin and noradrenaline re-uptake inhibition, lipophilic
Fentanyl	Potent MOR agonist, TLR4 receptor agonist, lipophilic
Buprenorphine	Partial MOR agonist, DOR antagonist, ORL1 agonist, TLR4 receptor agonist, lipophilic
Methadone	MOR agonist, may also have NMDA antagonist properties, TLR4 receptor agonist, lipophilic
Oxycodone	MOR agonist, also has KOR agonist properties, TLR4 receptor agonist, hydrophilic
Diamorphine	Pro-drug of morphine, converted by deacetylation. May have direct effect on MOR subtype, lipophilic, high water solubility
Codeine	Pro-drug of morphine, converted principally by CYP 2D6, hydrophilic

1.6 Expression of MOR on immune cells

As the principal effect of opioids is initiated by binding to the transmembrane and extracellular domains of the opioid receptor on the cell surface, for *in vitro* opioids to have an effect on immune cells, it is likely to be necessary for these cells to express

opioid receptors. However, certain opioids may also have direct effects on immune cells mediated via non-opioid mechanisms, such as those described in Table 1.4. *In vivo*, opioids may also modulate immune function via indirect effects, by actions on the CNS and the release of immune mediators (described in section 1.9), as well as by direct effects on the immune cells expressing opioid receptors (Wei et al., 2003).

In MOR knockout ($MOR^{-/-}$) mice, chronic morphine administration has been shown not to induce changes in the immune system, whereas their wild-type counterparts exhibited splenic and thymic atrophy, with decreased lymphocyte numbers. In the thymus there was also an alteration of lymphocyte distribution (Gaveriaux-Ruff et al., 1998, Roy et al., 1998a). NK cell cytotoxicity was also markedly decreased by morphine in mice with MORs. This indicates that the effects of opioids on lymphocytes and NK cells are likely to be mediated via MORs (Gaveriaux-Ruff et al., 1998, Roy et al., 1998a). Baseline immune parameters (including size and lymphocyte population of lymphoid organs, NK cell cytotoxicity, lymphocyte proliferation and immunoglobulin production), in the $MOR^{-/-}$ mice were no different to those in wild-type mice (Gaveriaux-Ruff et al., 1998), indicating that intrinsic immune function is not dependent on the MOR.

There is currently no definitive way to demonstrate the presence of functional opioid receptors on immune cells; data from radioligand binding studies using ^{3}H-naloxone as well as polymerase chain reaction (PCR) analysis are discrepant (Borner et al., 2009, Kraus, 2009, Williams et al., 2007). The most relevant evidence is that which is based on the demonstration of functional effects. As early as 1979, evidence demonstrating that morphine suppressed rosette formation by activated T cells, that this effect was enhanced by met-enkephalin, and that both effects were inhibited by naloxone, suggested the presence of opioid receptors on human peripheral blood T cells (Wybran et al., 1979). Despite this functional evidence for the presence of opioid receptors on immune cells, not all studies have confirmed this. One possibility for the discrepancy is that opioid receptor expression might depend upon the activation status of the immune cell. In a study investigating healthy volunteer-derived resting peripheral blood mononuclear cells (PBMCs), the classical opioid receptors could not be detected with PCR, antibody or ligand studies; ORL1 was expressed in these PBMCs (Williams et al., 2007). Another study in peripheral lymphocytes from healthy humans showed no specific binding of μ, κ and δ radioligands to their cell membranes (Palm et al., 1996). Some of the discrepancy might be due to a 5′ truncated splice variant (where the messenger ribonucleic acid [mRNA] has been shortened and not detected by certain

PCR primers) of the human MOR gene, OPRM1. This splice variant produces a 6 transmembrane μ3 receptor which preferentially binds morphine, but not endogenous opioid peptides; these receptors have been identified in a murine monocytes cell line (Makman et al., 1995) and human neutrophils and monocytes (Cadet et al., 2003).

Opioid receptors have been demonstrated on Mytilus (blue mussel) immunocytes and human monocytes, and on these cells morphine counteracts the stimulatory effect of TNF-α and IL-1α (Stefano et al., 1993). It has been suggested that morphine may have a role in calming or terminating the state of immune alertness. Using PCR and Western blot analysis of human and monkey lymphocytes, Suzuki and colleagues have shown that directly applying morphine to these cells increases OPRM1 gene transcription and protein synthesis (Suzuki et al., 2000). Using a MOR antibody and a Western blot analysis, MORs were detected on human neutrophils and their expression was increased by LPS stimulation (Glattard et al., 2010).

It has been reported that the OPRM1 gene is not transcribed in unstimulated or resting T cells and that these cells do not constitutively express MOR in this inactive state (Borner et al., 2008, Kraus et al., 2001). However, when activated by a physiological relevant stimulus (using mAbs that bind to CD3 and CD28) MOR mRNA is increased in both human T cells and Jurkat cells (an immortalised T cell line). This indicates that OPRM1 gene expression depends on the activation status of the T cells. Morphine has been shown to have a suppressive effect by inhibiting the amount of IL-2 mRNA production in anti-CD3 and anti-CD28 mAb activated human T cells. This effect of morphine was mediated via the MOR, both because of the need for prior activation to induce MOR expression and as its effect was blocked by αPhe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr amide (CTAP), a MOR specific antagonist (Borner et al., 2008).

Some of the discrepancies in these studies could be due to the immune cell type used, its level of activation, the methodology used to detect the opioid receptor and in the case of PCR, the primer sequence used. This is compounded by the non-constitutive expression of MOR in immune cells, and that when it is expressed by cytokine-induction, MOR mRNA is at a very low level (~1% of that in neurons) (Borner et al., 2007). Even if opioid receptors are detected, either as transcript or protein, it is functionality which is of primary importance. If the detection of MOR mRNA at very low levels requires nested PCR to produce sufficient amplification for detection, then its clinical relevance could be questioned. However, it has been shown that very low levels of mRNA can produce functional MOR proteins in T cells (Borner et al., 2007).

1.6.1 Regulation of opioid receptor expression

Various extracellular signals are involved in the control of OPRM1 gene transcription. These include hormones, such as oestrogen (Quinones-Jenab et al., 1997) and drugs, such as cocaine (Azaryan et al., 1996). The effects of cytokines on the regulation of OPRM1 gene transcription in lymphocytes and the pathways via which these effects are mediated have been extensively investigated. Cytokines are among the most potent physiological regulators of OPRM1 gene transcription and cell surface opioid receptor expression in immune and neuronal cells (Kraus, 2009). The transcriptional effects of cytokines on some genes may either be via direct effects on transcription factors or indirectly due to induction of secondary cytokines (Kraus et al., 2001, Kraus et al., 2003).

Opioid receptor expression and the effect of opioids might also be different depending on the subject group and the conditions studied. *In vivo* morphine and methadone induced a potent and persistent down-regulation of opioid binding sites in murine splenocytes (Patrini et al., 1996). Former heroin addicts on methadone maintenance treatment have been reported to exhibit a reduced lymphocyte binding of ^{3}H-naloxone, and decreased levels of MOR and DOR mRNA (Toskulkao et al., 2010). This could mean that the effects of opioids on immune cells could be modulated by previous exposure, and there could thus be a difference between different experimental groups. Further differences are possible between different groups studied, as they may produce different MOR splice variants (Vousooghi et al., 2009). Patients on methadone-maintained treatment have increased OPRM1 deoxyribonucleic acid (DNA) methylation in lymphocytes (Nielsen et al., 2009). Non-opioid receptor actions of opioids could also be differentially affected within different study cohorts, as opioid addiction or maintenance therapy groups may have different PBMC NMDA receptor subunit expression (Roozafzoon et al., 2010).

The OPRM1 gene has many polymorphisms, most of which are in the promoter region (Hoehe et al., 2000). If these affect the binding sites for transcription factors then they can alter the regulation of the gene. NF-κB is important in regulation of OPRM1 gene transcription and there are many NF-κB binding sites on this gene, including three in the promoter region. There are polymorphisms within the NF-κB binding site and within a STAT6 element, the latter of which impairs regulation of the gene by cytokines such as IL-4 (Borner et al., 2002, Kraus et al., 2001, Kraus et al., 2003). The MOR promoter contains cytokine response elements, including NF-IL6.

Induction of MOR expression has been shown to occur in response to IL-1 (Mohan et al., 2010), IL-4 (Kraus et al., 2001, Trafton et al., 2000), IL-6 (Borner et al., 2004) and TNF (Borner et al., 2002, Kraus et al., 2003). In contrast, IFN-γ down-regulates OPRM1 gene transcription (Kraus et al., 2006). These mechanisms are described below and in Figure 1-15.

1.6.2 Interleukin-1

IL-1 induces the expression of the MOR in endothelial cells, however both IL-1α and IL-1β are needed simultaneously (Vidal et al., 1998). IL-1 activates several transcription factors, including NF-IL6, NF-κB, c-Jun and c-Fos (O'Neill, 1995). IL-1β increased MOR mRNA in SK-N-SH cells (a human neuroblastoma cell line) (Mohan et al., 2010).

1.6.3 Interleukin-4

IL-4 induces OPRM1 gene transcription in human T cells, neutrophils, DCs, immune cell lines (Raji, U-937, and HMEC-1) and rat neurons. Transfection experiments in Raji and SH SY5Y cells (a human neuroblastoma cell line) with reporter gene constructs have directly linked the IL-4 effect to the cis-active MOR promoter (Kraus et al., 2001). IL-4, via STAT5 and STAT6, activates transcription of the OPRM1 gene (Borner et al., 2006, Hou et al., 1994). The IL-4-responsive elements of the human OPRM1 gene promoters are STAT6 binding sites. Furthermore, allelic variation within the IL-4–responsive element/STAT6 binding site of OPRM1 promoter reduces responsiveness of the gene to IL-4 to approximately 50% (Kraus et al., 2001).

In an *in vitro* model using Jurkat E6.1 cells, cannabinoids acting on the CB2 receptor induced OPRM1 gene transcription via IL-4 and the transcription factors STAT6 and GATA3. The MORs were shown to be functional, as morphine caused phosphorylation of MAPK in those cells stimulated by cannabinoids (Borner et al., 2006).

1.6.4 Interleukin-6

IL-6 has been shown to induce an up-regulation of MOR mRNA in SH SY5Y cells (Borner et al., 2004). IL-6 activates the Janus kinase (JAK) family of tyrosine kinases (Heinrich et al., 2003b) which phosphorylates and activates STAT1 and STAT3 transcription factors, which bind to the IL-6 response element, located on the promoter of the human OPRM1 gene. These increase OPRM1 gene transcription and MOR mRNA expression (Borner et al., 2004).

IL-6 can also activate the JAK pathway which involves the G-proteins Ras and Raf. This then activates MAPK, which stimulated the transcription factors STAT1, STAT3, NF-IL-6, NF-κB and AP-1 which can all be involved in the downstream signalling of IL-6 (Borner et al., 2004).

1.6.5 Tumour Necrosis Factor

TNF up-regulates the transcription of the OPRM1 gene via the transcription factor NF-κB in both neurons (Borner et al., 2002) and immune cell lines (Raji and U937) (Kraus et al., 2003). Stimulation of Raji cells with TNF has been reported to induce OPRM1 gene transcription within 6 hr, and a neutralising antibody approach has shown this to be mediated by TNF receptor 2 (Kraus et al., 2003).

1.6.6 Interferon-gamma

In T cells and in SH SY5Y cells, OPRM1 gene transcription is down regulated by IFN-γ, in which it inhibits the IL-4-mediated induction of gene transcription (Kraus et al., 2006).

1.6.7 Activator protein-1

The AP-1 transcription factor family, which includes c-Jun and c-Fos, have an important role in T cell differentiation and apoptosis. They may also be important in OPRM1 gene transcription, as there are AP-1 elements within the OPRM1 gene promoter (Borner et al., 2002, Kraus et al., 1995). c-Jun is activated by phosphorylation by JNK which in turn is activated by cytokines or stress. c-Fos is phosphorylated by PKC, PKA and MAPK.

1.6.8 Cannabinoid agonists

Like opioids, cannabinoids can also modulate immune functions, with tetrahydrocannabinol suppressing pro-inflammatory cytokine production from murine T helper cells (Klein et al., 2004). Whereas MORs are normally not expressed, cannabinoid receptors are constitutively expressed by immune effector cells (Borner et al., 2004, Kraus et al., 2001, Kraus et al., 2003). Activation of cannabinoid CB2 receptors has been shown to increase the transcription of the OPRM1 gene via induction of IL-4, STAT6 and GATA3 (Bidlack et al., 2006, Borner et al., 2006, Klein et al., 2004). The cannabinoid CB1 receptor might inhibit the effects of neuronal TNF-α (Rossi et al., 2011) and decrease MOR transcription (Paldyova et al., 2007).

1.6.9 Summary

It has been demonstrated that opioid receptor expression is under the regulatory control of numerous cell signals (Figure 1-15) and it is likely to be altered in states of immune activation such as inflammation and cancer. It is therefore possible that the detected effect of opioids on immunity is dependent on the study model. This would be particularly relevant in human healthy volunteer vs. clinical studies and with regards to the activation status of cell populations that are used for *in vitro* studies.

Although lymphocytes are capable of synthesising opioid receptors, this might only occur when they are activated. Thus, the effects of opioids on the immune system in health and disease might differ. In health, where there is no lymphocyte activation and no expression of opioid receptors on these cells, any influence that opioids have on immunity is likely to be indirectly mediated via neuro-immune mechanisms. In diseased conditions, such as cancer or infection, in which immune activation is present, lymphocytes might express low levels of MORs and prescribed opioids would have both direct peripheral immune modulating effects, as well as CNS mediated neuro-immune effects.

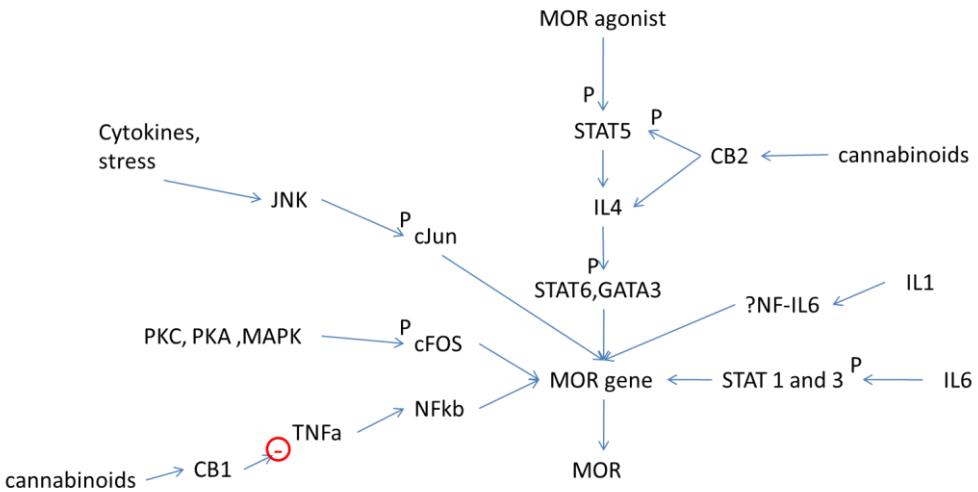

Figure 1-15: Illustration of the various promoters of OPRM1 gene transcription.
TNF-α, IL-1, IL-4 and IL-6 via specific activators of gene transcription, are all involved in the up-regulation of OPRM1 gene transcription. Cannabinoids acting on the cannabinoid CB2 receptor and opioids by phosphorylating STAT5, both act via IL-4, and subsequent phosphorylation of STAT6 and GATA3 to induce OPRM1 gene transcription. The AP-1 transcription factors (c-Jun and c-Fos) are activated by JNK (c-Jun terminal kinases) and PKC, PKA and MAPK. Cannabinoids via the cannabinoid CB1 receptor can inhibit TNF-α. P indicates activated by phosphorylation.

1.7 Neuro-immune interactions of opioids

There are bidirectional connections between the nervous and immune systems. The nervous system can communicate with the immune system via endocrine mediators, sympathetic nervous system (SNS) innervation of lymphoid organs and by the cholinergic parasympathetic nervous system. The reciprocal communication from the immune to the nervous system is principally mediated by cytokines (Kin and Sanders, 2006, Wrona, 2006).

Not only is there evidence that different types of immune cells can express the opioid receptor, but it has also been shown that certain immune cells, including neutrophils and T cells, can contain and release endogenous opioids, including endogenous morphine (Labuz et al., 2010, Zhu et al., 2005). These opioids might be involved in modulation of pain transmission, but could also have a role in immune modulation, with endogenous opioids acting as autocrine and paracrine immune signals (Zhu et al., 2005). From an evolutionary perspective, this action might have developed to modulate the local immune response to injury, as T cells can have a variable effect on pain modulation (Costigan et al., 2009, Labuz et al., 2009, Liang et al., 2008) which can be due to their neuroanatomical location, subtype of T cell and the cytokines released (Hutchinson et al., 2008, Liang et al., 2008, Mika, 2008).

As well as having central analgesic actions, opioids can also act at peripheral sites to modulate pain transmission (Stein and Lang, 2009). There is an interaction between immune cell-derived opioid peptides and opioid receptors on sensory nerves (Figure 1-16). During inflammation, opioid receptors are up-regulated in the peripheral nerve endings of sensory neurons (Stein and Lang, 2009). Immune cells migrate to injured tissues directed by chemokines and adhesion molecules and under stressful conditions or in response to corticotrophin-releasing factor and IL-1β, can secrete opioids which inhibit pain and decrease immune alertness (Machelska and Stein, 2006).

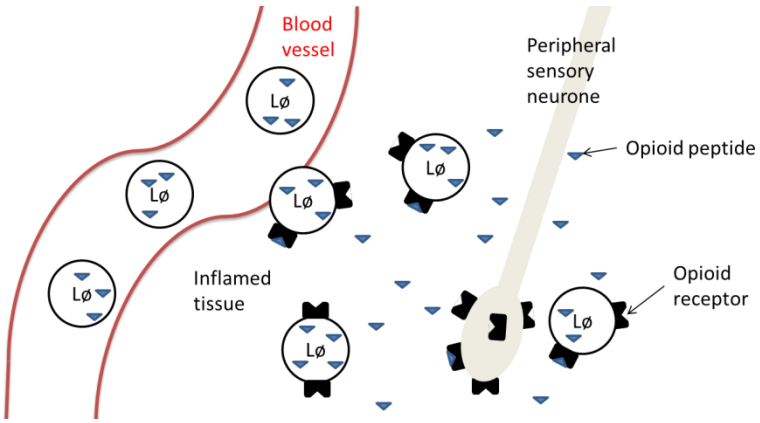

Figure 1-16: Illustration of the peripheral neuro-immune interaction.
Lymphocytes (Lø) migrate out of the blood vessels into areas of inflammation where they can secrete endogenous opioid peptides, including endogenous morphine. These can act on peripheral sensory neurons to reduce pain and also on opioid receptors on their cell membrane (autocrine) and nearby immune cells (paracrine) to reduce immune activation and inflammation. Adapted from Machelska and Stein, 2006, Stein and Lang, 2009, Zhu et al., 2005.

1.8 Immunomodulatory actions of opioids

It might seem unintuitive that pain-killing drugs which work by interfering with the endogenous opioid system can influence immunity. However, it becomes self-evident that this is likely to be the case given that the endogenous opioid system has multiple effects on homeostasis and immune defence mechanisms, in addition to the modulation of pain (Table 1.1). It has even been said that if opioids had not been used as analgesics and had been discovered by immunologists, then the endogenous opioids would have been classified as cytokines (Vallejo et al., 2004). This is because opioids exhibit the principal properties that are associated with cytokines; they are produced by immune cells and they have autocrine, paracrine and endocrine sites of action. They also exhibit functional redundancy and pleiotropy, and have effects that are dose- and time-dependent (Peterson et al., 1998). It is therefore possible that exposure to opioids can potentially have many effects on the immune system.

It has been known that morphine affects immune cells for over 100 years, when Cantacuzene showed that morphine administered to guinea pigs suppressed phagocytosis and leukocyte trafficking (Cantacuzene, 1898, Friedman et al., 2003, Vallejo et al., 2004). Since then, research has emphasised the often detrimental effect of opioids on the immune system (Brack et al., 2011, Budd, 2006, Sacerdote, 2008). These reports have been published across the spectrum of basic science and clinical medical journals and include cellular, animal and human studies. However, they have

principally used *in vitro* approaches and experimental animal models (Brack et al., 2011, Budd, 2006). The majority of this work has been conducted using a variety of methodological approaches, and has tended to evaluate the effect of morphine on one or two aspects of immunity without the ability to compare doses, duration of opioid therapy or immune activation status between studies (Brack et al., 2011, Budd, 2006, Sacerdote, 2008). There has been few well-designed clinical studies to test the *in vitro* and animal findings in patients, and until these have been done it is difficult to know the importance of opioid-induced immune modulation in relevant patient groups (Brack et al., 2011, Sacerdote, 2008).

Furthermore, as stated earlier, immune activation status may be critical for the expression of opioid receptors on T cells and their ability to respond directly to opioids (Borner et al., 2008). This might limit the value of studies using resting lymphocytes in the laboratory. It also potentially limits the value of using healthy volunteers, as they are likely to have a different immune activation status compared to patients with cancer or infection, with the result that any direct effect of opioids on lymphocytes might not be evident in healthy volunteers without lymphocyte activation (Borner et al., 2009).

The immunological properties of opioids are complex and their effects depend upon which opioid is used and the study methodology. Some opioids such as morphine have been reported to be immunosuppressive, whereas buprenorphine might be 'immune neutral' and tramadol may enhance immune function (Budd, 2006, Sacerdote et al., 1997). There is a clear need to systematically explore the effects of the commonly-used opioids on those key aspects of the immune system that are involved in immune surveillance and protective anti-tumour and anti-pathogen immunity.

The following sections outline the previous literature which describe the mechanisms of opioid-mediated immunosuppression (1.9) and the receptors by which this occurs (1.9.1); whether this is mediated by direct effect of opioids on the immune cells or via central mechanisms (1.10) and the kinetics of these effects (1.11). Then the other *in vivo* studies which have assessed the effects of opioids on NK cells, phagocytosis and oxidative burst, T cells and cytokine production that are not already described in earlier sections will be reviewed (1.12). *In vitro* studies, which are methodologically related to the current study, are principally discussed in the Results, in which they are critically evaluated in comparison with the current study.

1.9 Mechanisms of opioid-mediated immunosuppression

Opioids could influence immune function via direct effects on immune cells and/or indirectly via centrally-mediated mechanisms and the systemic production and release of immunomodulatory mediators. The direct mechanism would depend on responsive immune cells expressing appropriate opioid receptors or via non-opioid actions of opioid drugs (i.e. via TLR4). The indirect mechanism would depend on opioids influencing centrally-produced mediators such as immunosuppressive glucocorticoids that are released as a result of hypothalamic pituitary adrenal (HPA) axis activation.

Many of the studies dissecting the mechanisms of opioid-mediated immunomodulation, including if it is a direct effect on immune cells, whether it is MOR dependant or if it is mediated by release of immunomodulatory mediators, have used morphine. Many of the studies in this section will thus be based on morphine and may not be generalisable to all opioids. For example, in mice, a 75 mg pellet of subcutaneous morphine caused atrophy of the spleen and thymus, and to attenuate lymphocyte proliferative responses to T and B cell mitogens within 72 hr (Bryant et al., 1987). As well as causing thymic and splenic atrophy by suppressing hematopoietic cell development, morphine has also been shown, by inducing apoptosis, to reduce macrophage and B cell numbers in the mouse spleen (Singhal et al., 1998). In a healthy volunteer study, morphine has been shown to decrease NK cell cytotoxicity and antibody-dependent cellular cytotoxicity (Yeager et al., 1995). The evidence for whether these effects of morphine on the immune system are mediated by the MOR and the involvement of central and peripheral mechanisms will be explored in the following sections.

1.9.1 Opioid receptors subtypes mediating the effects of opioids on immunity

Many studies have evaluated the relative importance of the different opioid receptors in the mediation of the effects of opioids on immunity. The two principal methods used are gene knockout models and specific opioid receptor agonists or antagonists (Table 1.5).

Table 1.5: Selective opioid receptor agonists and antagonists.

	Agonists	Antagonists
MOR	Endomorphin 1 and DAMGO	CTAP, CTOP, β-funaltrexamine
KOR	U50,488H, U69,593	Nolbinaltorphimine, nor-binaltorphimine
DOR	DSLET (δ2) and DPDPE (δ1);	Naltriben, naltrindole

The different selective opioid receptor agonists and antagonists used in the studies exploring the relative roles of opioid receptors subtypes in immune effects. αPhe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP); αPhe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr amide (CTAP), [D-Pen^{2,5}]-enkephalin (DPDPE); D-Ser^{2}-Leu-Enkephalin-Thr^{6} (DSLET); [D-Ala^{2}, N-MePhe^{4}, Gly-ol]-enkephalin (DAMGO).

Gaveriaux-Ruff and colleagues assessed the capacity of splenic NK cells, from C57BL/6 mice, to kill YAC-1 cells (a NK cell sensitive murine T lymphoblastoma target cell line) using a ^{51}Cr release assay (Gaveriaux-Ruff et al., 1998). They also studied T and B cell proliferation in response to concanavalin A (Con A; a T cell mitogen) and LPS (a B cell mitogen) using a ^{3}H-thymidine uptake assay, and used an enzyme-linked immunosorbent assay (ELISA) to assess antibody production. Flow cytometry was used to assess the distribution of CD4^{+} T cells, CD8^{+} T cells and B cells in the thymus and spleen. Mice were given saline as a control or treated with increasing doses of morphine intraperitoneally every 12 hr for 6 days, up to a maximum of 100 mg/kg. Before morphine, there was no difference between wild-type and MOR$^{-/-}$ mice with regard to the above assays. Morphine-treated wild-type mice exhibited lymphoid organ atrophy, a diminished CD4^{+}:CD8^{+} cell ratio in the thymus and reduction in NK cell cytotoxicity. In MOR$^{-/-}$ mice, morphine administration had no effect on immunity, analgesia, reward or physical dependence (Gaveriaux-Ruff et al., 1998).

The effects of 0.6 mg of morphine, as a single intraperitoneal injection, on lymphocyte function in wild-type, MOR$^{+/-}$ and MOR$^{-/-}$ C57BL/6J female mice, 3 hr after morphine administration has been evaluated (Weber et al., 2006). Splenic NK cell cytotoxicity against YAC-1 cells was measured using a ^{51}Cr release assay. The responsiveness of splenic T cells to phytohaemagglutinin (PHA; a T cell mitogen), Con A, and anti-CD3 mAbs was determined on the basis of ^{3}H-thymidine uptake. Morphine suppressed all of these immune parameters in wild-type animals, had no effect on these in MOR$^{-/-}$ mice and partially inhibited NK cell cytotoxicity (but had no effect on T cell responsiveness) in MOR$^{+/-}$ mice (Weber et al., 2006).

Tomassini and colleagues assessed the role of opioid receptor subtypes in the effect of morphine on phagocytosis by murine peritoneal macrophages (Tomassini et al., 2003). Wild-type C3HeB/FeJ and C57B/L6 female mice and MOR$^{-/-}$ C57B/L6 mice, had

intraperitoneal injections of thioglycollate and were killed after 5 days when peritoneal macrophages were isolated. Phagocytosis was evaluated by the ability of macrophages to ingest opsonised sheep red blood cells (SRBCs) over 20 min, as measured by microscopic examination. Selective μ (DAMGO) and δ2 (D-Ser^2-Leu-Enkephalin-Thr^6; DSLET), but not κ (U50,488H) agonists, inhibited Fc-mediated phagocytosis, indicating that μ and δ2 opioid receptors are responsible for the transduction of the opioid signal (Tomassini et al., 2003).

This group further evaluated the role of opioid receptor subtypes in murine Fc-mediated phagocytosis, using a similar methodology (Tomassini et al., 2004). C3HeB/FeJ or C57B/L6 female mice had intraperitoneal injections of thioglycollate, were killed after 5 days and peritoneal macrophages were isolated. Phagocytosis was assessed by the ability of macrophages to ingest opsonised SRBCs over 20 min as measured by microscopic examination. Selective opioid receptor agonists (μ: endomorphin 1 and DAMGO; δ1: D-Pen^{2,5}-enkephalin [DPDPE]; δ2: DSLET and κ: U50,488H) were added 30 min prior to addition of SRBCs. In a subgroup of mice, opioid antagonists (μ: αPhe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide [CTOP]; δ: naltriben; and κ: nor-binaltorphimine) were added 30 min prior to the agonist. They found that μ and δ2 receptors, but not κ receptors, mediated the effects of opioids on Fc-mediated phagocytosis by murine peritoneal macrophages. Furthermore, μ and δ2 agonists interacted, suggesting a cooperative mechanism of action which might be due to μ/δ heterodimers (Tomassini et al., 2004).

Heterodimerisation of μ and δ opioid receptors have been reported in the nervous system (George et al., 2000), with this oligomerisation having a role in opioid synergy (Gomes et al., 2000). It might be that opioids have a variable effect on different immune parameters via a complex interaction with μ and δ opioid receptors. Dimerised opioid receptors behave differently than the individual receptors, such that morphine can induce internalisation of the GPCR complex when it binds to a μ/δ receptor chimera, whereas it cannot when it binds to a MOR monomer (Roth and Willins, 1999). This is likely to have implications in the development of opioid tolerance.

Roy and colleagues assessed the role of the MOR in the effects of morphine on immune function using MOR$^{-/-}$ mice (Roy et al., 1998a). Although morphine suppressed peritoneal macrophage phagocytosis and secretion of TNF-α in wild-type mice, it did not affect these in MOR$^{-/-}$ mice, indicating that these effects are MOR mediated.

Morphine also reduced spleen and thymus weight, PHA-induced T cell proliferation, and macrophage secretion of IL-1 and IL-6; however these effects remained in $MOR^{-/-}$ mice, suggesting they might be mediated by KOR, DOR or non-opioid receptors. It may also be a combination of these, as the unselective opioid antagonist naltrexone partly reversed the effects of morphine in both wild-type and $MOR^{-/-}$ mice (Roy et al., 1998a).

Szabo and colleagues used specific agonists and antagonists to assess the relative importance of the opioid receptors on the ability of peritoneal macrophages from female C3HeB/FeJ mice to phagocytose *Candida albicans* (Szabo et al., 1993). Morphine suppressed phagocytosis, which was reversed by CTAP, a MOR antagonist, but not by κ (nor-binaltorphimine) or δ (naltrindole) antagonists. However, selective μ (DAMGO), κ (U50,488H) and δ (DPDPE) opioid agonists, dose-dependently suppressed phagocytosis, and these effects were blocked by the respective receptor-selective antagonists. It was concluded that all three opioid receptors may be involved in the modulation of macrophage function and morphine's effect is via the MOR (Szabo et al., 1993).

1.10 Central and peripheral mediation of opioid-induced immunosuppression

The evidence described above indicates that all opioid receptor subtypes might be involved in the effect of opioids on immune function but the principal actions of morphine are mediated via the MOR. The CNS contains many opioid receptors and it is well established that opioids (both endogenous and prescribed) acting at these receptors modulate pain (Zubieta et al., 2001). The CNS and immune systems are intimately connected, with CNS outflow to the spleen (via the SNS) and adrenal glands (via the HPA axis) being able to release immune modulating agents to influence the immune system (Felten et al., 1985). As a consequence, as well as having a direct effect on immune cells, opioids might also have an indirect effect on immunity via an action in the CNS by releasing immunomodulatory mediators. Figure 1-17, below, illustrates the main pathways through which opioids can affect immune function. The indirect action can only be assessed when drug exposure takes place *in vivo*, as it is mediated by opioid receptors in non-immune cells. Thus, although *in vivo* studies enable all the potential actions of opioids to be evaluated, they have a complexity that is absent from *in vitro* studies, potentially making it more difficult to dissect out the underlying mechanisms.

1.10.1 Evidence that opioid-induced immunomodulation is peripherally mediated

In vitro experiments indicate that immune dysfunction by morphine is mediated via direct interactions with MORs on immune cells (Borner et al., 2008). It has been demonstrated that *in vitro* morphine decreases the phagocytic activity of macrophages and that this effect is blocked by the opioid antagonist naltrexone (Roy et al., 1998a, Szabo et al., 1993). *In vitro* μ (morphine and DAMGO) and κ (U50,488H and U69,593) opioid agonists inhibited antibody production, by acting directly on murine splenic cells, which was blocked by naloxone (Guan et al., 1994, Taub et al., 1991).

Wang and colleagues assessed the relative importance of the direct effects of chronic morphine on lymphocytes and macrophages compared with its effects via the SNS and HPA axis using wild-type and $MOR^{-/-}$ Balb/c X C57BL/6 mice (Wang et al., 2002). To assess the role of the HPA axis, mice were infused with corticosterone at concentrations found in morphine-treated mice. The role of the SNS and catecholamines was assessed by administering chlorisondamine (an SNS ganglionic blocker) to mice and implanting 75 mg morphine pellets subcutaneously 1 hr later. Animals were killed 48 hr after this and cell suspensions made from thymus, spleen, and peritoneal lymph nodes. These were incubated with 5 μg/ml Con A and proliferation was measured using a ^{3}H-thymidine uptake assay. Con A induced IL-1β, IL-2, IFN-γ, and TNF-α levels were measured using ELISAs. Morphine decreased splenocyte proliferation and IFN-γ levels and increased macrophage IL-1β and TNF-α levels. None of these effects depended on corticosteroids. Thymic cellularity and thymocyte proliferation were partially suppressed by corticosteroids. Blocking the SNS only partially reversed the suppressive effects of morphine on splenic-lymphocyte proliferation and IFN-γ levels. They also showed that in wild-type animals, the MOR was expressed in thymic, splenic and lymph node lymphocytes, and peritoneal macrophages. The authors concluded that the immune effects of morphine are mostly mediated directly by the MOR on lymphocytes and macrophages when delivered *in vivo* to mice, although there may be a contributing role from the SNS and HPA axis (Wang et al., 2002).

Rojavin and colleagues assessed the effects of *in vitro* and *in vivo* morphine on peritoneal macrophage phagocytosis of *Candida albicans* during a 30 min assay (Rojavin et al., 1993). A 75 mg morphine pellet implanted subcutaneous into mice inhibited peritoneal macrophage phagocytosis. *In vitro* morphine dose-dependently

decreased phagocytosis. The suppressive effects of morphine *in vitro* and *in vivo* were blocked by naltrexone, indicating that morphine can interact with opioid receptors on macrophages and directly decrease phagocytosis (Rojavin et al., 1993).

The μ3 receptor is a morphine sensitive and opioid peptide insensitive MOR, formed as a splice variant of the OPRM1 gene mRNA (Cadet et al., 2003). μ3 receptors have been detected on granulocytes and have been shown to mediate the inhibitory effects of *in vitro* morphine on granulocyte chemotaxis (Makman et al., 1995, Stefano et al., 1993). Furthermore, μ3 opioid receptors, by a NO dependent mechanism, mediate morphological changes in human monocytes, induced by a direct action of morphine (Magazine et al., 1996).

Previous *in vitro* studies have demonstrated effects of certain opioids on different immune cells, meaning the opioid must be having a direct action on the cell (Bonnet et al., 2008, Casellas et al., 1991, Sheen et al., 2007, Tomassini et al., 2003, Tsujikawa et al., 2009, Welters et al., 2000b, Wu et al., 2009). These *in vitro* studies are described in the Results.

1.10.2 Evidence that opioid-induced immunomodulation is centrally mediated

To evaluate the central vs. peripheral effects of opioids, the *in vivo* actions of morphine (which crosses the blood-brain barrier) and peripherally acting N-methylmorphine (which does not cross the blood-brain barrier), have been assessed in rodents (Hernandez et al., 1993, Shavit et al., 1986). Hernandez and colleagues have shown that subcutaneous morphine in the Sprague-Dawley rat suppressed Con A stimulated lymphocyte proliferation, increased corticosterone and was analgesic, whereas peripherally administered N-methylmorphine had no effect on these parameters. When applied directly into the anterior hypothalamus or intracerebroventricularly, both morphine and N-methylmorphine inhibited lymphocyte proliferation, elevated plasma corticosterone levels and induced similar analgesic responses (Hernandez et al., 1993). Subcutaneous and intracerebroventricular administration of morphine, but not subcutaneous N-methylmorphine, produced a naltrexone-reversible suppression of splenic NK cell cytotoxicity in the male Fischer 344 rat, using a ^{51}Cr release assay and YAC-1 target cells (Shavit et al., 1986).

Furthermore, by directly injecting morphine into specific neuroanatomical sites in the rat brain, it has been shown that opioid receptors in the caudal region of the peri-aqueductal grey (PAG) mediate morphine induced suppression of splenic NK cell function (Gomez-Flores and Weber, 2000, Hernandez et al., 1993, Lysle et al., 1996, Weber and Pert, 1989), whereas its inhibitory effects on blood lymphocyte proliferation to Con A stimulation are mediated via MORs in the anterior hypothalamus (Hernandez et al., 1993).

Lewis rats have been treated with intracerebroventricular DAMGO (MOR agonist), DPDPE (DOR agonist), or U69,593 (KOR agonist) and killed 1 hr later (Nelson et al., 2000). Single-cell splenic suspensions were generated and NK cell cytotoxicity, T cell proliferation to Con A and leukocyte numbers were evaluated. NK cell cytotoxicity was measured with a ^{51}Cr release assay using splenic leukocytes and YAC-1 target cells. Splenic T cells were stimulated with 0.5 and 5.0 μg/ml Con A and proliferation was measured using a ^{3}H-thymidine uptake assay. DAMGO suppressed NK cell cytotoxicity and Con A stimulated T cell proliferation. DPDPE and U69,593 had no effects. There was no difference in splenic leukocyte numbers between rats treated with saline or any of the opioid agonists. Intracerebroventricular N-methylnaltrexone (a peripherally acting MOR antagonist) attenuated the suppressive activity of DAMGO on NK cell cytotoxicity and stimulated T cell proliferation to Con A (Nelson et al., 2000). This indicates that the opioid-mediated inhibition in NK cell cytotoxicity and splenic T cell proliferation to Con A are at least partly mediated by CNS MORs.

Wistar rats received 40 μg of intrathecal morphine which caused an elevation of serum adrenocorticotropic hormone (ACTH) concentration and had a suppressive effect on splenic NK cell cytotoxicity (measured by a release of europium from labelled YAC-1 cells) and Con A induced IL-2 and TNF-β production (Zhang et al., 1998). TNF-α levels were not affected. Naloxone injected into the PAG, after the intrathecal morphine, partially antagonised the inhibition of NK cell cytotoxicity and the elevation of ACTH concentrations. However, it did not affect the Con A induced IL-2 production or splenic TNF-β activity, indicating that morphine can affect aspects of immune function by a spinal mechanism which is partly mediated via the PAG. Naloxone alone decreased ACTH levels which indicates that endogenous opioid peptides regulate ACTH secretion (Zhang et al., 1998).

Lysle and colleagues investigated the immune effects of morphine and N-methylnaltrexone injected into distinct positions in male Wistar rats brains (Lysle et al.,

1996). Single injections of morphine into the lateral ventricle of the rat caused a dose-dependent decrease in splenic T and B cell proliferation (to Con A, PHA and LPS), splenic NK cell cytotoxicity (using a ^{51}Cr release assay and YAC-1 target cells), IL-2 and IFN-γ production. Injection of morphine into the caudal PAG inhibited NK cell cytotoxicity, but not lymphocyte or cytokine function. N-methylnaltrexone injection into the PAG antagonised the inhibitory effect of morphine on NK cell cytotoxicity (Lysle et al., 1996). This indicates that opioid receptor activation in the caudal region of the PAG is involved in the effect of morphine on splenic NK cell cytotoxicity, and other brain regions regulate the effect of morphine on lymphocyte proliferation and cytokine production.

Fecho and colleagues investigated the effect of acute morphine (15 mg/kg injected subcutaneously) on the SNS and the HPA axis, in male Lewis rats (Fecho et al., 1996). Chlorisondamine (a SNS ganglionic blocker) and adrenalectomised rats were used to assess these responses. The adrenalectomised rats were given exogenous corticosterone and killed 1 hr after the morphine injection. Splenic and whole blood lymphocytes were activated with mitogens and proliferation measured using ^{3}H-thymidine assays. IFN-γ was measured using an ELISA. Splenic NK cell cytotoxicity was measured against ^{51}Cr-labelled YAC-1 cells. Morphine inhibited splenic lymphocyte proliferation to Con A, LPS and ionomycin with phorbol 12-myristate 13-acetate (PMA; an activator of PKC), which was blocked by both chlorisondamine and adrenalectomy. Morphine also inhibited splenic lymphocyte proliferation to PHA and IFN-γ production, which was blocked by chlorisondamine, but not adrenalectomy. Morphine inhibited Con A and PHA induced blood lymphocyte proliferation, which was blocked by adrenalectomy, but not chlorisondamine and splenic NK cell cytotoxicity, which was not affected by chlorisondamine or adrenalectomy (Fecho et al., 1996).

Overall, these results imply that both sympathetic and adrenal activity are involved in mediating the inhibitory effects of morphine on the proliferative response of splenic lymphocytes to Con A, LPS and ionomycin with PMA. The inhibitory effect of morphine on splenic lymphocyte proliferation to PHA and IFN-γ production is mediated by the SNS (rather than the HPA axis). The capacity of morphine to inhibit proliferative responses of peripheral T cells to Con A or PHA involves HPA activity (rather than SNS). However, neither sympathetic nor HPA activity seems to mediate morphine's inhibition on splenic NK cell cytotoxicity, suggesting that this might be a direct, rather than a centrally mediated, effect of morphine (Fecho et al., 1996).

In a series of experiments, Saurer and colleagues investigated the central effects of morphine on splenic NK cells in male Lewis rats. They showed that acute morphine (15 mg/kg subcutaneous) modulates splenic NK cell cytotoxicity (against ^{51}Cr-labelled YAC-1 cells) via central D_1 dopamine receptors in the nucleus accumbens shell, as microinjection of SCH-23390 (a D_1 receptor antagonist) into this area, antagonised the effect of morphine on NK cells. Furthermore, NK cell cytotoxicity was reduced when SKF-38393 (a D_1 receptor agonist) was microinjected (Saurer et al., 2006a). They then showed that this effect of SKF-38393 was reversed by the selective neuropeptide Y (NPY) Y_1 receptor antagonist, BIBP3226. BIBP3226 also blocked the inhibition of morphine on splenic NK cells (Saurer et al., 2006b). NPY is released upon sympathetic activation and produces direct and dose-dependent inhibitory effects on NK activity *in vitro* (Nair et al., 1993). Splenic lymphocytes in rats have been shown to express NPY Y_1 receptors (Bedoui et al., 2002).

There are two principal pathways which mediate the central immunosuppressive effects of morphine. First, acute administration enhances opioid activity in the PAG which in turn activates the SNS. The SNS innervates lymphoid organs, such as the spleen, and this activation of the SNS induces the release of immunosuppressive biological amines which suppress NK cell cytotoxicity (Irwin et al., 1988)**.** Second, prolonged use of opioids increases activity in the HPA axis and leads to the production of immunosuppressive glucocorticoids. This, in turn, decreases NK cell cytotoxicity (Mellon and Bayer, 1998).

There may however be a 3rd central pathway (or at least an additional arm to the SNS pathway) by which morphine may reduce splenic NK cell cytotoxicity. By activating D_1 receptors in the nucleus accumbens shell, morphine increases the release of NPY from SNS, which acts on peripheral NPY Y_1 receptors on NK cells, inhibiting their activity (Saurer et al., 2006a, Saurer et al., 2006b).

1.10.2.1 Sympathetic nervous system

Lymphoid tissues including the spleen, thymus, bone marrow, lymph nodes (and the lymphocytes they contain) and the adrenal medulla, are innervated by noradrenergic and peptidergic neurons of the SNS which can modulate their function (Felten et al., 1985). It has also been shown in healthy humans that the catecholamine adrenaline transiently reduced the responses of mononuclear cells to Con A and PHA (Crary et al., 1983).

The β-adrenergic receptor may be involved in the immunomodulatory effects of opioids. In rats, morphine, β-endorphin and DAMGO increase catecholamine release from the adrenal medulla and peripheral sympathetic nerves. This is due to MOR activation in specific brain areas, including the hypothalamic paraventricular nucleus and brainstem nucleus of the solitary tract (Appel et al., 1986, Van Loon et al., 1981). Radioligand binding studies have demonstrated the presence of β-adrenergic receptors on human peripheral blood lymphocytes (Borst et al., 1990, Khan et al., 1986, Williams et al., 1976) providing a physiological basis for this hypothesis.

Fecho and colleagues used male Lewis rats to investigate the role of β-adrenergic receptors in the immunoregulatory properties of morphine (Fecho et al., 1993). Nadolol (an unselective β-adrenoreceptor antagonist), atenolol (a β_1-selective antagonist) and ICI-118,551 (a β_2-selective antagonist) were administered prior to subcutaneous morphine (15 mg/kg). The proliferation of splenic leukocytes in response to Con A, PHA, LPS, and ionomycin with PMA were measured. Splenic NK cell cytotoxicity was evaluated with a ^{51}Cr release assay using YAC-1 target cells. The β-adrenoreceptor antagonists did not affect blood leukocyte proliferation (in response to Con A and PHA), splenic NK cell cytotoxicity, or total spleen and blood leukocyte counts. All of the β-adrenoreceptor antagonists completely blocked the suppressive effects of morphine on the proliferative response of lymphocytes to Con A, PHA, LPS and ionomycin with PMA. This suggests that β-adrenergic receptors are involved in the suppressive effects of morphine on splenic T and B lymphocytes. The authors postulated that in order to have these effects morphine has a central action on the SNS innervating the spleen via β-adrenoreceptors (Fecho et al., 1993).

1.10.2.2 Hypothalamic-pituitary-adrenal axis

The paraventricular nucleus of the hypothalamus, the anterior lobe of the pituitary gland and the cortices of the adrenal glands, and the feedback interactions between them constitute the HPA axis. This is an important part of the neuroendocrine system and is activated by stress. Morphine also activates this pathway by increasing hypothalamic corticotrophin-releasing factor levels, causing the secretion of ACTH from the anterior pituitary, stimulating the release of adrenal corticosteroids (Nikolarakis et al., 1989). Corticosteroids have an inhibitory effect on immune function (Cox et al., 1982). As described previously, Hernandez and colleagues have shown that both intracerebroventricular and subcutaneous morphine in the rat increased corticosterone and suppressed Con A stimulated lymphocyte proliferation (Hernandez et al., 1993).

Borman and colleagues assessed the effect of morphine on plasma cortisol concentration and NK cell cytotoxicity in pigs (Borman et al., 2009). Pietrain crossbreed male pigs were injected with morphine (0.5, 1 or 5 mg/kg) or saline. The effect of pre-treatment with 1 mg/kg naloxone was also evaluated. Samples were taken before morphine administration and 15, 60,120,180 and 240 min after morphine. PBMCs were isolated to assess NK cell cytotoxicity against YAC-1 target cells, which was measured using a ^{51}Cr-release assay. The percentage and total number of NK cells were counted using microscopy. Plasma cortisol concentration was measured using a radioimmunoassay. Morphine (0.5 mg/kg) induced a stimulation of NK cell cytotoxicity for at least 4 hr, whereas 1 mg/kg induced an initial stimulation of NK cell cytotoxicity (within 1 hr), with subsequent suppression. 5 mg/kg of morphine suppressed NK cell cytotoxicity at 3 hr without any initial stimulation. These effects were all naloxone sensitive. NK cell numbers decreased with 1 and 5 mg/kg of morphine from 2 hr onwards, with no effect of naloxone pre-treatment. All doses of morphine increased plasma cortisol levels at all time-points, with no effect of naloxone pre-treatment. However, naloxone alone also increased plasma cortisol concentration (Borman et al., 2009).

This study indicates that morphine increases plasma cortisol levels, but that there is a discrepancy in its effect on NK cell cytotoxicity. It is likely that cortisol is just one of several factors involved in mediating the effect of morphine and that cortisol's suppressive effects may be more important in chronic opioid administration, especially at higher doses of morphine.

1.10.3 Summary

As well as potentially having direct effects on immune cells, depending on their activation status, opioids may also have indirect effects on the immune system by two main nervous system pathways. In acute administration, opioids may activate the SNS, which not only directly innervates primary and secondary lymphoid organs but can also activate the release of immunosuppressive catecholamines. There might be a separate central effector mechanism via the release on NPY. During chronic opioid administration, activation of the HPA axis, mediated through immunosuppressive glucocorticoids, may be more important in the effect of opioids on immune function (Figure 1-17).

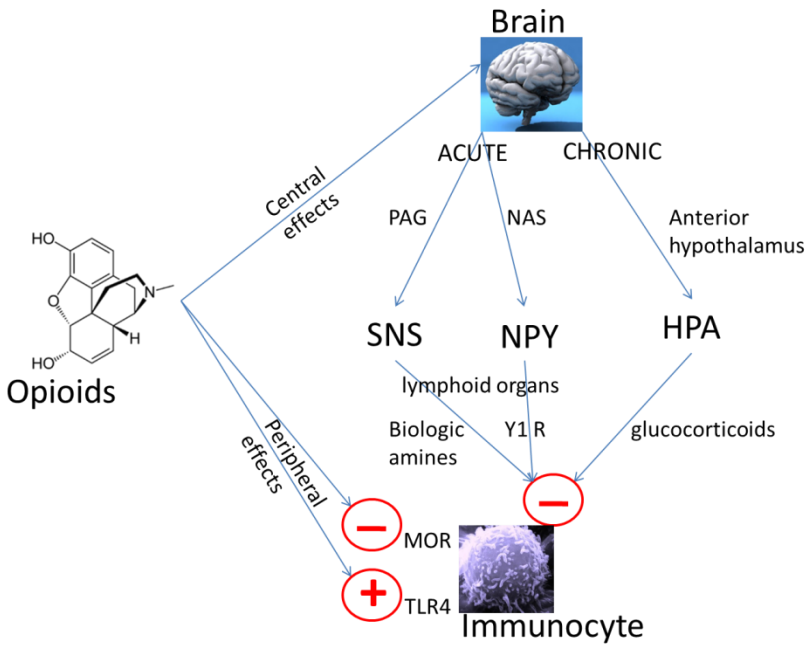

Figure 1-17: Peripheral and central mechanisms of opioid-induced immune suppression. Opioids can act directly on immune cells if they are expressing opioid receptors (especially μ opioid receptors; MOR), some opioids might also activate Toll like receptor 4 (TLR4). The centrally-mediated route of immune suppression depends on the duration of administration of the opioid. If opioids are administered acutely the potential immune effects are thought to be mediated via the peri-aqueductal grey (PAG) and sympathetic nervous system (SNS). Some opioids might also inhibit NK cells by an action on the nucleus accumbens shell (NAS) and release of neuropeptide Y (NPY) acting via Y1 receptors (Y1R). During chronic administration the effect is via the hypothalamic-pituitary-adrenal axis **(**HPA axis) releasing suppressive glucocorticoids.

1.11 Kinetics of opioid effects on immune function

The previous section outlined the studies which focused on the mechanism of the effect of opioids on immunity; this section includes those studies which primarily focus on the time course of opioid action. Studies not included in these sections are presented in section 1.12, where the literature on the effects of opioids on the different types of immune cells is discussed, this includes the majority of the *in vivo* studies.

The pharmacological, cellular and potential clinical effects of opioids and the time course for these have been studied. These studies assessed the rate of onset and offset of immune effects. Other studies have elucidated the effect of immune tolerance to opioids and also the effects of opioid withdrawal.

Bryant and colleagues investigated the time course of chronic treatment by subcutaneously implanting a 75 mg morphine pellet into male C3H/HeN mice (Bryant et al., 1988). Splenic and thymic weight and cellularity, as well as mitogen simulated

(1.0 μg/ml Con A or 0.5 μg/ml LPS) *in vitro* lymphocyte proliferation (using a ^{3}H-thymidine uptake assay) were assessed 6, 12, 24, 48, 72, 96 or 120 hr after morphine pellet implantation and compared to placebo-treated mice. T cell proliferation to Con A was reduced at 48 and 72 hr only. B cell proliferation to LPS was reduced at 24, 48 and 72 hr. Both responses had normalised by 96 hr. Splenic and thymic atrophy also occurred in morphine-treated mice which peaked at 48 to 72 hr (Bryant et al., 1988).

To assess tolerance to the immunological effects of morphine, Lewis rats consumed a drinking solution containing morphine (0.2, 0.4, or 0.6 mg/ml) or water without morphine for 20 days (West et al., 1997). They were then administered a subcutaneous injection of morphine (15 mg/kg) or saline and killed 1 hr later. Splenic lymphocytes were stimulated with Con A, PHA and LPS (all at 0.5, and 5.0 mg/ml) and proliferation measured using a ^{3}H-thymidine uptake assay. Splenocyte IFN-γ production to Con A was determined using an ELISA. NK cell cytotoxicity was evaluated against ^{51}Cr-labelled YAC-1 target cells after a 5-hr incubation. In the rats that were exposed to no morphine or 0.2 mg/ml of morphine, the injection of morphine suppressed splenic NK cell cytotoxicity, mitogen-stimulated splenic T and B cell proliferation and IFN-γ production. In the rats that consumed the two highest concentrations of morphine, the injection of morphine did not suppress NK cell cytotoxicity, but did suppress the mitogen-stimulated splenic T and B cell proliferation and IFN-γ production (West et al., 1997). These findings suggested that tolerance to the suppressive effect of morphine on NK cell activity, but not to mitogen-stimulated splenic T and B cell proliferation and IFN-γ production, occurs within 20 days.

The differential effect on immunity may be due to different pathways regulating the effect of morphine on the different immune cells. Whereas the SNS might be important for the effect of morphine on splenic immune cells, the effect of morphine on immune cells in the blood may occur via activation of the HPA axis (Fecho et al., 1996). The effect of morphine on splenic NK cells may be mediated by a separate pathway involving the opioid receptors in the caudal region of the PAG (Lysle et al., 1996, Weber and Pert, 1989), and not involve the sympathoadrenal pathways or the HPA axis (Fecho et al., 1993, Fecho et al., 1996).

Bhaskaran and colleagues assessed the effect of low-dose morphine on inducing tolerance to the immune modulating effects of larger morphine doses (Bhaskaran et al., 2007). For these experiments, male FVB/N mice were administered high-dose

morphine (40 mg/kg) every 12 hr for 10 days, with or without morphine priming (1 mg/kg) 3 hr before the high-dose morphine. Peritoneal macrophages were elicited using intraperitoneal thioglycollate on day 8 and removed 2 days later, when the mice were killed. Bone marrow was also removed at this point. The macrophages were incubated with opsonised *E.coli* for 1 hr at 37°C and, in order to assess the ability of macrophages to contain the phagocytosed bacteria, supernatants from control and morphine-treated cells were put onto agar plates to determine survival and bacterial colonies after incubation for 24 hr. High-dose morphine decreased the ability of macrophages to contain and kill bacteria and promoted apoptosis of macrophages. Morphine priming stopped all these effects of high-dose morphine. High-dose morphine enhanced macrophage P38 MAPK phosphorylation, which was attenuated by morphine priming. SB202190 (P38 MAPK phosphorylation inhibitor) attenuated high-dose morphine mediated macrophage apoptosis (Bhaskaran et al., 2007). This study suggests that morphine priming induces tolerance to the immune effects of high-dose morphine, via modulation of a P38 MAPK mechanism.

The effect of *in vitro* morphine and methadone on human macrophages previously exposed to methadone *in vivo* was assessed (Delgado-Velez et al., 2008). Blood was collected from previous intravenous heroin users who were on methadone (between 80 – 120 mg/day) for at least one year, and a healthy control population. Human monocyte-derived macrophages (hMDMs) were produced from both these groups. The monocytes from the methadone group were cultured with 400 ng/ml of methadone. The effect of 30 min and 24 hr exposure to 10^{-13} – 10^{-5} M of morphine (equivalent to 10^{-5} – 2853 ng/ml) and 10^{-12} – 10^{-6} M of methadone (equivalent to 10^{-4} – 309 ng/ml) on hMDMs from the control group was assessed. The effect of methadone withdrawal was tested by culturing hMDMs from the methadone patients in opioid-free media. Phagocytosis was measured by counting the number of macrophages which had internalised one or more opsonised SRBC. Acute exposure of hMDMs to morphine dose-dependently inhibited their phagocytic activity. Methadone exposure resulted in a U-shaped dose-response curve, with maximal inhibition occurring at 10^{-9} M of methadone (≈ 0.3 ng/ml). Exposure to morphine or methadone for 24 hr resulted in normalisation of phagocytosis, suggesting that tolerance to these opioids had developed. Phagocytosis was suppressed again when these opioids were withdrawn from chronically-exposed and opioid tolerant hMDMs (Delgado-Velez et al., 2008).

Pacifici and colleagues examined the time course of the immunomodulatory effects of *in vivo* morphine and methadone in male C57BL6 mice (Pacifici et al., 1994). Single

dose subcutaneous morphine (20 mg/kg) and methadone (12.5 mg/kg) were administered and blood was collected at 20, 40, and 80 min as well as 2 and 24 hr after drug treatment. Elicited peritoneal neutrophils and macrophages were collected. Neutrophil phagocytosis and killing was assessed radiochemically using ^{3}H-uridine labelled *Candida albicans*. Splenic leukocytes were used as effector cells in the NK cell cytotoxicity assay, which was measured by a 4-hr incubation with ^{51}Cr-labeled YAC-1 target cells. Morphine stimulated neutrophil phagocytosis and increased *Candida* killing at 20 and 40 min; both parameters returned to baseline at 2 hr, and were reduced at 24 hr. Neutrophil phagocytosis and killing were unaffected by methadone. Neither morphine nor methadone affected NK cell cytotoxicity at any time point (Pacifici et al., 1994). The peak immune activation of morphine correlates to peak plasma levels and the reduction in immune function occurred when morphine was no longer detectable, potentially representing a withdrawal state.

Martucci and colleagues studied the effects of acute and chronic fentanyl and buprenorphine administration on the immune responsiveness of splenocytes in male Swiss mice (Martucci et al., 2004). In the acute part of the study, subcutaneous fentanyl (0.25 mg/kg) or buprenorphine (5 mg/kg) were administered and the mice were killed 1 hr later. Several immune parameters were assessed: a ^{3}H-thymidine uptake assay was used to measure Con A-induced splenocyte proliferation (2 day incubation), a ^{51}Cr-release assay was used to evaluate the killing of YAC-1 target cells by splenic NK cells (4-hr incubation) and ELISAs were used to measure IL-2 and IFN-γ production in the supernatants of the activated splenocytes. The single dose of fentanyl rapidly decreased lymphocyte proliferation. Buprenorphine did not affect lymphocyte proliferation and neither opioid affected splenic NK cell cytotoxicity, IL-2 or IFN-γ production. In the chronic phase of this study, the effect of fentanyl and buprenorphine at 1, 3 and 7 days were determined, by implantation of subcutaneous pumps continuously infusing fentanyl (7.5 μg/hr), buprenorphine (12.5 μg/hr) or saline. After 24 hr, fentanyl reduced all measured parameters. By 3 days, NK cell cytotoxicity had returned to normal, whereas lymphocyte proliferation, IL-2 and IFN-γ production only returned to baseline levels by 7 days. Buprenorphine did not affect any of the measured parameters. In this model, tolerance to the fentanyl-induced reduction in NK cell cytotoxicity occurred after 3 days, whereas tolerance for all of the other immune parameters occurred within 7 days. In an additional arm to this study, mice were administered the 7 day infusion, as above, and then received twice the dose of fentanyl or buprenorphine for 1 or 3 days, before being killed. Neither fentanyl nor

buprenorphine affected any of the measured immune parameters in this experiment, suggesting that once immunological tolerance to fentanyl had developed, increasing the dose did not overcome it. This indicates that development of tolerance to opioid-induced immunosuppression may occur, at least in the case of fentanyl, as buprenorphine did not affect any of the immune parameters tested in this study (Martucci et al., 2004).

The time course of the development of tolerance to the various aspects of immune function and the effect of opioid withdrawal is vital for the evaluation of pre-clinical studies as well as the potential clinical impact. For example, it may be that immune function in heroin (diamorphine) abusers normalises once they commence methadone maintenance therapy (Novick et al., 1989, Sacerdote et al., 2008). This might be due to the regular dosing and longer half-life of methadone which results in a steady plasma concentration. However, the effect of lifestyle changes of people stopping a heroin addiction in normalising immune function cannot be underestimated. The studies described in the following section, help to elucidate the effects of opioids on key immune parameters that are involved in anti-tumour and anti-infective immunity, as well as in immune regulation.

1.12 Effects of opioids on specific aspects of immune function

This section summarises the data from previous *in vivo* studies which have assessed the influence of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on NK cell cytotoxicity, phagocytosis and oxidative burst of neutrophils and monocytes, T cell function and cytokine production. It is organised according immune cell function, rather than cell type (although there is overlap), as this division is more important for determining the potential effects of these opioids on disease processes. This is also reflected in the organisation of the experiments in the current study.

The immunomodulatory effect of morphine on certain aspects of the immune system has been extensively studied using many different methodologies. There is much less known about the effect of other opioids on the different components of immune function. Furthermore, as previously described, many of the potential mechanisms of opioid-induced immunomodulation have been elucidated using morphine.

1.12.1 Natural killer cell cytotoxicity

NK cell cytotoxicity is critical in the control of viral infections and in tumour immunosurveillance. There has been a range of studies assessing the effect of some opioids (and even comparing two opioids) on this parameter in animal and human models.

In two similar studies (in terms of NK cell cytotoxicity) by Carpenter and colleagues, female C3H/HeN mice were alloimmunised with C57BL/6J mouse splenocytes and administered subcutaneous morphine (50 mg/kg) daily. After 11 days the mice were killed, splenic lymphocytes removed and NK cell cytotoxicity evaluated using a 4 hr assay with ^{51}Cr-labelled YAC-1 cells. Morphine exposure had no effect on splenic NK cell cytotoxicity (Carpenter et al., 1995, Carpenter et al., 1994).

Gaspani and colleagues compared the effects of morphine and tramadol on NK cell cytotoxicity and tumour progression using a rat surgical model of tumour metastasis (Gaspani et al., 2002). For this, Fischer 344 male rats either underwent a sham laparotomy operation or were just handled, 5 hr after which MADB106 cells were injected into the tail vein. Three subcutaneous injections of morphine (10 mg/kg), tramadol (20 or 40 mg/kg) or saline were administered 30 min before surgery or handling, immediately after surgery (or 15 min after handling), and 5 hr after surgery/handling (at the time of tumour cell injection). 10 mg/kg morphine and 40 mg/kg tramadol have been shown to be equianalgesic in rats (Bianchi and Panerai, 1998). Twenty one days after surgery, rats were killed and visible surface lung metastases counted. Splenocyte NK cell cytotoxicity was measured against YAC-1 target cells using a ^{51}Cr release assay. In the non-surgical group, 40 mg/kg tramadol increased NK cell cytotoxicity (20 mg/kg of tramadol non-significantly increased NK cell cytotoxicity), whereas 10 mg/kg of morphine decreased NK cell cytotoxicity. Surgery itself reduced NK cell cytotoxicity and this correlated with increased numbers of lung metastases. Morphine did not influence surgery-induced effects, whereas tramadol dose-dependently increased NK cell cytotoxicity and reduced metastasis in the surgical group (Gaspani et al., 2002).

Overall, the effect of tramadol on NK cell cytotoxicity following surgery might be the summation of two independent effects. First, the stimulation of NK activity which is also present in non-operated rats, due to serotonin re-uptake inhibition; second, the prevention of surgical pain together with the decrease in NK cell cytotoxicity which pain itself would cause (Gaspani et al., 2002). It is the (+) enantiomer of tramadol which

inhibits serotonin uptake and its immunostimulatory effect is inhibited by metergoline, a serotoninergic antagonist (Sacerdote et al., 1999). Increased serotoninergic tone leads to stimulation of NK cell cytotoxicity and lymphocyte proliferation (Clancy et al., 1991, Mossner and Lesch, 1998). D-fenfluoramine and fluoxetine (serotonin re-uptake inhibitors) increase serotonin levels which stimulate NK cell activity and lymphocytes in young male and old female rats (Clancy et al., 1991, Sacerdote et al., 1999).

Shavit and colleagues studied the effect of fentanyl on NK cell cytotoxicity and how this influenced tumour metastasis in male Fischer 344 rats (Shavit et al., 2004). This study consisted of three separate experiments. In the first experiment, the rats were injected subcutaneously with 0.1 or 0.3 mg/kg fentanyl or saline; 2 hr or 6 hr before, at the same time as, or 1 hr after having radiolabelled MADB106 tumour cells injected into their tail vein. Lung tumour retention (LTR) was assessed 4 hr after the injection of the tumour cells, by killing the rats and removing their lungs to measure their radioactive content. LTR is the ratio of radioactivity measured in the lungs to the radioactivity in the injected tumour cell suspension and is a measure of tumour load. This showed that there was a significant, dose-dependent, increase in LTR when fentanyl was administered 2 hr before, at the same time as or 1 hr after having the tumour cells injected. Fentanyl administration 6 hr before tumour inoculation did not affect LTR. In a second experiment a single subcutaneous dose of 0.15 mg/kg fentanyl or saline was administered 1 hr before MADB106 tumour injection. Three weeks later, the rats were killed and the number of visible surface lung metastases was counted. In the rats receiving fentanyl there were significantly more metastases. In the third experiment, the impact of a single subcutaneous dose of 0.15 mg/kg fentanyl on the cytotoxicity of NK cells and on LTR within the same animal was studied. Blood was taken 1 hr after fentanyl administration to assess NK cell cytotoxicity (using a whole blood assay, with a 4-hr incubation with ^{51}Cr-labelled YAC-1 target cells). This was followed by an intravenous injection of radiolabelled MADB106 tumour cells. Fentanyl not only inhibited NK cell cytotoxicity, but also caused an increase in LTR within the same animal (Shavit et al., 2004). This series of experiments shows that fentanyl suppresses NK cell cytotoxicity and that this correlates with increased tumour growth and metastasis when fentanyl is administered around the time of tumour cell inoculation.

Male albino Wistar rats were administered oral morphine (20 or 40 mg/kg/day) or methadone (15 or 30 mg/kg/day) for 6 weeks (Van Der Laan et al., 1996). Lymphocytes from the spleen, lungs and peritoneal fluid were isolated. The number of NK cells in each organ was determined as was cytotoxicity for an individual NK cell and

the total NK activity in each of the organs. NK cell cytotoxicity of the lymphoid cells from these organs was measured using a ^{51}Cr labelled YAC-1 as target cells. Morphine reduced the number of NK cells in the spleen and overall splenic NK cell cytotoxicity, but cytotoxicity per NK cell was unaffected. Peritoneal NK cell cytotoxicity was increased by morphine, but there was no change in the number of NK cells in the peritoneum. Only high-dose morphine increased the total lung cytotoxicity and there was only a trend for increased lung NK cell cytotoxicity and NK cell number. High-dose methadone decreased total splenic NK cell cytotoxicity, although it did not affect individual cell NK activity, and there was only a trend for decreased cell numbers. Peritoneal NK cell number was dose-dependently reduced by methadone, as was total NK cell cytotoxicity. In the lungs, methadone reduced cell numbers, individual cell and total organ NK cell cytotoxicity (Van Der Laan et al., 1996). Chronic morphine and methadone reduced splenic NK cell cytotoxicity. Morphine stimulated peritoneal and pulmonary NK cell cytotoxicity, whereas methadone was inhibitory.

Yeager and colleagues investigated the *in vivo* effects of different dosing regimens of morphine on NK cell cytotoxicity in 16 healthy volunteers (Yeager et al., 1995). The volunteers took 30 mg of oral slow release morphine the night before intravenous morphine, in low dose (0.025 mg/kg loading dose followed by 0.015 mg/kg/h for 24 hr) and high dose (0.05 mg/kg loading dose followed by 0.03 mg/kg/h for 24 hr) arms of the study. Two out of seven volunteers in the low dose and three out of nine volunteers in the high dose arms needed a 50% dose reduction due to side effects. Peripheral blood was taken five times: 1 week before the morphine, during the morphine infusion (at 2 and 24 hr), and 1 and 7 days after the morphine administration. Both spontaneous NK cell cytotoxicity and IFN-γ stimulated NK cell cytotoxicity in PBMC preparations were assessed against ^{51}Cr-labelled K562 target cells (4-hr incubation). Suppression of NK cell cytotoxicity occurred at 2 hr and 24 hr after the onset of morphine administration in both groups. The reduction of NK cell cytotoxicity recovered within 24 hr in the low dose group, but remained at 24 hr after the infusion was stopped in the high dose group (Yeager et al., 1995).

Yeager and colleagues also evaluated the *in vivo* effects of short-term intravenous fentanyl (3 μg/kg bolus followed by 1.2 μg/kg/hr infusion over 2 hr) on multiple immune parameters in seven healthy human volunteers (Yeager et al., 2002). NK cell cytotoxicity was assessed before fentanyl administration, at the end of the infusion, and at 1 and 24 hr after completion of the infusion. PBMCs were isolated by density gradient centrifugation and NK cell cytotoxicity was evaluated using these PBMCs as

effector cells against ^{51}Cr-labelled K562 target cells. Fentanyl increased NK cell cytotoxicity at the end of the infusion, which returned to baseline within 1 hr. The increased NK cell cytotoxicity was due to an increase in the proportion of $CD16^+$ cells in peripheral blood (possibly due to recruitment of NK cells into the circulation), rather than the activity of the individual NK cells (Yeager et al., 2002).

Rapid transient increases in NK cell numbers and cytotoxicity have been described in healthy volunteers after a subcutaneous injection of adrenaline and noradrenaline due to a β adrenergic-mediated recruitment of NK cells (Schedlowski et al., 1996). NK cells are rapidly recruited from lymphoid organs such as the spleen and released from endothelia of the blood vessels (the marginal pool), which is dependent on β_2 adrenoceptors being expressed on NK cells (Benschop et al., 1997). Fentanyl does not affect peripheral catecholamine concentrations (Tandonnet et al., 1991), although stress has been shown to increase catecholamine-induced leukocytosis (Benschop et al., 1996). It might be that in this study (Yeager et al., 2002) that NK cells were released from the marginal pool, increasing total NK cell cytotoxicity and that this might not be directly related to the fentanyl, but the stress from the procedure.

Jacobs and colleagues assessed the *in vivo* effects of an intravenous bolus of 0.2 μg/kg fentanyl on cellular immune function in seven healthy male volunteers (Jacobs et al., 1999). Blood was taken before, and 15 and 30 min after the fentanyl. PBMCs were isolated by density gradient centrifugation and were either used as the effector cells in the NK cell cytotoxicity experiment (evaluated using a 4-hr incubation with ^{51}Cr labelled K562 target cells) or stained with anti-CD16 and anti-CD56 mAb for flow cytometric assessment of NK cell numbers. Fentanyl did not influence individual NK cell cytotoxicity, but increased the number of circulating NK cells ($CD16^+$ and $CD56^+$), by more than 250% within 15 min and this was maintained at 30 min (Jacobs et al., 1999).

NK cell numbers and cytotoxicity were evaluated in male current heroin (diamorphine) abusers, long-term (>10 yr) methadone maintained previous heroin abusers and healthy individuals (Novick et al., 1989). NK cell cytotoxicity was measured using ^{51}Cr labelled K562 as target cells and PBMCs (isolated by density gradient centrifugation) as effector cells. Heroin abusers exhibited a reduced NK cell cytotoxicity compared with methadone subjects and controls (which were similar), but NK cell numbers in all three groups were similar (Novick et al., 1989). The current heroin abusers were undergoing inpatient detoxification, which may have induced opioid withdrawal effects on the NK cells, rather than the diamorphine directly affecting the cells.

A clinical study of 30 patients undergoing surgery for uterine carcinoma compared the influence of morphine and tramadol on NK cell function in the post-operative period (Sacerdote et al., 2000). All patients were administered fentanyl as well as anaesthetic medications (thiopental, succinylcholine, isoflurane, nitrous oxide and pancuronium), and an intramuscular injection of 10 mg morphine or 100 mg tramadol immediately after surgery. Peripheral blood was taken both before opioid administration and 2 hr after. NK cell cytotoxicity, using isolated PBMCs, against ^{51}Cr-labeled K562 target cells (4-hr incubation) was assessed before the operation, immediately after surgery and 2 hr post-operatively. NK cell cytotoxicity was not affected by the surgery or by morphine (trend for inhibition only); however, patients receiving tramadol exhibited an increased NK cell cytotoxicity at 2 hr. The analgesic effects were similar at this time point (Sacerdote et al., 2000).

Beilin and colleagues investigated the effects of two doses of fentanyl on NK cell cytotoxicity in a randomised trial of 40 patients undergoing surgery for malignant or benign conditions (Beilin et al., 1996). For the high-dose fentanyl group, a single dose of 75-100 pg/kg intravenous fentanyl was used at the beginning of surgery, and for the low dose group 1 pg/kg intravenous fentanyl was administered with the option of a further 5 pg/kg of fentanyl being used during the surgery. The other anaesthetic medications also differed between groups. The high-dose fentanyl group received pancuronium at the beginning of surgery, with or without isoflurane during the surgery. The low dose fentanyl group received thiopental, succinylcholine and N_2O in addition to isoflurane and pancuronium. At the end of surgery, neostigmine and atropine were administered to both groups. Peripheral venous blood was collected at the time of induction for baseline values, 60 min after surgery had commenced, then at 24 and 48 hr post-operatively. PBMCs were isolated using gradient centrifugation and were frozen at -70°C until they were used as the effector cells for the NK cell cytotoxicity assay. NK cell cytotoxicity was measured using ^{51}Cr labelled K562 target cells (4-hr incubation). The effect of a 24 hr stimulation with IL-2 or IFN-α/β of the patients PBMCs on NK cell cytotoxicity was also studied *in vitro*. Initially, both concentrations of fentanyl induced a similar reduction in NK cell cytotoxicity, with a peak effect at 24 hr post-operatively. However, by 48 hr after surgery, NK cell cytotoxicity had returned to baseline levels in patients receiving low-dose fentanyl, whereas NK cell cytotoxicity was still significantly reduced after the higher dose of fentanyl. No differences were found between patients having surgery for malignant or benign conditions. The suppressive effect of fentanyl at 24 hr was reversed in the IL-2 or IFN-α/β stimulation experiments. These results indicate that despite different anaesthetic drugs being used, the high dose of fentanyl

caused a more prolonged reduction of NK cell cytotoxicity. The long-term impact or overall outcome of different doses of fentanyl were not determined (Beilin et al., 1996).

In summary, the *in vivo* effects of morphine tramadol, fentanyl, buprenorphine, methadone and diamorphine on NK cell function have been assessed in variable models, including animal, healthy human and patient studies (from the sections 1.9 - 1.12.1). There are discrepancies in the results of these studies which might, in part at least, be explained by the differing methodologies, species used, dose and duration of opioid administration. However, in general the literature reports that morphine, fentanyl, methadone and diamorphine (heroin) decrease NK cell cytotoxicity *in vivo* (although short-term *in vivo* exposure to fentanyl in humans increases NK cell numbers and cytotoxicity), buprenorphine does not affect NK cell cytotoxicity and tramadol enhances it. This change in NK cell cytotoxicity correlates with tumour growth and metastasis in rodent models (Gaspani et al., 2002, Shavit et al., 2004).

1.12.2 Phagocytosis

Phagocytosis is the process by which particles are engulfed and is one of the ways which neutrophils and monocytes control bacterial and fungal infection as well as remove foreign material. Several studies have assessed the effect of some opioids (and even compared two opioids) on phagocytosis in animal and human models.

Shirzad and colleagues evaluated the *in vivo* effects of tramadol and morphine on the phagocytic activity of murine peritoneal phagocytes in BALB/c mice (Shirzad et al., 2009). The mice were divided into three groups: the first were given twice daily intraperitoneal morphine: 20 mg/kg on day 1, 40 mg/kg on day 2, 60 mg/kg on day 3 and 80 mg/kg for the following seven days. The second group was treated with tramadol using the same dose regimen as for morphine. The third group was given saline. Although tramadol is reported to have 20% the potency of morphine, in a pilot study, mice treated with tramadol at equianalgesic doses to those of morphine experienced seizures and lower doses of tramadol were therefore used. On days 3, 5 and 10 the mice were killed, peritoneal fluid collected and the cells isolated and incubated with opsonised SRBCs. The number of phagocytes and the number of SRBCs engulfed by each individual peritoneal phagocyte (phagocytic index) were counted using a microscope. There was no difference between the three groups at days 3 and 5, but at day 10 tramadol increased the number of peritoneal phagocytes as well as the phagocytic index, whereas morphine decreased the number of peritoneal phagocytes as well as the phagocytic index compared with saline. The number of

phagocytic cells in the peritoneal cavity increased in all three groups over the 10 days, which may have been due to the repeated intraperitoneal injections. This study indicates that the chronic administration of tramadol stimulates peritoneal phagocytosis, whereas morphine suppresses phagocytosis in murine peritoneal macrophages. However, their acute administration is less likely to be significant (Shirzad et al., 2009).

Tubaro and colleagues assessed the *in vivo* effect of morphine and methadone on murine neutrophils and macrophages, and rabbit alveolar macrophages (Tubaro et al., 1987). Male CD_1 mice and male New Zealand rabbits were administered twice daily morphine at 10 mg/kg on day 1, 20 mg/kg on day 2, 30 mg/kg on day 3, 40 mg/kg on day 4; or methadone at 2.5 mg/kg on day 1, 5 mg/kg on day 2, 7.5 mg/kg on day 3, 10 mg/kg on days 4 to 6 days. Peritoneal exudates (containing neutrophils and macrophages) were elicited and obtained from the mice. Alveolar macrophages were obtained from the rabbit. Phagocytosis of *Candida albicans* was measured radiochemically by the uptake of ^{3}H-uridine. Morphine, but not methadone inhibited murine neutrophil and macrophage phagocytosis, both after a single dose and at 3 days. By 6 days, methadone had also begun to inhibit neutrophil phagocytosis, but not to the same extent as morphine. In rabbit alveolar macrophages, morphine reduced phagocytosis more than methadone (Tubaro et al., 1987).

The *in vivo* effect of morphine on neutrophil phagocytosis and killing was evaluated in 16 male rhesus monkeys (Liu et al., 1992). All animals were initially given intramuscular injections of saline, with nine monkeys subsequently being administered morphine. The 8-hourly morphine dose for the 1st week was 1 mg/kg, followed by 3 mg/kg for the 2nd week and 5 mg/kg for the 3rd and 4th weeks. Blood was taken 2 hr after the first daily injection, neutrophils were isolated and their ability to phagocytose *Candida albicans* blastospores over a 45-min incubation was assessed using fluorescent microscopy. Phagocytosis was not affected by morphine (Liu et al., 1992).

Yeager and colleagues assessed the *in vivo* effects of short-term intravenous fentanyl (3 μg/kg bolus followed by 1.2 μg/kg/hr infusion over 2 hr) in seven healthy human volunteers (Yeager et al., 2002). Neutrophils were isolated from whole blood and their phagocytosis was assessed before fentanyl, at the end of the infusion, and 1 and 24 hr after completion of the infusion. The proportion of neutrophils phagocytosing Candida in 15 min was microscopically counted. Fentanyl did not affect neutrophil phagocytosis (Yeager et al., 2002).

Tubaro and colleagues studied neutrophil and monocyte activity in intravenous drug users on regular prescribed morphine (approximately 170 mg/day) or methadone (approximately 39 mg/day) treatment (Tubaro et al., 1985). Although not a “clean” study owing to non-compliance in the methadone group (as detected by drug screening), this likely reflects the clinical reality. Neutrophils and monocytes were isolated from peripheral blood using density centrifugation. Opsonised *Candida albicans* was used to assess phagocytosis, by microscopically counting the number of fungi phagocytosed. Intravenous drug users maintained on morphine exhibited a reduction in both neutrophil and monocyte phagocytic activity relative to controls. In contrast, phagocytosis was unaffected in those maintained on methadone (Tubaro et al., 1985).

In summary, the *in vivo* effect of opioids on phagocytosis has not been extensively studied. From the few previous *in vivo* studies, it seems that morphine suppresses phagocytosis more than methadone, that fentanyl might be immune-neutral in terms of phagocytosis and that tramadol might enhance phagocytosis. However, in view of differing methodological approaches, leading to sometimes conflicting results, comparative conclusions about the effect of opioids on phagocytosis cannot be made.

1.12.3 Oxidative burst reaction

After foreign materials, including bacteria and fungi, are phagocytosed into an intracellular phagosome, they are degraded or killed, in part by the oxidative burst reaction. This reaction involves the rapid release of reactive oxygen species (O_2^- and H_2O_2) and is critical in the control of bacterial and fungal infections. Limited studies have assessed the effect of morphine, fentanyl and methadone (and even compared morphine and methadone) on the oxidative burst reaction in animal and human models.

Tubaro and colleagues investigated the *in vivo* effects of morphine and methadone on murine neutrophils and macrophages, and rabbit alveolar macrophages (Tubaro et al., 1987). Male CD_1 mice and male New Zealand rabbits were administered twice daily morphine at 10 mg/kg on day 1, 20 mg/kg on day 2, 30 mg/kg on day 3, 40 mg/kg on day 4; or methadone at 2.5 mg/kg on day 1, 5 mg/kg on day 2, 7.5 mg/kg on day 3, 10 mg/kg on day 4 to 6 days. Neutrophil- and macrophage-rich peritoneal exudate cells were elicited and removed from the mice. Alveolar macrophages were obtained from the rabbit. O_2^- production was evaluated in Con A and Cytochalasin E stimulated neutrophils and PMA stimulated macrophages using a superoxide dismutase

inhibitable reduction of ferricytochrome *c*. Morphine inhibited the oxidative burst reaction of murine neutrophils and macrophages, and rabbit macrophages. Although methadone also inhibited the oxidative burst reaction in murine neutrophils and rabbit macrophages, this was less pronounced than morphine; methadone had no effect on murine macrophages (Tubaro et al., 1987).

Liu and colleagues evaluated the effect of morphine on neutrophil killing in 16 male rhesus monkeys (Liu et al., 1992). All animals were initially given intramuscular injections of saline, with nine animals subsequently being administered morphine. The 8-hourly morphine dose for the 1st week was 1 mg/kg, followed by 3 mg/kg for the 2nd week and 5 mg/kg for the 3rd and 4th weeks. Blood was taken 2 hr after the first daily injection, neutrophils were isolated and their ability to kill *Candida albicans* blastospores over a 45-min incubation was assessed using fluorescent microscopy. Morphine caused a transient reduction (between 3 and 5 weeks) in the ability of neutrophils to kill blastospores, which returned to baseline after this time (Liu et al., 1992).

Jacobs and colleagues assessed the *in vivo* effects of an intravenous bolus of 0.2 μg/kg fentanyl on cellular immune function in seven healthy male volunteers (Jacobs et al., 1999). Blood was taken before, and 15 and 30 min after the fentanyl. Neutrophils were isolated by sedimentation, and activated by a 20-min incubation with PMA, *E.coli* or a combination of TNF and N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP; a synthetic, chemotactic peptide which mimics the activity of bacterially-derived peptides). Flow cytometry was used to assess oxidative burst activity, as measured by the oxidation of dihydrorhodamine to fluorescent rhodamine. Fentanyl had no effect on the oxidative burst reaction of neutrophils (Jacobs et al., 1999).

Tubaro and colleagues evaluated neutrophil and monocyte activity in intravenous drug users taking regularly prescribed morphine (~170 mg/day) or methadone (~39 mg/day) (Tubaro et al., 1985). In the methadone group, drug screening revealed that other opioids were also taken, reflecting the clinical reality. Neutrophils and monocytes were isolated from peripheral blood using density centrifugation. O_2^- production was evaluated both in PMA stimulated neutrophils and monocytes using a superoxide dismutase inhibitable reduction of ferricytochrome *c*. *Candida albicans* viability was assessed by using methylene blue staining. Intravenous drug users taking morphine had a reduction in the ability of neutrophils and monocytes to kill *Candida albicans*. Methadone reduced the killing ability of neutrophils, to a lesser degree than morphine,

but increased the killing by monocytes. The authors speculate this latter effect may be due to other unspecified substances taken by this group. Superoxide anion production was decreased only in neutrophils by morphine and to a lesser extent by methadone (Tubaro et al., 1985).

In summary, the *in vivo* effect of opioids on the oxidative burst reaction has not been extensively studied. From the limited data available it might be that morphine and methadone suppress the ability of neutrophils and monocytes/macrophages to perform the oxidative burst reaction and that fentanyl might not affect this response.

1.12.4 T cell function

The T cell population comprises both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells, which help to coordinate the immune response and provide the adaptive arm of immune cytotoxicity, respectively. Several studies have assessed the effect of certain opioids on these adaptive immune cells in various *in vivo* conditions which are described below.

In two similar studies (in terms of cytotoxic T cell activity) by Carpenter and colleagues, female C3H/HeN mice were alloimmunised with C57BL/6J mouse splenocytes and treated with subcutaneous morphine (50 mg/kg) daily. A subgroup of mice also received the MOR alkylating agent β-funaltrexamine. After 11 days the mice were killed and splenic lymphocytes and peritoneal leukocytes were removed. $CD8^+$ cytotoxic T cell activity was assayed using a 4 hr assay with ^{51}Cr-labelled EL-4 cells. Chronic morphine exposure decreased splenic and peritoneal cytotoxic T cell activity. β-funaltrexamine reversed the morphine-induced suppression of $CD8^+$ cytotoxic T cell activity, indicating that this is a MOR mediated effect (Carpenter et al., 1995, Carpenter et al., 1994).

The effect of burns and morphine on splenic T cell proliferation has been investigated in C57BL/6 mice (Alexander et al., 2005). The mice were given a 6.25, 12.5 or 25% total body surface area burn or a sham procedure. The mice were either untreated or administered a 2 mg/kg/day subcutaneous morphine infusion. Splenocytes were isolated for *in vitro* analysis 1, 4, or 7 days later. The splenocytes were incubated with anti-CD3 for 48 hr and T cell proliferation was determined spectrophotometrically by measuring 5-bromo-2-deoxyuridine uptake by splenocytes. Neither the 6.25% burn nor morphine alone altered splenic T cell proliferation to anti-CD3. In contrast, morphine treatment of mice with a 6.25% burn suppressed splenic T cell proliferation at 4 and 7

days. Morphine treatment did not increase the suppressed T cell proliferative responses associated with larger burns of 12.5% and 25%. It is possible that the larger burns have maximal suppressive effects on T cells, which cannot be added to by morphine (Alexander et al., 2005).

Yeager and colleagues evaluated the *in vivo* effects of short-term intravenous fentanyl (3 μg/kg bolus followed by 1.2 μg/kg/hr infusion over 2 hr) in seven healthy volunteers (Yeager et al., 2002). Blood was taken before fentanyl administration and at 1 and 24 hr after completion of the infusion. PBMCs were isolated by density gradient centrifugation and kept in liquid N_2 until assayed. The proportions of leukocyte sub-populations (CD3, CD4, CD8, CD14, CD16, CD19, CD57, CD64 and HLA class I) was determined using flow cytometry of PBMCs. T cell proliferation in response to a 3-day incubation with Con A was assessed using a ^{3}H-thymidine uptake assay. The effect of fentanyl on antibody response to pneumococcal vaccination was determined using an ELISA. Fentanyl increased the percentage of $CD8^+$ T cells in peripheral blood (at the end of the infusion, which remained at 24 hr), but had no other effect on any of these immune parameters (Yeager et al., 2002).

Jacobs and colleagues assessed the *in vivo* effects of an intravenous bolus of 0.2 μg/kg fentanyl on cellular immune function in seven healthy male volunteers (Jacobs et al., 1999). Blood was taken before, and 15 and 30 min after the fentanyl. PBMCs were isolated by density gradient centrifugation and stained with mAbs to CD3, CD4 and CD19 and measured by flow cytometry. These lymphocyte subtypes were not affected by fentanyl (Jacobs et al., 1999).

Lymphocyte subset composition in current heroin (diamorphine) abusers, long-term (>10 yr) methadone maintained previous heroin abusers and healthy individuals have been compared (Novick et al., 1989). Lymphocyte subsets were measured using mAbs and flow cytometry in whole blood. Heroin abusers had higher absolute numbers of $CD2^+$, $CD3^+$, $CD4^+$ and $CD8^+$ cells compared with methadone patients and controls, which were similar (Novick et al., 1989). The current heroin abusers were undergoing inpatient detoxification, which might have induced withdrawal effects on the lymphocyte subsets rather than the diamorphine directly affecting the cells.

In a clinical study of 30 patients undergoing surgery for uterine carcinoma, the effect of morphine and tramadol on T cell function was compared in the post-operative period (Sacerdote et al., 2000). All patients had anaesthetic medications as well as fentanyl.

Patients were given an intramuscular injection of 10 mg morphine or 100 mg tramadol immediately post-operatively. Peripheral blood was taken before surgery, immediately post-operatively (before morphine and tramadol administration, but after fentanyl) and 2 hr post-surgery. PBMCs were isolated by density gradient centrifugation. PHA-induced T cell proliferation was assessed at these time points using a ^{3}H-thymidine uptake assay. Although anaesthesia/surgery suppressed T cell proliferation (measured immediately post-operatively, before the administration of tramadol or morphine), patients receiving morphine continued to exhibit suppressed T cell proliferation at 2 hr, whereas patients administered tramadol had pre-surgical responses at this time. There was no difference in the analgesic effects (Sacerdote et al., 2000).

In summary, there are a few studies assessing the *in vivo* effect of morphine, tramadol, fentanyl, methadone and diamorphine on T cell function, across a range of experimental conditions. Morphine has been shown to have suppressive effects on T cells and fentanyl might be neutral. There have not been enough previous *in vivo* studies into the effects of the other opioids on T cells to draw any definitive conclusions about their effects.

1.12.5 Cytokines

Cytokines are cell signalling molecules that are secreted from a variety of cells, including immune cells. They have several roles, including being involved in the regulation of humoral and cellular immune responses. As discussed in section 1.6, cytokines play a key role in the expression of opioid receptors on immune cells (Kraus, 2009). However, opioids also impact on cytokine production. Several studies have assessed the effect of opioids on cytokines. The *in vivo* studies are described below.

In a murine model of chronic morphine exposure and *Streptococcus pneumoniae* infection, $CB6F_1$ male mice were implanted with a 75 mg morphine or placebo pellet 24 hr before nasal *S. pneumoniae* inoculation (Wang et al., 2005b). Mice were killed 4, 24 and 48 hr after inoculation and TNF-α, IL-1β, and IL-6 were measured in lung lavages and tissue using ELISAs. Morphine reduced TNF-α, IL-1β, and IL-6 levels at 4 hr, but increased them at 24 and 48 hr. Mice administered morphine had a greater bacterial load and higher mortality (87% for morphine vs. 20% for placebo at 1 week). Morphine impaired the host immune response, altered cytokine production and increased susceptibility to *S. pneumoniae* infection (Wang et al., 2005b).

Female B6C3F1 mice were administered subcutaneous morphine for 5 days (20 mg/kg

twice a day on day 1, then on day 2, a 75 mg morphine pellet was implanted) before being killed (Bhargava et al., 1994). Splenocytes were removed and stimulated *in vitro* with anti-CD3 mAb for 2 days. Peritoneal macrophage were elicited with thioglycolate and stimulated by LPS for 2 days. Cytokine secretion was measured by spectroscopy. Morphine decreased splenic IL-2 and IL-4, but did not affect peritoneal macrophage TNF-α production (Bhargava et al., 1994).

Lysle and colleagues administered a single dose of subcutaneous morphine (0, 5, 10, 15 or 25 mg/kg) to male Lewis rats which were killed 1 hr later (Lysle et al., 1993). Blood, spleen and mesenteric lymph nodes were removed. Splenic and lymph node cells were incubated *in vitro* with Con A for 48 hr to activate T cells. IFN-γ was measured using an ELISA and IL-2 using a CTLL-2 proliferation assay. Morphine induced a dose-dependent suppression of IL-2 and IFN-γ production in splenic lymphocytes, whereas IL-2 and IFN-γ production by the mesenteric lymph node cells were not affected (Lysle et al., 1993).

The effect of a single subcutaneous injection of 20 mg/kg morphine and diamorphine on the production of IL-1β, IL-2, IL-10, TNF-α and IFN-γ by cultured splenocytes from C57/BL mice were assessed using ELISAs (Pacifici et al., 2000). Both opioids increased production of IL-1β, IL-2, TNF-α and IFN-γ at 20 and 40 min after administration. At 24 hr, production of these cytokines was inhibited, with only a partial recovery at 48 hr. Morphine and diamorphine had a stimulatory effect on the release of IL-10 at 24 hr, decreasing it by 48 hr (Pacifici et al., 2000).

Martucci and colleagues administered 20 mg/kg subcutaneous morphine to C57BL/6 mice, from which peritoneal macrophages and splenocytes were harvested 1 hr later (Martucci et al., 2007). Peritoneal macrophages were incubated with or without LPS and splenocytes with or without Con A. Production of IL-1β, IL-2, IL-4, IL-10, IL-12p70, TNF-α and IFN-γ were measured using ELISAs. Morphine reduced unstimulated and LPS stimulated macrophage production of the pro-inflammatory cytokines IL-1β, IL-12 and TNF-α, and the anti-inflammatory cytokine IL-10. Morphine decreased Con A stimulated splenocytes production of IL-1β, IL-2, IL-4, IL-10 and IFN-γ, and also unstimulated splenocyte production of IL-1β and IL-2 (Martucci et al., 2007).

The effect of burns and morphine on T_H cell cytokine production was assessed in C57BL/6 mice (Alexander et al., 2005). The mice were given a 6.25% total body

surface area burn or a sham procedure and were either untreated or treated with a 2 mg/kg/day subcutaneous morphine infusion. Plasma and splenocytes were isolated for *in vitro* analysis 1, 4 or 7 days later. The splenocytes were incubated with anti-CD3 for 48 hr and the stimulated T cell production of IL-2, IL-10 and IFN-γ were measured using ELISAs. There was an increase in IL-10 production to morphine, both in the sham and burnt mice, whereas morphine increased the IFN-γ levels in sham animals, but not the burnt mice. The relative increase in IL-10 to IFN-γ concentrations in burnt mice receiving morphine suggests a shift in the T cells towards a T_H2 type phenotype, based on cytokine production profiles (Alexander et al., 2005). The T_H2 cell phenotype could be immunosuppressive, as the cytokines produced by T_H2 cells promote humoral immunity and suppress the differentiation towards the T_H1 cell phenotype which activates the cellular immune response.

Sacerdote and colleagues compared the effects of buprenorphine and methadone maintenance treatment on cytokine levels in heroin addicts, using PHA stimulated PBMCs collected from these groups (Sacerdote et al., 2008). Current heroin addicts exhibited a reduced secretion of IL-4, IFN-γ and TNF-α, whereas the secretion of IL-2 was normal. All of these parameters were normal in methadone and buprenorphine groups, demonstrating that both buprenorphine and methadone maintenance preserves immune function (Sacerdote et al., 2008). This could be due to lifestyle changes, as well as differential modulatory effects of the opioids on immunity.

Blood taken from heroin (diamorphine) addicts and healthy volunteers, was stimulated *in vitro* with PHA or LPS and levels of IFN-γ and IL-10 were measured by ELISAs (Azarang et al., 2007). In heroin addicts, there was a decrease in IFN-γ and an increase in IL-10 production. This suggests that heroin addiction leads to a polarisation of $CD4^+$ cells towards the T_H2 response (Azarang et al., 2007).

The effects of morphine, tramadol and fentanyl have been compared in a post-operative study (Liu et al., 2006). For this, 150 patients were randomised to receive morphine, fentanyl or tramadol (all in combination with droperidol) via a patient controlled analgesia (PCA) device after upper abdominal surgery. IL-2 was measured pre-operatively and 1, 3 and 24 hr after surgey. The patients were all administered midazolam, propofol, fentanyl, vecuronium and either isoflurane or enflurane during surgery. NF-κB was measured using an Eletrophoretic Mobility Shift Assay, and IL-2 using an ELISA. Despite showing similar analgesia, morphine decreased IL-2 levels,

whereas tramadol and fentanyl increased it. The inhibitory effect of morphine on IL-2 production was related to its suppression of NF-κB. These findings indicate that morphine is likely to be immune suppressing, whereas fentanyl and tramadol might have an immune enhancing effect. (Liu et al., 2006).

In a randomised, placebo-controlled trial, the effect of tramadol on the production of pro-inflammatory and anti-inflammatory responses in 40 lung cancer patients undergoing pulmonary lobectomy was assessed (Wang et al., 2005a). Patients were administered intravenous tramadol (1.5 mg/kg bolus with a 0.5 mg/kg/hr infusion) or saline 5 min before surgery. All patients had fentanyl (2 μg/kg) as well as midazolam, propofol, vecuronium, diazepam, atropine and enflurane general anaesthesia intra-operatively. Epidural lidocaine, bupivacaine and fentanyl (5 μg/kg) were used for 3 days post-operatively. Blood was taken before surgery, 90 min after the start of surgery and at 4 and 24 hr after surgery. The blood was centrifuged, serum removed and stored at -80°C for measurement of IL-2, sIL-2R (soluble IL-2 receptors – a marker of immune activation), IL-6, and IL-10 using ELISAs. IL-2 was increased at 4 hr after surgery in patients receiving tramadol, but was reduced at 4 and 24 hr after surgery in the saline group. sIL-2R was increased at 4 and 24 hr after surgery in patients receiving saline, but only at 24 hr in the tramadol group. IL-6 and IL-10 were increased during and after surgery similarly in both groups. Tramadol did not alter the IL-6/IL-10 response, but enhanced IL-2 production and delayed the increase of sIL-2R (Wang et al., 2005a). This could be due to a direct effect of tramadol or due to a reduced requirement for enflurane as a consequence of tramadol's analgesic action given that inhaled anaesthetic drugs contribute to post-operative immunosuppression (Brand et al., 1997). Malignancy and the other medications might also influence cytokine release.

In summary, there have been many studies assessing the levels of some cytokines in response to several opioids. These studies have a varied methodology, with different species studied in different settings. In general, morphine has mixed effects on pro- and anti-inflammatory cytokines in murine models making it difficult to draw definitive conclusions about its overall effect. However, it did decrease IL-2 production following surgery and could potentially be clinically deleterious. The data on the other opioids are sparse, but buprenorphine appears to be immune neutral in terms of cytokine production and tramadol enhanced IL-2 levels and might even have beneficial clinical effects on the immune system. These clinical assumptions would need confirmation in long term patient studies.

1.13 Conclusion

The interactions of opioids with the immune system are highly complex and their differential immunological effects depend on their molecular structure, receptor binding profiles and non-opioid mediated effects (i.e. serotinergic re-uptake inhibition and TLR4 agonism). Numerous *in vivo* studies have already been conducted assessing the effect of some commonly used opioids on certain aspects of immune function.

The immunomodulatory effects of morphine, a MOR agonist, are the most comprehensively studied. Although the literature described above shows mixed results, due to differing methodologies, in general it tends to suppress NK cell cytotoxicity, phagocytosis, oxidative burst, T cell activation, and IL-1, IL-2, IL-4, IL-6, IL-12 and IFN-γ (Shirzad et al., 2009, Tubaro et al., 1987, Van Der Laan et al., 1996).

Tramadol binds with low affinity to the MOR and inhibits neuronal uptake of serotonin and noradrenaline. These actions convey the analgesic action of tramadol, and its serotonergic effect also appears to confer unique immunomodulatory properties on tramadol. In general the literature reports that tramadol increases NK cell cytotoxicity, phagocytosis, lymphocyte proliferation and IL-2 production (Gaspani et al., 2002, Sacerdote et al., 2000).

Fentanyl, a potent MOR agonist, has limited studies, showing mixed effects on NK cell cytotoxicity and $CD8^+$ cells, decreasing IL-2 and IFN-γ production and not influencing phagocytosis and oxidative burst (Beilin et al., 1996, Jacobs et al., 1999, Shavit et al., 2004, Yeager et al., 2002).

Buprenorphine, with its mixed opioid receptor binding profile, does not influence NK cell cytotoxicity and cytokine production (Martucci et al., 2004). This might be as it does not activate the SNS or the HPA axis, and does not affect the release of monoamines, corticosterone and corticosterone-binding globulin levels (D'Elia et al., 2003, Gomez-Flores and Weber, 2000).

Methadone is a MOR agonist which induces MOR internalisation and also has NMDA antagonistic properties. It has shown mixed effects on NK cell cytotoxicity, phagocytosis, oxidative burst and does not affect the T cell response (Tubaro et al., 1987, Van Der Laan et al., 1996).

Oxycodone, a MOR and KOR agonist, has not previously been studied.

Diamorphine, a pro-drug of morphine, reduces NK cell cytotoxicity in heroin abusers (Novick et al., 1989). It has mixed effects on cytokines, but tends to increase IL-1 and IL-10, and decrease IFN-γ levels (Azarang et al., 2007).

Codeine, a naturally occurring opiate alkaloid and pro-drug of morphine, has not been studied, although after *in vivo* conversion to morphine, it is likely to have the same effects as morphine.

There are many experimental differences between the studies and often the methods used for stimulating the different types of immune cells are not physiological and the overall effect of these opioids has to be taken in the context of the clinical situation and the most common reason for their prescription, pain (discussed further in section 4.8).

Methodological differences between the studies include:

- different animals and species
- male vs. female
- immune activation status
- peripheral whole blood vs. PBMCs vs. isolation of cells vs. splenic cells
- the method of cell removal and cell activation status
- incubation times with opioids at dissimilar concentrations
- the effect of opioid tolerance and withdrawal
- total cell numbers
- E:T cell ratios
- effector and target cells
- incubation times
- immune outcome measurement
- different mitogens at variable concentration and durations
- opsonisation

Unfortunately, not all studies mention all the relevant variables in their methodology making it even more difficult to compare and extrapolate their results.

To fully elucidate the effect of each of the opioids on the different immune cells and the consequence of this in disease necessitates a range of studies and methodologies ranging from cellular to clinical. *In vitro* studies enable very tight control over the cells assessed and potential intracellular pathways, but might tell little about the whole organism. Animal studies can use a specific strain of rodent which is known to have a certain response and several of these “identical” animals can be studied under various conditions. Although the use of healthy participants avoids the confounding effects of coexisting diseases, surgical trauma or pain, they might have a different immune

activation status. In the clinical setting in which opioids are administered, their interactions with other components of the human pain and inflammatory response need to be considered, it is also not possible to control all the variables which might affect their immunity (see section 4.8, Effect of pain on immune function).

Despite the wealth of studies for certain opioids, especially morphine, the potential impact of other opioids, like oxycodone and codeine have never been assessed on any immune parameter. Most other opioids have been evaluated to some extent on certain aspects of immune function, but there is not always consistency between these effects which might result from the wide ranging methodologies which have been used. More comprehensive and systematic studies assessing and comparing the effects of a range of opioids on many aspects of immune function are needed. Until there is definitive clinical data of these possible consequences, immunomodulation is another potential adverse effect of opioids that clinicians should take into consideration when deciding the “best” opioid for an individual patient.

1.14 Study Hypothesis, Aim and Objectives

Study Hypothesis: That morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine have differential effects on NK cell activation and cytotoxicity; neutrophil and monocyte phagocytosis and oxidative burst responses; T cell responsiveness; and cytokine production.

Study Aim: To evaluate the effects of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on multiple aspects of innate and adaptive immune function.

Study Objectives: To systematically compare the influence of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on:

1. NK cell cytotoxicity against K562 human erythroleukaemic target cells
2. NK cell activation (measured by CD69 expression) to anti-CD3/anti-CD28 mAbs and IL-2
3. Phagocytosis of *E.coli* by peripheral blood neutrophils and monocytes
4. Neutrophil and monocyte oxidative burst responses to *E.coli*, PMA (an activator of PKC) and fMLP (a chemotactic peptide)
5. $CD4^+$ and $CD8^+$ T cell activation to anti-CD3/anti-CD28 mAbs, as measured on the basis of CD25 and CD69 expression.
6. Cytokine production by unstimulated as well as IL-2 and anti-CD3/anti-CD28 mAb activated PBMCs

The analysis of NK cell cytotoxicity provides insight into the potential effects of these opioids on innate immune protection against tumours and viral infection.

The analysis of neutrophil and monocyte function provides insight into the potential effects of opioids on innate immune protection against bacterial and fungal infections.

The analysis of $CD4^+$ T cells and cytokine production provides insight into the potential effects of these opioids on the regulation of the immune system.

The analysis of $CD8^+$ T cells provides insight into the potential effects of these opioids on adaptive immune protection against tumours and viral infection.

CHAPTER 2

MATERIALS AND METHODS

2.1 Ethics

Ethical approval for this study was obtained from the University of Sheffield Medical School Research Ethics Committee (Reference Number: SMBRER102).

2.2 Blood samples

Peripheral blood was obtained from healthy volunteers by appropriately trained staff following written informed consent. Venepuncture was performed using a 21-Gauge needle and blood was collected into lithium heparin BD Vacutainers® (BD Biosciences, Oxford, UK). An aliquot of whole blood was used for the determination of neutrophil and monocyte phagocytosis and oxidative burst responses. PBMCs were isolated from the blood for the analysis of NK cell cytotoxicity, NK and T cell phenotype/activation status and cytokine studies.

2.3 Volunteers

To minimise the effect of interindividual variability both with the immune assays and in the response to opioids a small cohort of male and female volunteers were used throughout the study. One concern was that the interindividual variability could be so large that any effect of opioids on immune function would be hidden. Using the same volunteers for the range of immune assays also enables comparison of individuals across these tests. The fixed cohort of volunteers was healthy and not on any medications. The age range was 25 – 33 years old, with four males and three females. Gender-related differences in the proliferative ability of murine T and B cells *in vivo* have been reported (Gabrilovac and Marotti, 2000) and although this is also likely to be true in the volunteers, a mixed sample was more representative of the clinical situation.

All volunteers were of a similar age, as advancing age affects innate and adaptive immune function (immunosenescence). This primarily affects T cells, but can also influence NK cells (Bird, 2010, Desai et al., 2010). Although many patients receiving opioids are older, for this study a relatively young, healthy cohort was used in order to minimise the potential influence of immunosenescence on the variability of the measured parameters. Healthy volunteers had not undertaken any recent exercise

prior to blood sampling, as this could influence immune function by affecting both the endogenous opioid and the serotonergic systems (Mazzardo-Martins et al., 2010), as well as cytokine and TLR expression (Gleeson, 2007). Although moderate exercise is immune enhancing, strenuous exercise is suppressive (Gleeson, 2007, Shephard and Shek, 1999). Exercise also increases myokine (such as IL-6) production (Pedersen, 2011). None of the volunteers had recent vaccinations. They did not smoke, as this might negatively impact on immunity, especially innate immune function (Molloy, 2010).

We did not control for menstrual cycle in the female volunteers, although oestrogen levels might be important, especially with KOR agonists (Lawson et al., 2010). Although none of the volunteers had overt infection, subclinical infection, which is very prevalent and likely to lead to immune activation, was not excluded. Although none of the volunteers were fasting, as this can increase the production of endogenous opioids, including morphine (Molina et al., 1995), overall nutrition, which is vital for effective immune functioning (Chandra, 1999), was not assessed. Differing levels of stress, which tends to impair immune response (Ben-Eliyahu et al., 2007) by glucocorticoid (Elftman et al., 2010) and endogenous opioid release (Shavit et al., 1984) were not controlled for, either between the volunteers or for each volunteer at different time points. As there could be alterations in immune responses at different times of the day (Keller et al., 2009), all blood samples were all taken in the morning or early afternoon. However, there was no strict time schedule. Although all these factors are important and controlling for them will decrease variability and improve the reliability of the results, clinically there is likely to be differences in many of these factors.

2.4 Opioids

Morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine were obtained from the Pharmacy Department at the Royal Hallamshire Hospital, Sheffield. Each opioid was diluted to the appropriate concentration, as described in the following section, either with growth medium or phosphate buffered saline (PBS) prior to each experiment.

2.4.1 Relevance of opioid concentrations to immune cell activation

In order to ensure that the experimental regimen is as applicable to the clinical scenario as possible, the literature regarding plasma concentrations of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine in volunteer studies and in patients treated with opioids was reviewed.

The activation of opioid receptors and the triggering of downstream events by opioids and their potential non-opioid receptor mediated mechanism(s) of action are likely to depend on the concentration of opioid in the cellular microenvironment (Liu et al., 1992). Although plasma concentrations of opioids can be measured in patient and human volunteer groups, generally the dose of opioid prescribed is titrated to efficacy/toxicity, and only rarely are plasma concentrations measured. Even when plasma opioid concentrations are measured (usually in early clinical trials), their relationship to opioid concentrations present in the microenvironment of immune effector cells is unknown. Despite these limitations, it is important to consider the potential concentrations of opioid in the *in vivo* immune cell environment and use opioid concentrations that equate with those found in patients treated with exogenous opioids.

In vivo, pharmacologically-active, free opioid concentrations are lower because they are protein bound; for example, 80% of fentanyl is bound to plasma protein (Meuldermans et al., 1982) and is taken up by erythrocytes (Bower, 1982).

T cells normally reside in the lymph nodes until such time as APCs/DCs present specific antigen to them, at which time they become activated. The activated population clonally expands, initially in the lymph nodes, before emigrating into the circulation and migrating to the site of inflammation. Thus for T cells, the opioid concentration in the lymph node is potentially critical if it is to affect them during activation, especially if this is the point at which opioid receptors are transcribed and expressed by the T cell (Borner et al., 2008). In contrast, plasma and tissue opioid concentrations are relevant to circulating NK cells, neutrophils and monocytes. As it is difficult to precisely quantify the peri-T cell opioid concentration *in vivo* on the basis of currently available information, this *in vitro* study used concentrations of opioids which approximate to the *in vivo* plasma concentrations that are found in patients. It is accepted that interindividual variability of patient responses to opioid analgesics, including genetic and drug-drug interactions might result in wide variations in effective levels between individuals.

This interindividual variability is, in part, due to opioid receptor gene polymorphisms (Hoehe et al., 2000) and splice variants of opioid receptors (Pan, 2005, Pan et al., 2001). As more than half of the opioid receptor gene polymorphisms are within the promoter region, their primary influence is on gene transcription (Kraus et al., 2003). This is of prime importance as the potential effect of the opioid on immune cells will not

only depend on its local concentration, but also on the level of expression of appropriate receptors on the cells and the affinity at which the opioid binds to each of these receptors.

Tumour vascularisation will influence the numbers of NK and T cells able to penetrate into the tumour as well as intratumoural opioid levels, further impacting on their immunoregulatory potential. As there is poor circulation and perfusion in the tumour microenvironment there are likely to be low opioid levels here. Although the literature is mixed, recent studies suggest that opioids can inhibit angiogenesis (Afsharimani et al., 2011, Koodie et al., 2010, Martin et al., 2010) and might have a tumour-promoting effect by phosphorylating the epidermal growth factor receptor (Fujioka et al., 2011), making the opioid-tumour interaction even more complex in the clinical setting.

2.4.2 Clinically relevant plasma concentrations of opioids

Plasma concentrations of opioids in clinical practice, as reported from published trials, were reviewed. Below are summaries of the data found clinically, which were used to determine the opioid concentrations to be used in this study. These concentrations were then compared to previous immunological *in vitro* studies. This enabled the concentrations used in the study to approximate those that would be found in patients.

2.4.2.1 Morphine

In a post-operative trial of four patients using a PCA device, plasma morphine levels in venous blood samples were measured at 3, 6, 12, 18, 24 and 36 hr post operation. The median total morphine dose delivered in the 36 hr was 19.5 mg. Median plasma concentrations of morphine are shown in Table 2.1 (Santos et al., 2008).

Table 2.1: Median morphine plasma concentration over time in four patients using PCA.

Hours post operation	3	6	12	18	24	36
Median morphine plasma level (ng/ml)	13.1	18.9	53.0	28.9	13.9	15.4

A randomised controlled trial of 14 patients with cancer compared a 5 mg bolus of either intraosseous or intravenous morphine, the peak plasma morphine concentration (± SD) was 235 (± 107) and 289 (± 197) ng/ml respectively (Von Hoff et al., 2008).

In a study of 50 post-operative patients on morphine, with 225 concentration-time data points, the measured concentrations of morphine varied widely, although these peaked in the region of 200 ng/ml (Mazoit et al., 2007).

In a double-blind randomised controlled trial for the treatment of cancer pain, controlled-release morphine was compared with controlled-release oxycodone. Mean plasma concentrations (± SE) were measured at 0 (trough) and 3 hr after last dose of morphine, and were 21.3 (± 2.8) and 57.0 (± 9.7) ng/ml respectively (Mucci-LoRusso et al., 1998).

Opioid plasma concentrations were measured in nine patients who were being converted from morphine to methadone. The maximum morphine concentration was 51.8 ng/ml, with a range of 17 – 86 ng/ml (Mercadante et al., 2003).

These clinically relevant *in vivo* plasma concentrations were evaluated in the context of those used in previous *in vitro* immunological studies:

- 20, 40 and 80 ng/ml (Beilin et al., 2005)
- 3 pg/ml – 32 μg/ml (0.003 – 32,000 ng/ml) (Fuggetta et al., 2005)
- 10 – 1,000 nM (2.85 – 285.4 ng/ml) (Greeneltch et al., 2005)
- 10^{-8} M; (3 ng/ml) (Wang et al., 2006)
- 100 and 500 nM (28 and 143 ng/ml) (Lazaro et al., 2000)
- 0 – 10^{-5} M (0 – 2,853 ng/ml) (Delgado-Velez et al., 2008)
- 10^{-10} – 10^{-6} M (0.03 – 285 ng/ml) (Szabo et al., 1993)
- 38 mg/ml (3.8 x10^{7} ng/ml) (Liu et al., 2006)
- 2,000 and 200,000 ng/ml (Rao et al., 2004)

2.4.2.2 Tramadol

In a volunteer study of tramadol pharmacokinetics in relation to CYP2D6 phenotype, five subjects who were poor metabolisers and 19 subjects who were extensive metabolisers were given a single oral 100 mg dose of racemic tramadol; the plasma concentrations are shown in Table 2.2 (Garcia-Quetglas et al., 2007).

Table 2.2: Maximal tramadol concentrations in volunteers with differing CYP2D6 phenotypes

CYP2D6 phenotype	Tramadol C_{max} (mg/l)	SD
Extensive metabolisers	1.136	0.048
Poor metabolisers	1.145	0.086

In a post-operative study, 3 mg/kg of intravenous tramadol was administered to 174 patients. This resulted in plasma tramadol levels ranging between 55 and 1,100 ng/ml (Stamer et al., 2007).

In a randomised trial of 98 patients using racemic tramadol PCA after gynaecological surgery, serum tramadol levels of 590 ± 410 ng/ml were detected (Grond et al., 1999).

The reported serum concentrations in 40 patients using a tramadol PCA for post-operative analgesia (average dose administered in 4 hr was 102.8 mg) after major surgery, has been reported to be between 20.2 and 986.3 ng/ml, with a median concentration of 287.7 ng/ml (Lehmann et al., 1990).

After a single intravenous dose of 100 mg tramadol in 30 otherwise healthy patients undergoing elective orthopaedic surgery, the serum concentration of tramadol was in the region of 1 µg/ml (Gan et al., 2002).

These clinically relevant *in vivo* plasma concentrations were evaluated in the context of those used in previous *in vitro* immunological studies:

- 5, 10 and 20 µg/ml (5,000, 10,000 and 20,000 ng/ml) (Beilin et al., 2005)
- 7.5 mg/ml (7.5 x10^6 ng/ml) (Liu et al., 2006)

2.4.2.3 Fentanyl

In an immunological *in vivo* study in seven healthy volunteers, serum fentanyl levels were analysed 15 and 30 min after the intravenous administration of 0.2 µg/kg fentanyl. After 15 min the mean fentanyl concentration (± SD) was 1.91 ng/ml (± 1.17) and 30 min after injection it was 0.67 ng/ml (± 0.23) (Jacobs et al., 1999).

The pharmacokinetic properties of effervescent fentanyl buccal tablets have been evaluated in a phase I, open-label, crossover study of single-doses of 100, 200, 400 and 800 µg in adult volunteers. The plasma fentanyl concentrations remained reasonably stable for around 4 hr following administration and are presented in Table 2.3 (Darwish et al., 2006).

Table 2.3: Maximum plasma fentanyl concentrations in volunteers administered buccal fentanyl.

Fentanyl tablets	C_{max} (ng/ml)	SD
100 µg	0.25	0.14
200 µg	0.40	0.18
400 µg	0.97	0.53
800 µg	1.59	0.90

Plasma opioid concentrations have been measured at 5-hr intervals during the first 25 hr during conversion from transdermal fentanyl to methadone in 18 patients receiving

palliative care. The plasma concentrations are in Table 2.4 (Mercadante et al., 2007).

Table 2.4: The plasma concentration of fentanyl after cessation of therapy.

Time after stopping fentanyl (hours)	Fentanyl concentration median (ng/ml)	Fentanyl concentration range (ng/ml)
5	15	9 – 21
10	11	7 – 16
15	10	7 – 14
20	10	8 – 13
25	8	5.7 – 10.8

Serum fentanyl concentrations in a randomised controlled trial of 10 patients undergoing cardiac surgery were in the region of 5.8 ng/ml (Thomson et al., 2000).

Plasma fentanyl concentrations in a perioperative study of elderly and middle-aged patients have been measured**.** Fentanyl was given as a bolus (7.5 μg/kg) then as a continuous intravenous infusion (0.1 μg/kg/min) during cardiac surgery to two groups of patients, those over 75 and those under 60 years old. The plasma concentrations of fentanyl in an arterial blood sample at the point of stopping the infusion was 6 ng/ml in the over 75 group and 2 ng/ml in the under 60 group. Two hours after the cessation of the infusion, the fentanyl concentration was 4 and 2 ng/ml in the over 75 and under 60 group respectively (Pesonen et al., 2009).

These clinically relevant *in vivo* plasma concentrations were also evaluated in the context of those used in previous *in vitro* immunological studies:

- 5, 10 and 20 ng/ml (Schneemilch et al., 2005)
- 0.01, 0.1, 1, 10, 100 μM (3, 30, 300, 3,000, 30,000 ng/ml) (House et al., 1995)
- 4.16 x10^{-8} M (14.0 ng/ml) (Larsen et al., 1998)
- 5 and 50 ng/ml (Jaeger et al., 1998)
- 20 and 40 μM (5,700 and 11,400 ng/ml) (Jacobs et al., 1999)
- 20 ng/ml (Liu et al., 2006)
- 2, 20 and 200 ng/ml (Wu et al., 2009)
- 20 and 2,000 ng/ml (Rao et al., 2004)

2.4.2.4 Buprenorphine

A single intravenous dose of 0.002 mg/kg of buprenorphine was administered to 12 healthy male volunteers in a randomised, double-blind, placebo-controlled, crossover trial. Blood samples were collected at 0.5, 1, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, and 8 hr.

Plasma buprenorphine concentration peaked at 10 ng/ml at 30 min, and was 1 ng/ml after 1 hr (Escher et al., 2007).

In a randomised clinical trial of 57 opioid-dependent volunteers on opioid maintenance therapy, plasma concentrations with sublingual liquid or tablet formulations of buprenorphine have been compared. Subjects received between 8 mg liquid sublingual liquid and 32 mg tablets sublingual. Peak plasma buprenorphine concentrations were dose- and formulation-dependent, but were between 3.4 and 6.2 ng/ml (Chawarski et al. 2005).

In a healthy volunteer study, either 1 mg intravenous or 8 mg sublingual buprenorphine were administered. The maximum plasma buprenorphine concentration was in the region of 10 ng/ml (Everhart et al., 1997).

Watson and colleagues compared single-dose 0.3 and 0.6 mg intravenous buprenorphine given to 19 patients post-operatively and showed a peak plasma buprenorphine concentration at just over 10 ng/ml (Watson et al. 1982).

There are no previous *in vitro* immunological studies with which these clinically relevant *in vivo* plasma concentrations can be compared.

2.4.2.5 Methadone

Plasma opioid concentrations have been measured at 5-hr intervals during the first 25 hr during conversion from transdermal fentanyl to methadone in 18 patients receiving palliative care. The results are presented in Table 2.5 (Mercadante et al., 2007).

Table 2.5: Plasma methadone concentration after commencement of therapy.

Time after starting methadone (hours)	Methadone concentration median (ng/ml)	Methadone concentration range (ng/ml)
5	2	0–5
10	119	70–169
15	140	78–202
20	169	113–223
25	245	162–327

The maximum methadone concentration in a study of nine patients which were being converted from morphine to methadone was reported to be 172 ng/ml; with a range of 46 – 298 ng/ml (Mercadante et al., 2003).

In a study of patients on methadone maintenance therapy, their peak plasma methadone concentration was in the region of 450 ng/ml (Dyer et al., 2001).

These clinically relevant *in vivo* plasma concentrations were evaluated in the context of those used in previous *in vitro* immunological studies:

- $0 – 10^{-5}$ M (0 – 3,094 ng/ml) (Delgado-Velez et al., 2008)
- $10^{-18} – 10^{-6}$ M ($3x10^{-10}$ – 309 ng/ml) (Molitor et al., 1992)
- 0.0001 – 100μM (0.03 – 30,940 ng/ml) (Thomas et al., 1995b)

2.4.2.6 Oxycodone

Oxycodone was used for post-operative pain treatment at a dose of 0.05 mg/kg intravenously. This dose was used three times and oxycodone concentration was checked at 0, 15 and 45 min after each dose. Levels were 5 – 15 ng/ml at time 0 (trough), 20 – 30 ng/ml at 15 min and 10 – 20 ng/ml at 45 min post dose (Pesonen et al., 2009).

In a randomised, double-blind study for the treatment of cancer-associated pain, the plasma concentrations of controlled-release oxycodone were measured at point 0 (trough) and 3 hr after last dose of oxycodone. The dose range of oxycodone was 40-400 mg/day. The mean plasma concentrations (± SE) were 33.3 (4.2) and 58.5 (6.9) ng/ml at 0 and 3 hr respectively (Mucci-LoRusso et al., 1998).

In a cross-sectional study of over 400 patients with cancer pain taking oral oxycodone (median daily dose 60 [range 10 – 760] mg), the mean serum oxycodone concentration was 161 nM (≈ 51 ng/ml), with a 95% confidence interval of 139 – 183 nM (≈ 44 – 58 ng/ml) (Andreassen et al., 2011).

There are no previous *in vitro* immunological studies with which these clinically relevant *in vivo* plasma concentrations can be compared.

2.4.2.7 Diamorphine

As diamorphine is rapidly hydrolysed by sequential deacetylation after administration *in vivo*, few studies have measured systemic diamorphine concentrations (Rook et al., 2006). Those that have showed that diamorphine is only present in the systemic circulation for minutes. It is considered a pro-drug of morphine, with many of its pharmacological effects coming from this metabolite (Cone et al., 1993, Inturrisi et al., 1984). As diamorphine is converted by rapid deacetylation to morphine and is reported

to be twice as potent as morphine (Robinson et al., 1991), diamorphine concentrations that were half those of morphine were used in this study.

Some studies have assessed morphine concentration after diamorphine administration (Table 2.6). In a paediatric study, 0.1 mg/kg of diamorphine was administered by both the intravenous (n=12) and intranasal routes (n=12) and the morphine concentration over time was measured using a radioimmunoassay. The median (and range) maximum plasma concentration (C_{max}) for morphine was 36.1 (13.7 – 82.7) μM (≈ 10,260 [3,905 – 23,570] ng/ml) for the intranasal group and 108.6 (76.5 – 480.1) μM (≈ 30,951 [21,803 – 136,829] ng/ml) for the intravenous group (Kidd et al., 2009).

Table 2.6: Plasma morphine concentrations following diamorphine administration.

Diamorphine dose (mg)	Route	N	C_{max} (μM)	Reference
6-12	intranasal/ intramuscular	6	60 – 66	(Cone et al., 1993)
4-52	Intramuscular/ intravenous/ oral	11	53 – 490	(Inturrisi et al., 1984)
40	Intranasal/ intravenous	4	701 – 1,157	(Mitchell et al., 2006)

These clinically relevant *in vivo* plasma concentrations were evaluated in the context of that used in a previous *in vitro* immunological study:

- 0.0001 – 100μM (0.0369 – 36,940 ng/ml) (Thomas et al., 1995b)

2.4.2.8 Codeine

Single doses of oral codeine (60 or 120 mg) administered to 19 volunteers resulted in a mean (± SE) peak codeine plasma concentration of 214.2 ± 27.6 ng/ml for the 60 mg dose and 474.3 ± 77.0 ng/ml for the 120 mg dose (Kim et al., 2002).

In a study of 10 volunteers, plasma concentrations of codeine and morphine were measured after a single oral dose of 60 mg codeine. This resulted in a mean (± SD) peak codeine plasma concentration of 88.1 ng/ml (± 25.1) (Shah and Mason, 1990).

In a healthy volunteer study, a single dose of 60 mg oral codeine has been shown to result in a mean peak codeine plasma concentration of 159 ng/ml (Findlay et al., 1978).

These clinically relevant *in vivo* plasma concentrations were also evaluated in the context of that used in a previous *in vitro* immunological study:

- 0.1 – 5 μg/ml (100 – 5,000 ng/ml) (Sheen et al., 2007)

2.4.3 Summary

The findings presented above (and summarised in Table 2.7) defined the concentrations of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine which were used in the *in vitro* studies described herein (Table 2.8). Although the concentrations used in the current study were centred on those which have been reported/detected in volunteer and clinical studies, five-fold higher and lower concentrations were also used in order to explore dose-dependent effects. Five-fold dilutions were used to enable a wide range of concentrations to be assessed, whilst still being able to potentially detect differences around the clinically relevant range.

Table 2.7: Summary of opioid concentrations in previous studies.

Opioid	Plasma concentrations	C_{max}
Morphine	13 – 53, 21 – 57, 289 ng/ml	289 ng/ml
Tramadol	288, 1,145ng/ml	1,145 ng/ml
Fentanyl	2 – 6, 8 – 15 ng/ml	15 ng/ml
Buprenorphine	3, 6, 10 ng/ml	10 ng/ml
Methadone	2 – 245 ng/ml	245 ng/ml
Oxycodone	5 – 20, 33 – 59, 44 – 58 ng/ml	59 ng/ml
Diamorphine	Rapidly hydrolysed to morphine	n/a
Codeine	88, 159, 214 ng/ml	214 ng/ml

Table 2.8: Opioid concentrations used in this study.

Opioid	Stock concentrations	Final concentrations used in this study (ng/ml)			
Morphine	10 mg/ml	20	100	500	2,500
Tramadol	50 mg/ml	80	400	2,000	10,000
Fentanyl	50 μg/ml	0.8	4	20	100
Buprenorphine	300 μg/ml	0.8	4	20	100
Methadone	10 mg/ml	20	100	500	2,500
Oxycodone	10 mg/ml	4	20	100	500
Diamorphine	5 mg/ml	10	50	250	1,250
Codeine	60 mg/ml	16	80	400	2,000

The concentrations used in this study relate to those that are found clinically, with the maximal clinical concentration falling in the middle of the concentrations used, with 5x dilutions above and below the clinical maximum plasma concentration. In the NK cell cytotoxicity assay, the central two concentrations were used. In the phagocytosis and oxidative burst assay the maximum concentration was used in addition to this. In the T cell and cytokine assays all concentrations were used (with the addition of a supra-maximal concentration, approximately 10x that of the top concentration, shown in the T cell section). The diamorphine concentration was extrapolated from the morphine plasma concentration as it is approximately twice as potent.

It is interesting to note that the relative concentrations detected in clinical studies and used in this study, do not necessarily relate to the relative potency of the opioids used clinically (which are used when converting patients between the opioids). This is because the oral dose does not directly correlate to the plasma concentration, due to first pass metabolism, protein binding, excretion and *in vivo* enzymatic activity. There are also differences in whether the opioids are administered as a single dose or are in steady state, and due to differing half-lives, which is variable between the opioids.

2.5 Influence of opioids on natural killer cell cytotoxicity

NK cells are critical in the control of cancer and viral infections and this study examined the influence of opioids on their cytotoxic capacity and responsiveness to activation. The NK cell cytotoxicity assay assesses the ability of NK cells (effector cells within the PBMC population) at different effector:target (E:T) ratios to kill target cells. The target cell used in these studies is the K562 human erythroleukaemic cell line, which is highly-sensitive to NK cell killing, but resistant to killing by $CD8^+$ cytotoxic T cells as it is MHC class I negative. The killing of K562 cells by unstimulated and IL-2 stimulated PBMCs following a 3-hr incubation is determined using a multicolour flow cytometry assay.

For this, K562 cells are labelled with MitoTracker™ Green (MTG; Invitrogen Ltd., Paisley, UK), a fluorescent mitochondrial dye, prior to inclusion in the assay, and at the end of the assay cells are incubated with propidium iodide (PI; Sigma-Aldrich, Gillingham, UK) a fluorescent DNA stain which only labels non-viable cells. The proportion of killed K562 target cells (MTG^+ and PI^+) is then determined by flow cytometry (Hopkinson et al., 2007). The effector cell population used in these assays are PBMCs, of which 10 – 15% are $CD16^+/56^+$ NK cells.

2.5.1 Isolation of peripheral blood mononuclear cells

This study used PBMCs as the effector cell population. PBMCs were isolated from whole blood using a density gradient method. For this, whole blood was collected from healthy volunteers and diluted 1:1 with PBS at room temperature. Diluted blood (10 ml) was carefully layered onto 10 ml Nycoprep 1.077 (Axis-Shield POCAS, Oslo, Norway) density gradient separation medium. Samples were centrifuged at 300 xg for 30 min at 4°C with the brake off (so as not to disrupt the layers generated during the centrifugation). Aggregated erythrocytes and the more dense granulocytes sediment to leave a layer of PBMCs and platelets at the interphase (Figure 2-1). The interface was removed using a Pasteur pipette. As platelets are sensitive to activation and can be

activated by the Vacutainer® blood collection system and anticoagulant (Mody et al., 1999), and as platelet-derived secretory products can inhibit NK cell cytotoxicity (Skov Madsen et al., 1986) and cause a variable response in this assay (Hopkinson et al., 2007), platelets were removed from the PBMC preparation using a second density gradient separation step. For this, 10 ml of Nycoprep 1.068 (made by diluting 4 parts of Nycoprep 1.077 with 1 part PBS) was layered under 10 ml of the interface using a needle and syringe. Samples were centrifuged at 400 xg for 15 min at 4°C (break off). The supernatant containing the platelets was removed and the pellet containing the PBMCs was re-suspended and washed twice in PBS (300 xg, 5 min, 4°C).

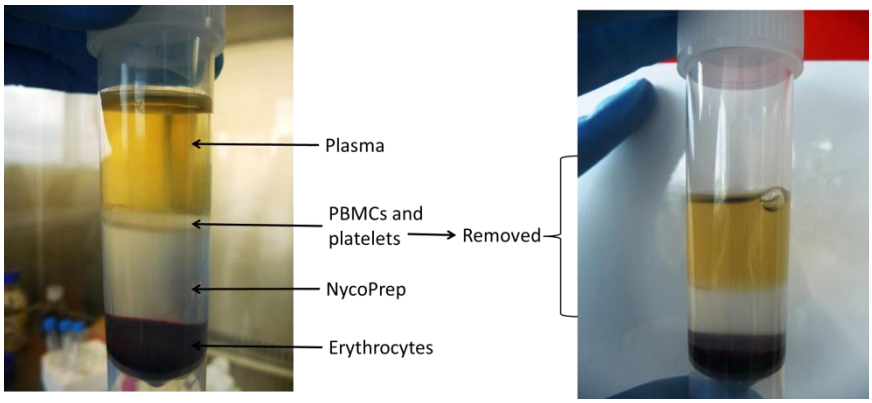

Figure 2-1: Isolation of PBMCs from whole blood.
Illustration of the different layers after centrifugation using a density separation media (**left**), and after the removal of the PBMC interface layer (**right**). Platelets are found throughout the different layers after density gradient centrifugation with NycoPrep 1.077. As they are also present in the PBMC layer, they are removed by a second density gradient centrifugation step.

PBMCs were re-suspended in 1 ml of Roswell Park Memorial Institute (RPMI) 1640 growth medium containing 10% v/v fetal bovine serum (FBS; Invitrogen Ltd., Paisley, UK), 100 μg/ml streptomycin and 100 U/ml penicillin (termed RPMI*), counted using a Neubauer haemocytometer and re-suspended at $5x10^6$ viable cells/ml in RPMI*.

PBMCs ($2x10^5$ cells/well) were incubated with media alone (unstimulated) or stimulated with a final concentration of 100 U/ml (50 ng/ml) recombinant human IL-2 (Miltenyi Biotec) in the presence or absence of different opioid concentrations (Table 2.9) for 3 days (37°C, 5% v/v CO_2, 100% humidity) in 96-well microtitre plates (final volume 250 μl). The cytotoxic potential of these cells and their activation status was then determined.

PBMCs were harvested and the contents of replicate wells were pooled. Cells were then centrifuged at 300 xg, for 5 min at 4°C and washed in order to remove the opioids,

as these might directly affect the K562 cells (Sergeeva et al., 1993) and have been shown to induce apoptosis at clinical concentrations (10^{-8} M of morphine; equivalent to 3 ng/ml) (Hatsukari et al., 2007). PBMCs were re-suspended at $5x10^6$/ml in RPMI*.

Table 2.9: Opioid concentrations used for the NK cell assays.

Opioid	Final opioid concentrations used for the NK cell assays (ng/ml)	
Morphine	100	500
Tramadol	400	2,000
Fentanyl	4	20
Buprenorphine	4	20
Methadone	100	500
Oxycodone	20	100
Diamorphine	50	250
Codeine	80	400

2.5.2 K562 cells

The target cell for the NK cell cytotoxicity assay is the highly NK cell sensitive human erythroleukaemic K562 cell line, purchased from the Health Protection Agency Culture Collection (formally European Cell and Culture Collection, Porton Down, UK). K562 stocks were stored in liquid N_2 in 90% FBS and 10% dimethyl sulfoxide (DMSO). Once thawed, cells were incubated at 37°C, 5% v/v CO_2 in RPMI* growth medium containing 2 mM L-glutamine (Invitrogen Ltd., Paisley, UK) and passaged (1:43 dilution) twice weekly. K562 cells were fluorescently labelled with MTG on the day of each experiment. For this, $8x10^6$ K562 cells were incubated with MTG (final concentration 75 nM) in 2 ml RPMI growth medium at 37°C for 20 min (Hopkinson et al., 2007). Cells were then centrifuged at 500 xg for 5 min at 4°C, washed with 5 ml of growth medium three times before being re-suspended in 1 ml of growth media and then counted using a haemocytometer. Cells were finally re-suspended at $1x10^6$ cells/ml.

2.5.3 Natural killer cell cytotoxicity assay

For the cytotoxicity assay, 25 μl of MTG-labelled K562 target cells ($1x10^6$ cells/ml) were added to 500, 250, 125 or 62.5 μl of the PBMC preparation ($5x10^6$ cells/ml) in 12x75 polycarbonate tubes, to give E:T ratios of 100:1, 50:1, 25:1 and 12.5:1 respectively. The incubation volume was made up to 525 μl with RPMI*. Tubes were vortexed then centrifuged at 1,000 xg for 3 min at 4°C. Samples were then incubated at 37°C for 3 hr and then kept on ice in the dark until analysis by flow cytometry. PI was added to the samples 10 min before flow cytometric analysis. The proportions of viable (MTG^+PI^-) and non-viable (MTG^+PI^+) K562 target cells was determined using a BD™ LSRII flow

cytometer (Hopkinson et al., 2007) (Figure 2-2). Unstained and MTG-labelled K562 cells that had not been incubated with PBMCs were used as controls for setting up the negative fluorescence and compensation values respectively. After the addition of PI, the MTG-labelled K562 cells were used as a measure of background death.

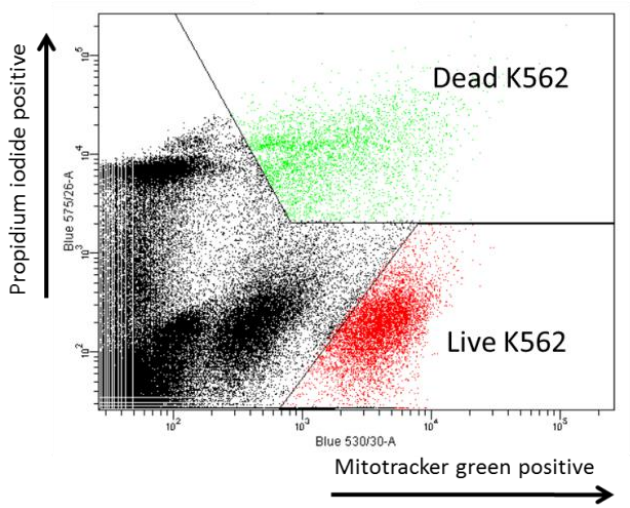

Figure 2-2: Representative dot plot for the NK cell cytotoxicity assay.
This shows MitoTracker Green vs. Propidium Iodide fluorescence and indicates the live (lower right, red) and dead (upper right, green) MTG^+ K562 target cell populations. The proportion of dead target cells, which reflects NK cell cytotoxicity, is calculated from these two populations.

Traditionally NK cell cytotoxicity has been measured using ^{51}Cr labelled target cells, with the level of ^{51}Cr released into the assay supernatant determined by a γ counter. Flow cytometry, which accurately determines the number of live/dead target cells has been demonstrated to correlate with the ^{51}Cr release assay (Kane et al., 1996, Shi et al., 1987).

NK cells can be influenced by a number of factors, including immunological and endocrinological, and there is potential inter- and intra-individual variability for this assay over time (Hopkinson et al., 2007, Jacobs et al., 1999, Motzer et al., 2003). Using the same methodology as the current study, baseline NK cell cytotoxicity between individuals (n=5) varied between 15 and 60% (at the 100:1 E:T ratio) and interassay variability over a 3 week period were up to 32% (Hopkinson et al., 2007). In another study, interassay variabilities for NK cell cytotoxicity were up to 46% (Motzer et al., 2003). These effects were minimised in the current study by using a small cohort of healthy volunteers and by removing the platelet-mediated effects.

2.5.3.1 Calculation of natural killer cell cytotoxicity

For each E:T cell ratio, NK cell cytotoxicity was represented by the proportion of K562 cells which were dead (number of MTG^+PI^+ K562 cells / total number of MTG^+ labelled K562 cells x 100) (Figure 2-2). This value was then corrected for background target cell death (i.e. the proportion of dead cells in the control K562 sample, without any NK cells). In this way, only actual NK cell killing is evaluated.

NK cell cytotoxicity is typically expressed as the degree of cytotoxicity (proportion of dead target cells) at the different E:T ratios (usually 100:1, 50:1, 25:1 and 12.5:1), or at a single E:T ratio. NK cell cytotoxicity has also been expressed as area under the cytotoxic curve (AUCC), a composite calculation of cytotoxicity which incorporates all four E:T ratios (Sheeran et al., 1988) as this approach increases the reproducibility and reduces the variability of the assay (Hopkinson et al., 2007). Sheeran first calculated the AUCC using Simpson's rule, in which the cytotoxicities at the E:T cell ratios of 12.5:1, 25:1, 50:1 and 100:1 are expressed as A, B, C and D respectively, Figure 2-3 (Sheeran et al., 1988). The following formula is then used to calculate the AUCC:

$$AUCC = [(12.5xA)+(12.5xB)+(25xC)+(50xD)+(12.5xA)+(25xB)+(50xC)]/2$$

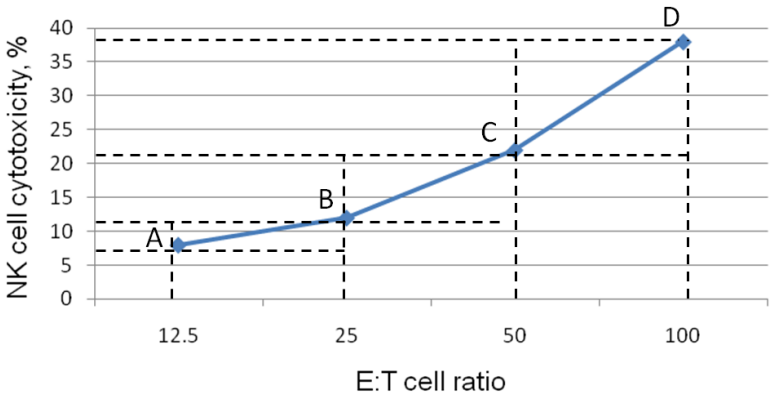

Figure 2-3: Calculating the area under the NK cell cytotoxicity curve (AUCC).
Using Simpson's rule (Sheeran et al., 1988), AUCC was calculated using AUCC = [(12.5xA)+(12.5xB)+(25xC)+(50xD)+(12.5xA)+(25xB)+(50xC)]/2. In which A, B, C and D are the measured cytotoxicities at effector:target (E:T) cell ratios of 12.5:1, 25:1, 50:1, and 100:1, respectively. Modified from Hopkinson et al., 2007.

In the current study, NK cell cytotoxicity was determined using PBMC populations, not purified NK cells. The proportion of NK ($CD16^+/56^+$) cells in this population was therefore determined by flow cytometry (see next section) and the value for the AUCC was adjusted for its NK cell content (to correct for individual variability in the prevalence of NK cells) by dividing the AUCC by the proportion of cells in the PBMC preparation which are $CD16^+/56^+$, expressed as decimal:

Corrected AUCC = AUCC(1/Z)

Where Z is the proportion of cells in the PBMC preparation which are $CD16^+/56^+$, expressed as decimal (i.e. for a sample containing 11% $CD16^+/56^+$ cells, Z=0.11)

The uncorrected AUCC provides a measure of the overall cytotoxic potential of the samples, whereas the corrected AUCC provides insight into differences in NK cell cytotoxic potential which are not related to changes in the proportion of NK cells in the sample under investigation. Both parameters have been calculated in the current study.

2.5.4 Effect of opioids on the activation status of lymphocyte subpopulations

In addition to assessing the influence of opioids on the cytotoxic potential of NK cell cytotoxicity, the influence of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on unstimulated and IL-2 induced expression of the activation markers CD25 and CD69 by $CD4^-CD8^-CD16^+CD56^+$ viable NK cell populations was determined by multiparameter flow cytometry. By labelling the PBMCs with mAbs to six CD antigens (Table 2.10), identification of specific cells and the activation status of those cells can be determined.

Table 2.10: The CD antigens analysed, their expression and function.

Target antigen	Immune cell expression	Functions
CD4	T cell subsets: T_H1, T_H2 and T_{reg} cells (two thirds of peripheral T cells). Low expression on monocytes and macrophages	MHC class II co-receptor. Binds Lck on cytoplasmic side of membrane
CD8	T cell subsets: cytotoxic T cells (one third of peripheral T cells)	MHC class I co-receptor. Binds Lck on cytoplasmic side of membrane.
CD16/56	NK cells	CD16 is a low affinity FcγRIII receptor CD56 (also known as N-CAM) mediates cell-cell contact and binding
CD25	Expressed on activated T cells, B cells, NK cells and monocytes	Alpha chain of the IL-2 receptor. Activation antigen
CD69	Expressed on activated T cells, B cells, NK cells and macrophages	Early activation antigen. Functional role in immune regulation. Contributes to lymphocyte retention in secondary lymphoid tissue

The monoclonal antibodies to target antigens, used to identify lymphocyte subtypes and their activation status by flow cytometry. Lck (leukocyte-specific protein tyrosine kinase), N-CAM (Neural Cell Adhesion Molecule), T_{reg} (regulatory T cell).

CD4 is a glycoprotein which is expressed on the surface of T helper cells, T_{reg} cells, monocytes, macrophages, and DCs. In T cells it is a co-receptor, which along with the TCR initiates an intracellular activation cascade via Lck, after interaction with a MHC class II molecule on an APC. IL-2 produced by $CD4^+$ T cells controls the activation of $CD8^+$ cytotoxic T cells via CD25 up-regulation (Obar et al., 2010).

CD8 is principally expressed on the surface of cytotoxic T cells and, to lesser degrees, on NK and DCs. CD8, like CD4, is a transmembrane glycoprotein that acts as a co-receptor with the TCR, however, CD8 binds to, and recognises antigen presented in the context of MHC class I molecules. Functionally, CD8 is a dimer and binds with high affinity to MHC class I ensuring that the cytotoxic T cell and target cell are tightly bound during antigen induced activation.

CD16 is the low affinity FcγRIII receptor, which binds the Fc region of immunoglobulins. CD56 or N-CAM (Neural Cell Adhesion Molecule) is involved in cell-cell interactions and growth. A mAb mix containing both CD16 and CD56 was used to identify NK cells in this study. The majority of NK cells (about 95%) express low levels of CD56 and high levels of CD16. There is also a smaller proportion of NK cells which express high levels of CD56 and are CD16 negative, these NK cells cannot bind immunoglobulin as they lack the low affinity Fc receptor, CD16; they are involved in cytokine release and have been termed regulatory NK cells (Yoshida et al., 2010). These two types of NK cells were not distinguished in the current study.

The assessment of lymphocyte activation provides information about the potential ability of the immune system to function normally and is a means to evaluate how this could be affected by opioids. Traditionally, lymphocyte activation *in vitro* has been assessed using proliferation markers such as DNA synthesis. Although requiring fewer cells, these techniques do not enable the responsiveness of individual cell subsets to be determined. The detection of activation antigens such as CD25 and CD69 on cell subsets by flow cytometry is a more definitive approach for measuring lymphocyte responsiveness in mixed cell populations (Maino et al., 1995, Simms and Ellis, 1996).

CD25 is the α-chain of the IL-2 receptor, which heterotrimerises with the β-chain, and common γ-chain to form the complete IL-2 receptor. When activated by the binding of IL-2, this receptor is involved in the activation and maintenance of immune responses and lymphocyte development. It is also critical in the promotion of proliferation and differentiation of T cells, NK cells and B cells, as well as being involved in the

elimination of self-reactive T cells (Castriconi et al., 2007, Curfs et al., 1997).

CD69 is an early activation antigen which is a member of the NK cell gene complex family (Testi et al., 1994). Unactivated NK cells express very low levels of CD69, but it is expressed by NK cells activated by IL-2, IL-12 and IFN-α (Gerosa et al., 1993). CD69 can be expressed by T cells within 30 min of TCR activation (Maino et al., 1995). Although ^{3}H-thymidine uptake lymphocyte proliferation assays are still often considered the standard for assessing T cell activation, CD69 expression by flow cytometry has been demonstrated to be correlate well with ^{3}H-thymidine and also provides definitive information on the responsiveness of cell subsets in a complex mixture (Mardiney et al., 1996, Simms and Ellis, 1996). The measurement of CD69 expression by flow cytometry has also been shown to be a more sensitive measure of T cell activation than proliferation studies such as the ^{3}H-thymidine uptake assays, although this depends on the method of activation used, as polyclonal stimuli (e.g. anti-CD3 mAb, staphylococcal enterotoxin B) induce CD69 expression, whereas oligoclonal stimuli (such as tetanus toxoid) do not (Simms and Ellis, 1996).

Although used as a lymphocyte activation marker, CD69 might also function as an immunoregulator. Initial *in vitro* data indicated that CD69 might act as a stimulatory receptor, yet more recent *in vivo* studies have disputed this (Sancho et al., 2005). CD69 might be a negative regulator of the immune response, in part by increasing the production of transforming growth factor (TGF)-β by $CD4^{+}$ T cells (Sancho et al., 2003). Evidence for this inhibitory effect of CD69 has come from studies in which the down-regulation of CD69 expression by CD69 mAbs reduces TGF-β levels, increases NK cell cytotoxicity and leads to a reduction in tumour growth (Esplugues et al., 2005). CD69 also contributes to lymphocyte retention in secondary lymphoid tissue (Shiow et al., 2006).

For the multi-parameter flow cytometry, $1.5x10^{5}$ PBMCs were washed with 1 ml of PBS containing 10% v/v FBS (staining buffer) and centrifuged at 300 xg, for 5 min at 4°C. The supernatant was removed using a vacuum aspirator and the cell pellet was re-suspended in 10 μl of staining buffer. The potential for non-specific mAb binding was reduced by pre-incubating cells with 0.5 μl normal mouse serum for 15 min at room temperature, following which a mixture of fluorescently-labelled mAbs was added (Table 2.11). All of the volumes of mAbs used were based on the manufacturers' recommendations and produced definitive separations of the cell populations under

investigation. Control samples either had no mAb added or were incubated with appropriate, fluorescently-conjugated isotype-matched control immunoglobulins. All tubes were incubated for 30 min in the dark at 4°C, after which cells were washed twice in 1 ml staining buffer (300 xg, 5 min, 4°C) and then re-suspended in 250 μl staining buffer and kept on ice prior to flow cytometry. Either 2.5 μl (1 μl/100 μl cell suspension) of 7-Aminoactinomycin D viability stain (Cambridge Biosciences, Cambridge, UK) was added to the sample 5-10 min before analysis, or 1.5 μl per $1.5x10^5$ cells (after a 1 in 10 dilution in PBS) of blue fixable live/dead viability stain (Invitrogen Ltd., Paisley, UK), was incubated with the sample for 30 min in the dark at 4°C.

The 7-Aminoactinomycin D viability stain was used in the initial studies. However, its broad emission spectrum resulted in spectral overlap into other detectors which required considerable compensation. The blue fixable live/dead viability stain is excited by the UV laser and its emission spectrum is distinct from that of the other flurochromes used. This reduced the amount of compensation that was required and allowed cells to be fixed prior to analysis.

Table 2.11: Details of the fluorescently-labelled monoclonal antibodies used.

Specificity	**Fluorochrome**	**Volume added** (manufacturers' recommendations)	**Clone number (supplier)**
CD4	Pacific Blue (PB)	1.5 μl (after 1:10 dilution in PBS)	RPA-T4 (BioLegend)
CD8a	Alexa Fluor™ 700 (AF700)	1.5 μl (after 1:20 dilution in PBS)	RPA-T4 (BioLegend)
CD16/56	Fluorescein isothiocyanate (FITC)	1.5 μl	LNK16/MEM-188 (AbD Serotec)
CD25	R-Phycoerythrin (PE)	1.5 μl	MEM-181 (AbD Serotec)
CD69	Allophycocyanin (APC)	3 μl	FN50 (BioLegend)

Samples were analysed using a BD™ LSRII flow cytometer and BD Biosciences FACSDiva™ acquisition and data analysis software. PBMCs were identified on the basis of their size and granularity, as represented by their forward and side light scatter characteristics (FSc vs. SSc) respectively (Figure 2-4). Spectral overlap between fluorochromes was corrected for using compensation beads. For this, each of the mAbs were added to 1 drop of anti-murine IgG-coated beads and 1 drop of negative control beads (BD Biosciences) in 12x75 mm polycarbonate tubes. An additional control tube was incubated in the absence of mAb. The fluorescent emission of the mAb stained beads were determined and used to set the compensation values using the FACSDiva™ software.

During flow cytometry the lymphocytes are identified on their size and granularity. Doublets (two adherent cells) and dead cells are then excluded in order to ensure the CD fluorescent marker analysis is performed on single, viable cells (Figure 2-4). NK cells are detected by the further gating of these PBMCs on the basis of CD16/56 expression (Figure 2-5). The expression of the appropriate antigens by these cells is then determined (Figure 2-6). Initial studies revealed that IL-2 stimulation alone had no effect on the expression of CD25 by $CD4^{-}CD8^{-}CD16^{-}CD56^{+}$ NK cells and as a consequence only data on CD69 expression are presented in the Results (Figure 2-6).

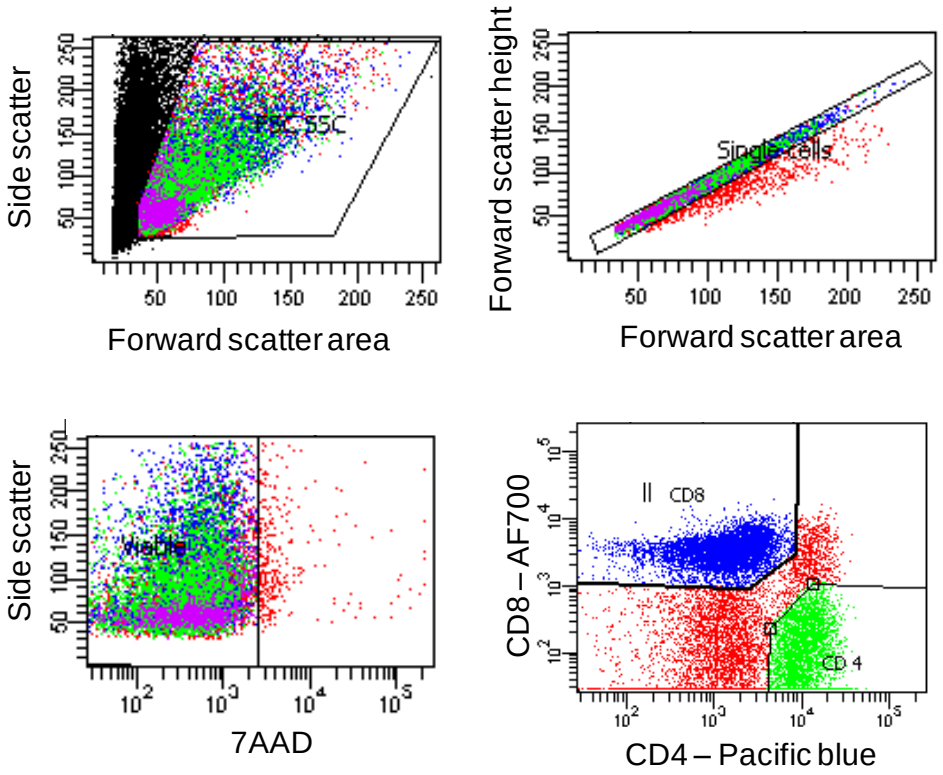

Figure 2-4: Representative flow cytometry plots for PBMCs.
This shows the PBMCs (gated population) defined by their size (forward scatter - FSc) and granularity (side scatter – SSc; **top left**). These cells are then gated by size (forward scatter height) and area (forward scatter area), removing doublets (which can have mixed CD antigen binding properties), to allow only single cells to be analysed (**top right**). These single PBMCs are then assessed for viability with 7-Aminoactinomycin D (7AAD; viability stain), removing dead cells from the analysis (which can have atypical CD antigen binding properties), to allow only viable single cells to be analysed (**bottom left**). These single viable PBMCs are then analysed for CD4 (conjugated to Pacific blue) and CD8 (conjugated to Alexa Fluor™ 700; AF700) binding (**bottom right**), before each of these lymphocyte subtypes have their activation status assessed by CD69 and CD25 expression.

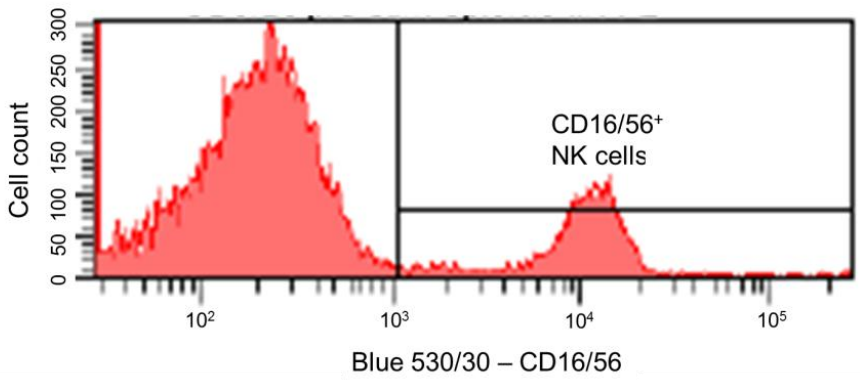

Figure 2-5: Histogram of CD16 and CD56 expression by PBMCs.
The expression of CD16 and CD56 as detected by a anti-CD16/CD56 mAb. The population of the cells expressing CD16 or CD56 are NK cells.

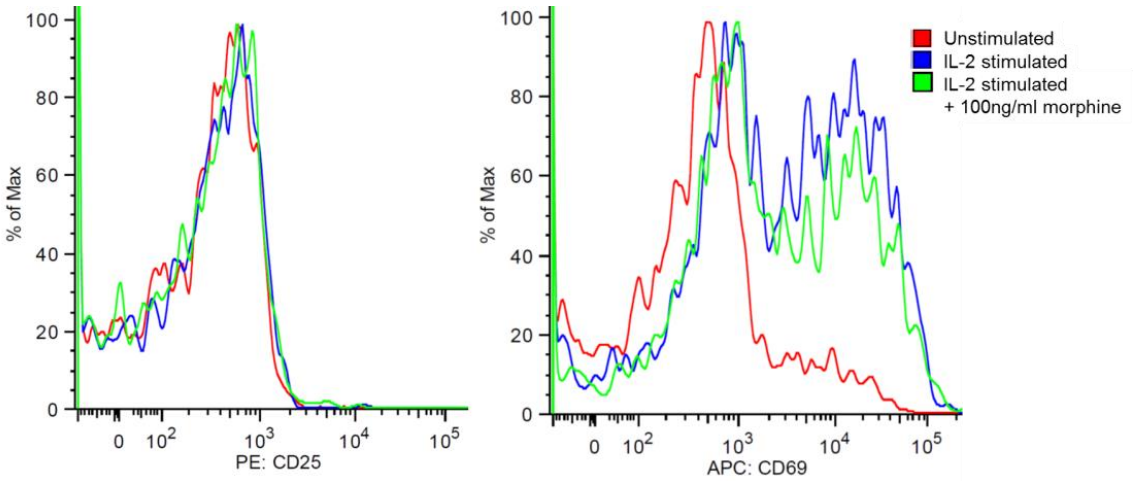

Figure 2-6: Overlay histograms of CD25 and CD69 expression by NK cells.
CD25 (**left panel**) and CD69 expression (**right panel**) by $CD16^{+}/56^{+}$ NK cells in unstimulated and IL-2 stimulated PBMC preparations, with and without 100 ng/ml of morphine.

2.6 Influence of opioids on neutrophil and monocyte function

Neutrophils and monocytes are innate immune cells which play a central role in the control of bacterial and fungal infection, as well as in the removal of dead cells and foreign material. The control of infection principally involves phagocytosis of the target cell/material and the subsequent generation of a cytotoxic oxidative burst reaction.

Initially, the effect of different incubation times with one concentration of morphine, tramadol and fentanyl on neutrophil and monocyte phagocytosis and oxidative burst responses were assessed. Subsequent experiments evaluated the influence of a single incubation time with morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine at variable concentrations (Figure 2-7 and Table 2.12).

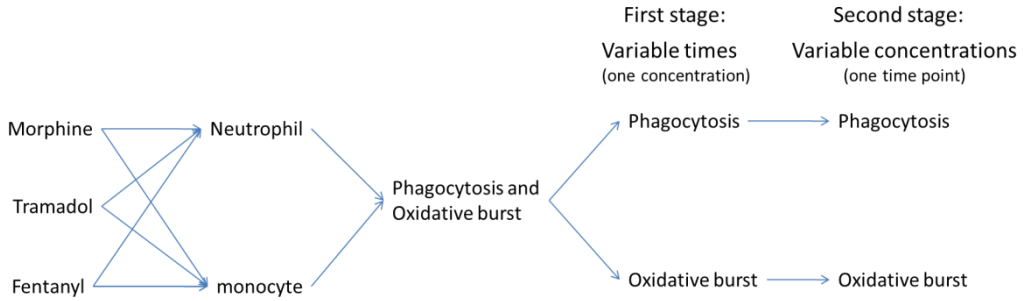

Figure 2-7: Sequence for the phagocytosis and oxidative burst assays.
Sequence of studies into the effects of different pre-incubation times with, and concentration of morphine, tramadol and fentanyl on neutrophil and monocyte phagocytosis and oxidative burst responses. Buprenorphine, methadone, oxycodone, diamorphine and codeine were assessed at the single time point of 60 min, as per the second stage of this experiment.

Previous studies have reported on inter- and intra-individual variability in neutrophil and monocyte and phagocytosis and oxidative burst responses. These effects were minimised in the current study by using a relatively fixed cohort of healthy volunteers and by using a validated commercial kit PHAGOBURST® test kit (Orpegen Pharma) with high intra-assay precision (manufacturer's information from data sheet). However, other factors, both genetic and environmental (i.e. diet) which were not compensated for, as well as different individual responses to opioids might also influence the perceived reproducibility of the data generated.

2.6.1 Effects of opioids on neutrophil and monocyte phagocytosis

To investigate the effect of opioids on the phagocytic response of neutrophils and monocytes, a whole blood, flow cytometric approach was performed using the PHAGOBURST® test kit (Orpegen Pharma). For this, heparinised whole blood (50 μl) was incubated for 0, 30, 60 and 120 min (section 2.6.1.2) at 37°C with 50 μl of opioid or

PBS (as a control). Samples were then incubated with 10 μl FITC-labelled opsonised *E.coli* for 10 min at 37°C. Control tubes containing 10 μl FITC-labelled opsonised *E.coli* were incubated for 10 min at 4°C. This made the incubation time with the opioid 10 min longer than specified, with the 0 min opioid incubation being for 10 min, along with the stimulation. At the end of the incubation period, all tubes were transferred to ice and 50 μl of quenching solution was added. This quenches the fluorescence of adherent, non-phagocytosed FITC-*E.coli*. Erythrocytes were then lysed by incubating samples with 3 ml of FACSLyse™ (BD Biosciences) for 20 min at room temperature, after which samples were washed twice in PBS (5 min, 250 xg, 4°C). This test has been demonstrated to have high intra-assay precision with Coefficients of Variation of 0.2% for the proportion of neutrophils phagocytosing *E.coli* and 1.5% for the MFI of this response, and 1.1% for the proportion of monocytes phagocytosing *E.coli* and 3.6% for the MFI of this response (PHAGOBURST® test kit data sheet, Orpegen Pharma).

The proportion of cells phagocytosing FITC-*E.coli* and the amount of *E.coli* that has been phagocytosed (median fluorescent intensity, MFI) were then analysed using a BD Biosciences FACSCalibur™ flow cytometer utilising BD Biosciences CELLQuest™ acquisition and analysis software. It has been demonstrated that the phagocytic index (by microscopically counting the number of erythrocytes ingested by 100 phagocytes) is a more sensitive parameter of phagocytic activity than just the number of phagocytes ingesting erythrocytes (Tomassini et al., 2004). In the current study this is taken further by using flow cytometry to accurately analyse (Kampen et al., 2004) at least 5,000 cells and measuring the absolute uptake of *E.coli* per cell and per population of cells, as well as the proportion of cells ingesting *E.coli*. Opsonised *E.coli* is also physiologically relevant in terms of host defence against infection.

2.6.1.1 Flow cytometric acquisition and analysis

Neutrophil and monocyte populations were identified on the basis of their size and granularity (FSc and SSc light scatter characteristics respectively) and confirmed using CD11b and CD14 mAb staining (for neutrophils and monocytes respectively). Data acquisition regions were plotted around these two populations (Figure 2-8) and data on a minimum of 5,000 cells in the monocyte region and more than 10,000 cells in the neutrophil region were collected. The proportion of cells which were positive for fluorescence and the intensity of this fluorescent signal (MFI) were recorded (Figure 2-9). Prior to acquisition, control samples were used to set the voltages (sensitivity) for the fluorescence signal.

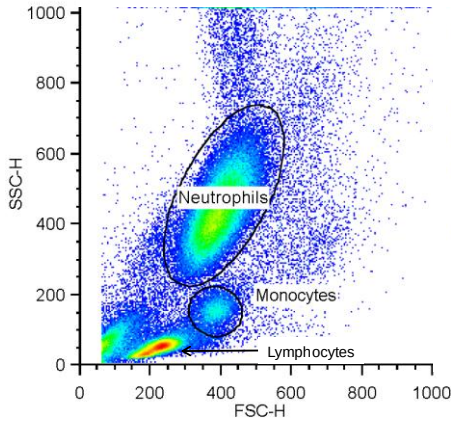

Figure 2-8: Representative flow cytometry dot plot for lysed whole blood.
This shows the three main populations of leucocytes in whole blood defined by their different size, forward scatter (FSc) and granularity, side scatter (SSc). Although the monocytes are gated during acquisition (to ensure at least 5,000 events), data on the more granular neutrophils are also acquired in order to enable the fluorescent signals generated by these two cell types to be analysed separately.

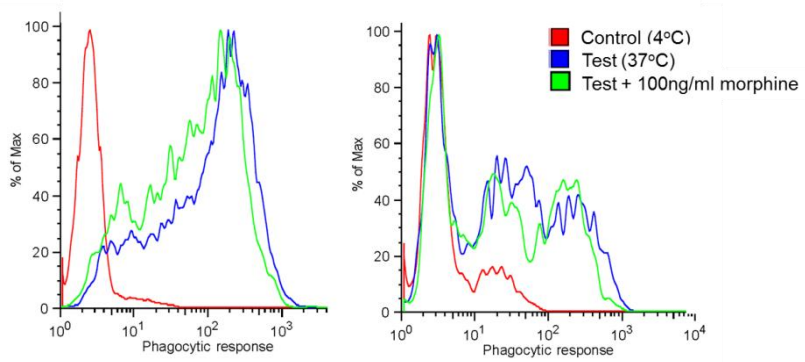

Figure 2-9: Representative overlay histograms illustrating the phagocytosis of *E.coli* by neutrophils and monocytes and the influence of morphine.
Fluorescent histograms for neutrophils (**left panel**) and monocytes (**right panel**) following incubation with FITC-*E.coli* for 10 min at 4°C and 37°C, with and without 100 ng/ml of morphine.

2.6.1.2 Phagocytosis time course experiments

To optimise the incubation time with opioids, initial kinetics experiments were performed. For these, heparinised whole blood (50 μl) was incubated at 37°C for 0, 30, 60 and 120 min with 50 μl of morphine (100 ng/ml final concentration), tramadol (400 ng/ml final concentration), fentanyl (4 ng/ml final concentration) or 50 μl of PBS (as a control). As neutrophils do not survive well *in vitro*, cultures needed to be kept as short as possible. However, up to 5 hr is unlikely to have any effect on viability (Welters et al., 2000a).

The findings relating to the influence of incubating whole blood for different times with morphine (Figure 2-10), tramadol (Figure 2-11) and fentanyl (Figure 2-12) on the ability of neutrophils and monocytes to phagocytose *E.coli* are presented below.

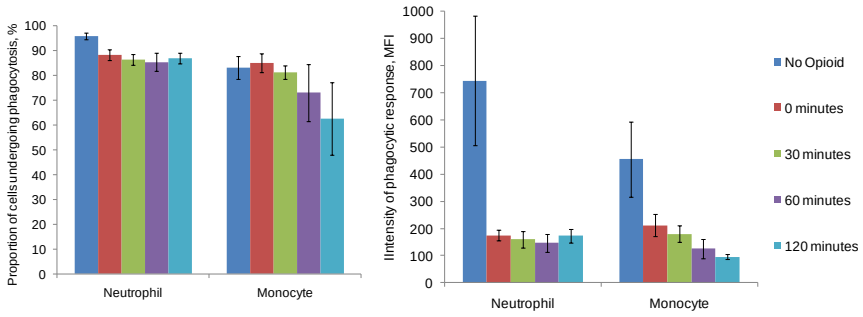

Figure 2-10: **Effect of incubation time with morphine on neutrophil and monocyte phagocytosis.**
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 100 ng/ml of morphine for the indicated times (0, 30, 60 and 120 min) prior to assessing the phagocytosis of FITC-*E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

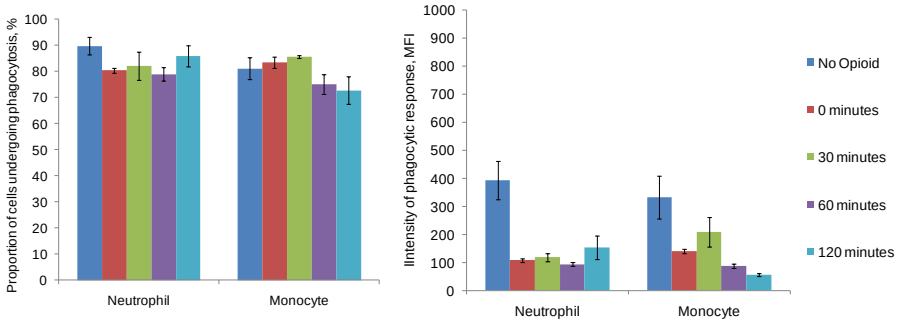

Figure 2-11: **Effect of incubation time with tramadol on neutrophil and monocyte phagocytosis.**
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 400 ng/ml of tramadol for the indicated times (0, 30, 60 and 120 min) prior to assessing the phagocytosis of FITC-*E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

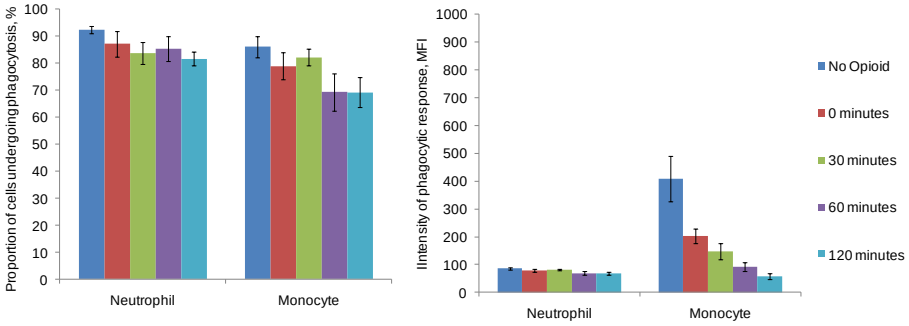

Figure 2-12: **Effect of incubation time with fentanyl on neutrophil and monocyte phagocytosis.**
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 4 ng/ml of fentanyl for the indicated times (0, 30, 60 and 120 min) prior to assessing the phagocytosis of FITC-*E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

Incubation of whole blood for 0, 30, 60 and 120 min with clinically relevant concentrations of morphine, tramadol and fentanyl had a greater effect on the intensity of the neutrophil and monocyte phagocytic response to *E.coli* (which correlates to the number of bacteria phagocytosed) than on the proportion of neutrophils and monocytes that phagocytosed the *E.coli*. Although there was a trend for a decrease in the proportion of neutrophils and monocytes performing phagocytosis with longer incubation times, this was less dramatic when compared to the effect on the intensity of the phagocytic response, which occurred with even the shortest incubation times. The exception to this was the effect of fentanyl on neutrophil phagocytosis, where the no opioid control was at a very low level. Although the short incubation times inhibited the total number of *E.coli* that were phagocytosed by monocytes, the longer incubation times tended to inhibit this further. For neutrophils, longer incubation times had no additional impact on the ability of these cells to phagocytose the bacteria.

These results informed the next series of experiments which investigated the effect of different concentrations the opioids (Table 2.12) at a fixed pre-incubation time of 60 min at 37°C on neutrophil and monocyte phagocytosis (presented in the Results). An incubation time of 60 min was used, as 120 min did not induce a consistently greater inhibition of phagocytosis and to minimise the total incubation period.

Table 2.12: Opioid concentrations used for phagocytosis and oxidative burst assays.

Opioid	Final opioid concentrations used for phagocytosis and oxidative burst assays (ng/ml)		
Morphine	20	100	500
Tramadol	80	400	2,000
Fentanyl	0.8	4	20
Buprenorphine	0.8	4	20
Methadone	20	100	500
Oxycodone	4	20	100
Diamorphine	10	50	250
Codeine	16	80	400

2.6.2 Effects of opioids on neutrophil and monocyte oxidative burst responses

Once internalised, the phagosome containing the pathogens fuses with a lysosome containing digestive enzymes to form a phagolysosome. These digestive enzyme (oxygen-independent) and oxygen-dependent (oxidative burst reaction) degradation become the next vital step in the elimination of the pathogen.

The effect of opioids on the various pathways that are responsible for inducing the oxidative burst in neutrophils and monocytes was assessed using the PHAGOBURST® test kit (Orpegen Pharma). Oxidative burst responses were induced using opsonised *E.coli* (physiological stimulus), PMA (a potent stimulus directly activating PKC) and fMLP (a synthetic peptide which mimics the activity of bacterial peptides; a relatively weak stimulus). This test has been shown to have high intra-assay precision with Coefficients of Variation of 0.1% for the proportion of neutrophils undergoing the oxidative burst reaction to *E.coli* and 4.8% for the MFI of this response, and 1.1% for the proportion of monocytes undergoing the oxidative burst reaction to *E.coli* and 6.5% for the MFI of this response (PHAGOBURST® test kit data sheet, Orpegen Pharma).

The oxidative burst reaction is characterised by the rapid release of reactive oxygen species (superoxide radical, H_2O_2) as part of the oxygen-dependent degradation in phagolysosomes. Neutrophils and monocytes use nicotinamide adenine dinucleotide phosphate oxidase to reduce O_2 to oxygen free radicals and then H_2O_2. They then use myeloperoxidase to further combine H_2O_2 with Cl^- to produce hypochlorite. This reaction is vital for the killing of potential pathogens, as well as for degrading internalised particles. A failure of phagocytic cells to kill organisms that they have engulfed because of defects in the enzymes that produce free radicals results in chronic granulomatous disease. This consists of a range inherited disorders which present in childhood with severe, recurrent bacterial and fungal infections (Dinauer, 2007).

Neutrophils and monocytes are able to detect the presence of infection by binding bacterial peptides containing N-formylmethionine, or fMet, a modified amino acid that is used for the initiation of all proteins synthesised in prokaryotes. The receptor which recognises these peptides is known as the fMLP receptor.

Pathogen-associated molecular patterns, such as bacterial LPS, are conserved small molecular motifs that are associated with pathogens. They are recognised by TLRs, TLR4 in the case of LPS, and other pattern or pathogen recognition receptors, located on cells of the innate immune system. LPS binds to TLR4 (in a complex with MD2), which then activates the MyD88-dependent pathway, recruiting IRAKs and activating NF-κB and MAPK. TLR4 also has an effect via a TRIF-dependent pathway that activates IRF3 and NF-κB. Both these pathways lead to the production of pro-inflammatory cytokines (Kawai and Akira, 2010). The MAPK pathway activates the ERK and JNK pathways and the early response transcription, AP-1 (c-Jun/c-Fos combination) (Aderem and Ulevitch, 2000).

Neutrophils and monocytes are activated by similar intracellular pathways to those that are modulated when opioid receptors are activated. G-protein subunits, dissociated by activation, stimulate adenylyl cyclase and PLC, producing the secondary messenger's cAMP, inositol trisphosphate and Ca^{2+}. These activate intracellular pathways affecting cell metabolism, motility, gene expression, and cell division. Opioids could potentially interfere with these G-protein targets or the secondary messengers, as there is overlap in the intracellular transduction pathways of opioids and in the activation pathways via which fMLP, PMA and *E.coli* trigger neutrophils and monocytes.

PMA is a very potent stimulus and PMA-induced neutrophil and monocyte responses might be influenced if an opioid interferes with PKC signal transduction. *E.coli* is of an intermediate potency and the stimulus which most closely mimics the mechanism of activation to which neutrophils or monocytes have evolved to elicit an oxidative burst. *E.coli* are gram negative bacteria which contain LPS in their cell wall and will, among other mechanisms, bind to and activate TLR4. In the clinical situation, and in this assay, *E.coli* are opsonised and thus they will also bind to Fc receptors and C3b complement receptors on neutrophils and monocytes. By using fMLP as a weak stimulus (designated as a 'low' control in the assay kit), the potential for opioids to increase or prime neutrophils or monocytes to perform the oxidative burst reaction can be assessed. Furthermore, these three stimuli activate the cells via different pathways and modifications in their stimulatory effects might provide some insight into the different pathways and mechanisms involved in the effects of opioids on neutrophils and monocytes.

When stimulated to undergo the oxidative burst, neutrophils and monocytes produce reactive oxygen species (superoxide anion, H_2O_2, hypochlorous acid) which oxidise

dihydrorhodamine 123 to its fluorescent derivative rhodamine 123 (Rothe and Valet, 1994). This is then detected by flow cytometry analysis. Reactive nitrogen intermediates such as peroxynitrite, which might be important in bacteria killing, were not evaluated and the synthesis of the oxygen intermediates O_2^- and HO_2 cannot be assessed (van Pelt et al., 1996).

Flow cytometric acquisition and analysis was the same as for phagocytosis (described in section 2.6.1.1), with the proportion of neutrophils and monocytes positive for the fluorescence of the oxidative burst reaction and the intensity of this signal being recorded (Figure 2-13 - Figure 2-14). This correlated to the activity of the oxidative burst reaction. Prior to acquisition, control samples were used to set the voltages (sensitivity) for the fluorescence signal.

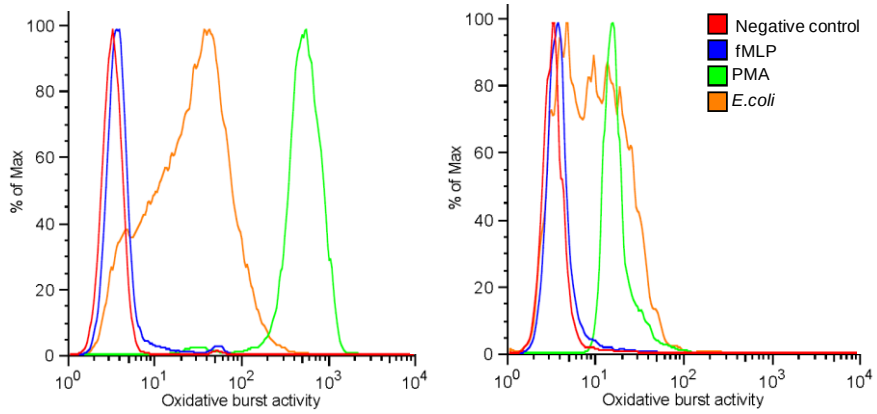

Figure 2-13: Overlay histograms for the oxidative burst reaction.
Comparison of the stimulatory effect of the negative control (no stimulation), fMLP (weak stimulus), PMA (potent stimulus) and *E.coli* (physiological stimulus) on the oxidative burst activity for neutrophils (**left panel**) and monocytes (**right panel**).

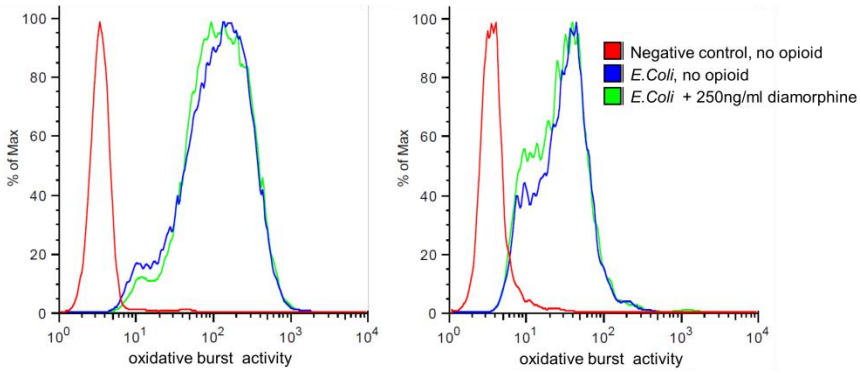

Figure 2-14: Representative overlay histograms illustrating the oxidative burst activity and the influence of diamorphine.
Fluorescent histograms of the oxidative burst reaction to *E.coli* for neutrophils (**left panel**) and monocytes (**right panel**) with and without 250 ng/ml of diamorphine. Whole blood was incubated (37°C) with diamorphine or saline for 1 hr before assessing the oxidative burst response to *E.coli* (10-min incubation) using flow cytometry (negative control included for comparison).

2.6.2.1 Oxidative burst time course experiments

As with the phagocytosis experiments, in initial experiments 50 μl of heparinised whole blood were pre-incubated with 50 μl of morphine (100 ng/ml final concentration), tramadol (400 ng/ml final concentration), fentanyl (4 ng/ml final concentration) or 50 μl of PBS (as a control) for 0, 30, 60 or 120 min prior to the addition of the stimulus. Samples were then incubated for 10 min at 37°C with 10 μl fMLP (5 μM final concentration), 10 μl PMA (8.1 μM final concentration) or 10 μl opsonised *E.coli* ($1x10^9$ bacteria/ml) in 12x75 mm Falcon polycarbonate tubes. 10 μl of washing solution was added to the negative control. This made the incubation time with the opioid 10 min longer than specified above, with the 0 min opioid incubation being for 10 min, whilst being stimulated.

At the end of the incubation period, 10 μl of dihydrorhodamine 123 substrate solution (one disk reconstituted with 1 ml washing solution 60 min prior to use) was added to each tube, after which tubes were mixed and incubated at 37°C for 10 min. Erythrocytes were lysed by incubating samples with 3 ml of FACSLyse™ for 20 min, then samples were washed twice in PBS (5 min, 250 xg, 4°C). The proportion of cells undergoing the oxidative burst and its intensity were then analysed using a BD Biosciences FACSCalibur™ flow cytometer utilising BD Biosciences CELLQuest™ acquisition and analysis software.

The influence of varying incubation time with morphine (Figure 2-15, Figure 2-16), tramadol (Figure 2-17, Figure 2-18) and fentanyl (Figure 2-19, Figure 2-20) on the ability of neutrophils and monocytes to perform the oxidative burst reaction is illustrated below.

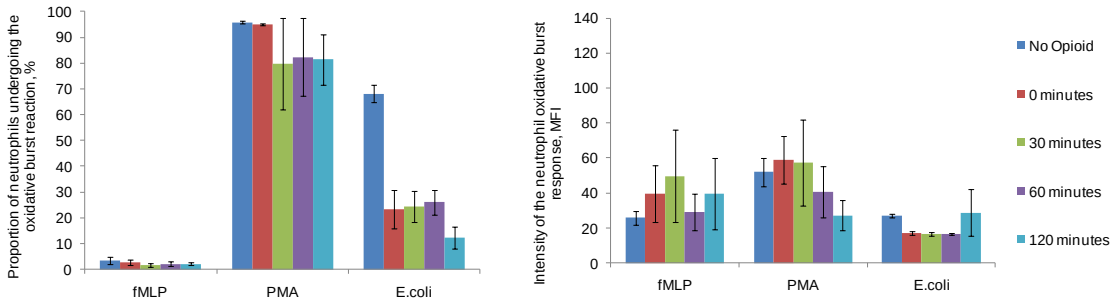

Figure 2-15: **Effect of incubation time with morphine on neutrophil oxidative burst reaction.**

The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of the oxidative burst response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 100 ng/ml of morphine for the indicated times (0, 30, 60 and 120 min) prior to assessing the oxidative burst reaction stimulated by fMLP, PMA and *E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

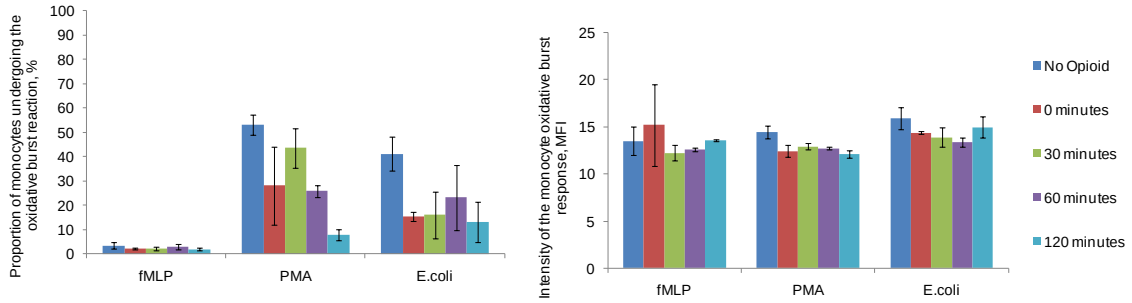

Figure 2-16: **Effect of incubation time with morphine on monocyte oxidative burst reaction.**

The proportion of monocytes undergoing the oxidative burst reaction (**left panel**) and the intensity of the oxidative burst response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 100 ng/ml of morphine for the indicated times (0, 30, 60 and 120 min) prior to assessing the oxidative burst reaction stimulated by fMLP, PMA and *E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

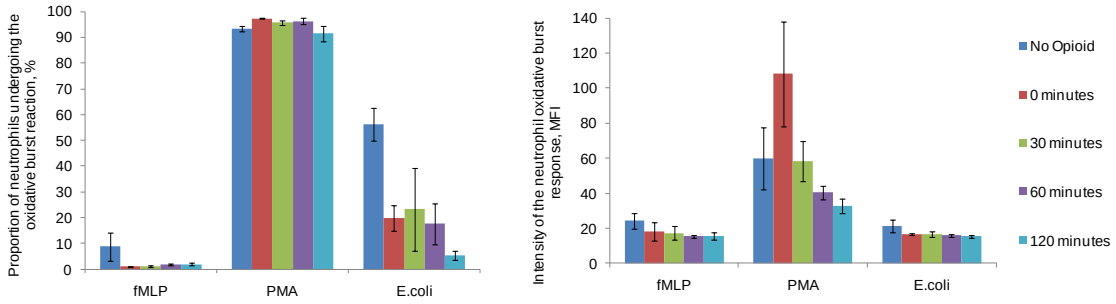

Figure 2-17: **Effect of incubation time with tramadol on neutrophil oxidative burst reaction.**

The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of the oxidative burst response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 400 ng/ml of tramadol for the indicated times (0, 30, 60 and 120 min) prior to assessing the oxidative burst reaction stimulated by fMLP, PMA and *E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

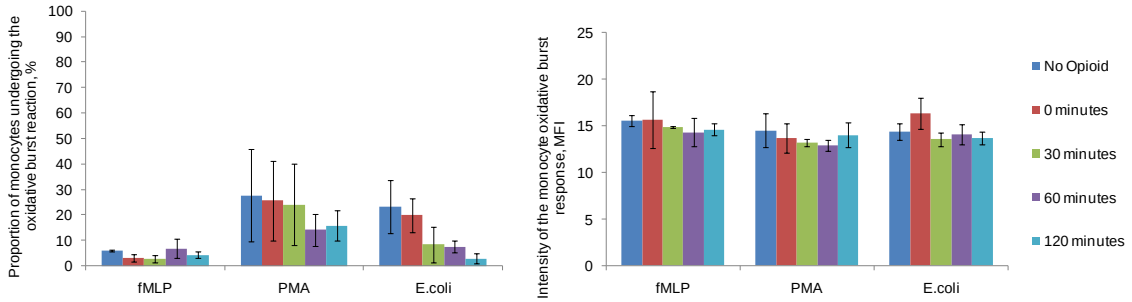

Figure 2-18: **Effect of incubation time with tramadol on monocyte oxidative burst reaction.**

The proportion of monocytes undergoing the oxidative burst reaction (**left panel**) and the intensity of the oxidative burst response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 400 ng/ml of tramadol for the indicated times (0, 30, 60 and 120 min) prior to assessing the oxidative burst reaction stimulated by fMLP, PMA and *E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

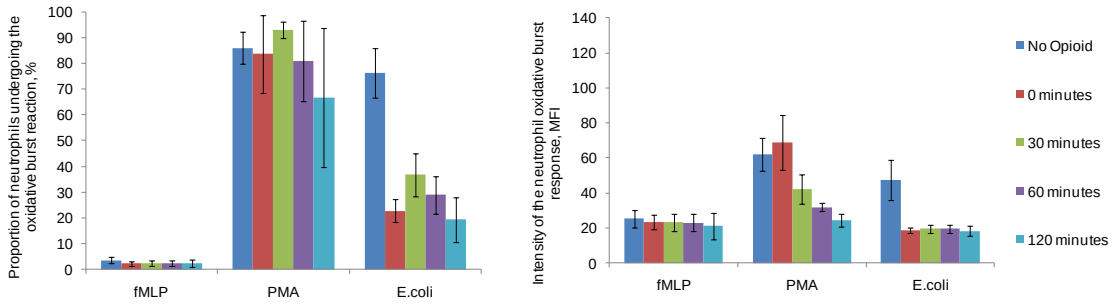

Figure 2-19: **Effect of incubation time with fentanyl on neutrophil oxidative burst reaction.** The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of the oxidative burst response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 4 ng/ml of fentanyl for the indicated times (0, 30, 60 and 120 min) prior to assessing the oxidative burst reaction stimulated by fMLP, PMA and *E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

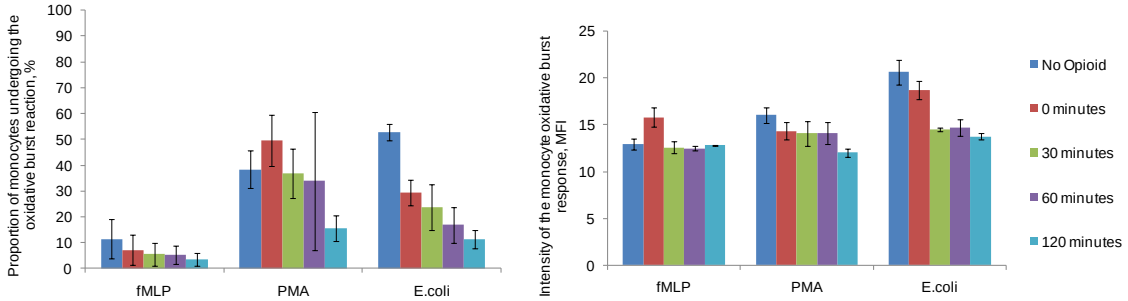

Figure 2-20: **Effect of incubation time with fentanyl on monocyte oxidative burst reaction.** The proportion of monocyte undergoing the oxidative burst reaction (**left panel**) and the intensity of the oxidative burst response (median channel of fluorescent intensity, MFI; **right panel**). Whole blood was incubated with 4 ng/ml of fentanyl for the indicated times (0, 30, 60 and 120 min) prior to assessing the oxidative burst reaction stimulated by fMLP, PMA and *E.coli*, with a further 10-min incubation. Data are means ± SEM from 3 independent experiments.

Clinically relevant concentrations of morphine, tramadol and fentanyl over 0, 30, 60 and 120 min tended to inhibit neutrophil and monocyte oxidative burst reactions, although the effects were dependent on the stimulus, opioid and, in some cases, the exposure time.

Morphine only inhibited the stimulatory effect of *E.coli* on the proportion of neutrophils undergoing the oxidative burst reaction, which was independent of incubation time. The intensity of this response (MFI) was also inhibited (except at 120 min). For longer incubation times, morphine inhibited the intensity of the neutrophil oxidative burst response to PMA activation. Although monocytes produced a less vigorous response compared to neutrophils, morphine inhibited the proportion of monocytes undergoing the oxidative burst reaction to *E.coli* at all-time points and the effect of PMA at longer incubation times. The magnitude of this response, although small at baseline, was also marginally inhibited when the monocytes were stimulated with PMA or *E.coli*.

Tramadol inhibited the proportion of neutrophils undergoing the oxidative burst reaction in response to both fMLP and *E.coli*, which was independent of the incubation time. Although the intensity of this response to all stimuli was very low, there might be a trend for this to be inhibited by tramadol. The only effect on monocytes was to time-dependently inhibit the proportion of cells undergoing the oxidative burst response to *E.coli*.

Fentanyl inhibited the proportion of neutrophils undergoing *E.coli*-induced oxidative burst and its intensity at all-time points. It also decreased the intensity of response to PMA in a time-dependent manner. Fentanyl reduced the proportion of monocytes undergoing the oxidative burst in response to *E.coli*, at all-time points, although the effects were more pronounced as the incubation time increased. The intensity of this response was also decreased, except at 0 min pre-opioid incubation.

On the basis of these results the subsequent series of experiments assessed the effect of different concentrations of opioids at a fixed pre-incubation time of 60 min at 37°C on the oxidative burst reaction by neutrophils and monocytes. Three concentrations of each opioid were evaluated (Table 2.12), these are presented in the Results. An incubation time of 60 min was used, as 120 min did not induce a consistently greater inhibition of the oxidative burst response and to minimise the total incubation period.

2.7 Effect of opioids on the responsiveness of peripheral blood mononuclear cells populations to polyclonal stimulation

$CD4^+$ T cells are central to the coordination of immune responses and $CD8^+$ T cells are involved in the killing of virally infected cells or tumour cells expressing MHC class I. The influence of opioids on the susceptibility of these lymphocyte populations to polyclonal activation using anti-CD3/anti-CD28 mAb coated beads (on the basis of CD25 and CD69 activation marker induction) was determined. As evidence suggests that opioid receptors are only expressed on activated lymphocytes (Borner et al., 2008, Kraus, 2009), these experiments compared the influence of opioids on the responsiveness of activated T cell populations. This also might more closely reflect the potential effects of opioids on the immune status of patients with cancer or infection. During the incubation of PBMCs with anti-CD3/anti-CD28 mAb coated beads, the T cells become activated, secreting cytokines and activating other cells in the culture (Figure 2-21), again more closely reflecting what might be happening physiologically.

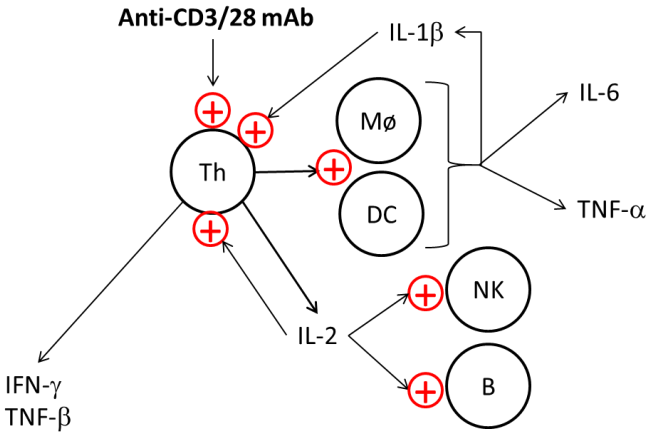

Figure 2-21: **Effect of CD3 and CD28 receptor activation on T cells, in PBMC preparations.** Using anti-CD3/anti-CD28 mAb coated beads, T helper (Th) cells were activated in a physiological manner, leading to cytokine production. This can activate other immune cells, including dendritic cells (DCs) monocytes (Mø), natural killer cells (NK) and B cells (B), producing further cytokines and activation of the immune response.

Anti-CD3/anti-CD28 mAb coated beads activate T cells by binding to the CD3 subunit of the TCR with concomitant signalling via the CD28 co-stimulatory receptor. Mitogens, such as Con A, activate signal transduction pathways and promote mitosis. Although mitogens are often used to activate lymphocytes, they do not mirror the *in vivo* activation pathways as closely as anti-CD3/anti-CD28 mAb coated beads.

Two types of commercially-available anti-CD3/anti-CD28 mAb coated beads are Dynal Dynabeads® (Invitrogen Ltd., Paisley, UK) which are potent activators of the TCR

pathway inducing cell expansion and activation, and the less potent T_{reg} Suppression Inspector beads (Miltenyi Biotec). The latter are designed for the analysis of T_{reg} cell activation and subsequent suppression of T responder cells, hence their name. Both these beads activate cells via the same pathways, as occurs physiologically. It is the concentration of anti-CD3/anti-CD28 mAbs on the surface of these beads which dictates their different potencies, with the Miltenyi beads being coated with lower concentrations of both mAbs.

For these experiments, PBMCs were isolated as described above (Section 2.5.1), viable cells were counted using trypan blue dye exclusion and re-suspended at $2x10^6$ cells/ml. Anti-CD3/anti-CD28 mAb coated beads at a 1:1 bead:cell ratio were added. In order to reduce error between samples, the beads were added to the all the cells that required stimulating, these were mixed and subsequently aliquoted. To the unstimulated cells, an equivalent volume of medium was added. 105 μl of the bead/cell mix ($2x10^5$ cells) or the unstimulated cells were pipetted into 96-well round-bottomed plates. RPMI* (100 μl) was added to the unstimulated and 'no opioid' control stimulated wells and 100 μl of opioid (diluted in RPMI* to the concentrations in Table 2.13) were added to the appropriate wells containing stimulated cells. Plates were then incubated for 1, 2, 3, 4 or 5 days at 37°C, 5% v/v CO_2 and 100% humidity.

Table 2.13: Opioid concentrations used in experiments assessing the influence of opioids on Miltenyi anti-CD3/anti-CD28 mAb stimulated PBMC populations.

Opioid	Final concentrations used for the T cell assays (ng/ml)				
Morphine	20	100	500	2,500	25,000
Tramadol	80	400	2,000	10,000	100,000
Fentanyl	0.8	4	20	100	1000
Buprenorphine	0.8	4	20	100	750
Methadone	20	100	500	2,500	25,000
Oxycodone	4	20	100	500	25,000
Diamorphine	10	50	250	1,250	12,500
Codeine	16	80	400	2,000	150,000

These assays used the lower 4 concentrations, representing 5 times dilutions around the therapeutic range (the lower 4 concentrations in the table). In some of the experiments a much larger concentration (the concentration on the right in the table) was also used in order to assess if very high concentrations had an effect on T cell activation.

After the appropriate incubation time, cells were harvested, counted using a haemocytometer and $1.5x10^5$ cells were transferred to 1.5 ml microfuge tubes for flow cytometric staining and analysis. After the first centrifugation, supernatants were removed and stored at -80°C for subsequent cytokine analyses. The expression of CD25 and CD69 by $CD4^+$ and $CD8^+$ T cells was determined using multi-parameter flow

cytometry as described previously (Figure 2-22). The expression of CD25 and CD69 on $CD16^{+}CD56^{+}$ NK cells which is induced as a bystander effect of T cell activation in the PBMC preparations were also examined. All experiments included a viability stain and only the data derived from viable single-cell populations was analysed due to non-specific mAb binding to non-viable and doublet cell populations (Figure 2-4).

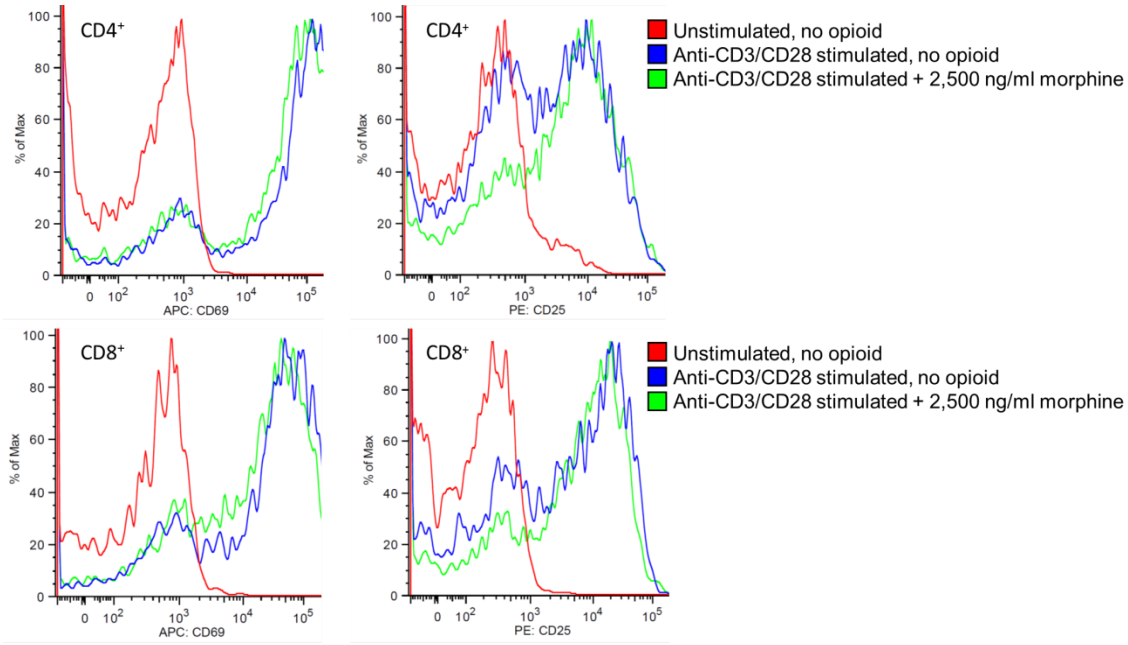

Figure 2-22: Representative overlay histograms illustrating the expression of CD25 and CD69 on unstimulated and stimulated T cell subpopulations and the effect of morphine.
Comparison of CD69 expression (**left panels**) and CD25 expression (**right panels**) on unstimulated and Miltenyi anti-CD3/anti-CD28 mAb stimulated $CD4^{+}$ (**top panels**) and $CD8^{+}$ (**bottom panels**) T cells, and how this is influenced by 2,500 ng/ml of morphine in a 3 day PBMC culture.

Appropriate controls (unstained and non-reactive isotype matched) were used for unstimulated, stimulated no opioid and opioid tubes. Table 2.13 indicates the concentrations of the opioids used for these assays. In subsequent assays, the influence of extremely high concentrations of each opioid (morphine 25,000 ng/ml; tramadol 100,000 ng/ml; fentanyl 1000 ng/ml; buprenorphine 750 ng/ml; Methadone 25,000 ng/ml; oxycodone 25,000 ng/ml; diamorphine 12,500 ng/ml; codeine 150,000 ng/ml) on T cell responsiveness to polyclonal stimulation and the consequential bystander activation of NK cells were assessed.

Initial experiments explored CD25 and CD69 expression on $CD4^{+}$ and $CD8^{+}$ T cells in response to activation with Dynal and Miltenyi anti-CD3/anti-CD28 mAb coated beads at different bead:cell ratios and the kinetics of this activation.

2.7.1 Kinetics of anti-CD3/anti-CD28 mAb coated bead-induced activation

Preliminary experiments assessed the kinetics of $CD4^+$ and $CD8^+$ T cell activation in response to Dynal and Miltenyi anti-CD3/anti-CD28 mAb coated beads by examining the activation over 5 days. The proportion of cells expressing CD25 and CD69 and the intensity of this expression were assessed in two volunteers in order to characterise the activation response profile to these stimuli. These are presented separately for each volunteer below (Figure 2-23 - Figure 2-26).

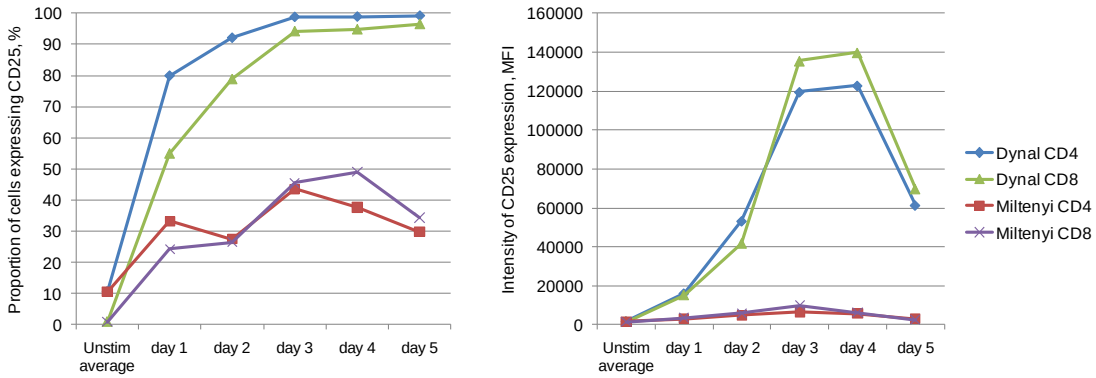

Figure 2-23: Effect of Dynal and Miltenyi anti-CD3/anti-CD28 mAb coated beads on CD25 expression by $CD4^+$ and $CD8^+$ T cells (Volunteer 1).
The proportion (%) of $CD4^+$ and $CD8^+$ T cells expressing CD25 (**left panel**) and the intensity of this expression on the positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with either Dynal or Miltenyi anti-CD3/anti-CD28 mAb coated beads for between 1 and 5 days prior to assessing CD25 expression. The unstimulated (unstim) average is a composite of the unstimulated control value from each of the 5 days.

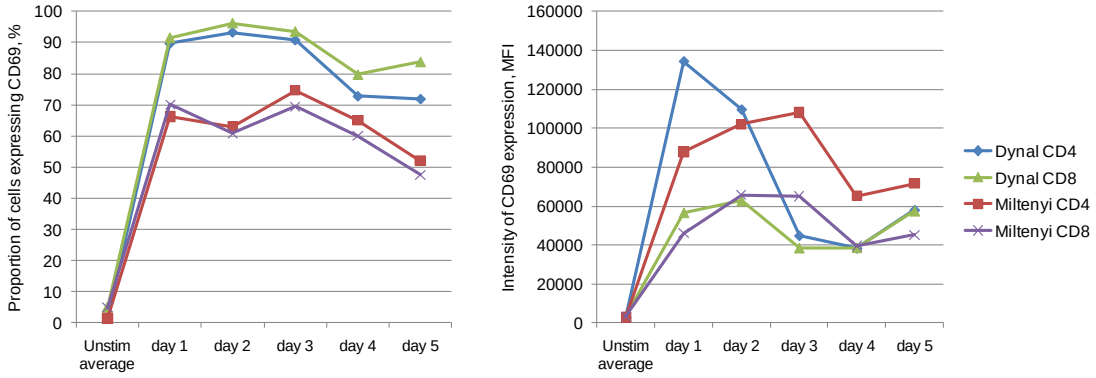

Figure 2-24: Effect of Dynal and Miltenyi anti-CD3/anti-CD28 mAb coated beads on CD69 expression by $CD4^+$ and $CD8^+$ T cells (Volunteer 1).
The proportion (%) of $CD4^+$ and $CD8^+$ T cells expressing CD69 (**left panel**) and the intensity of this expression on the positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with either Dynal or Miltenyi anti-CD3/anti-CD28 mAb coated beads for between 1 and 5 days prior to assessing CD69 expression. The unstimulated (unstim) average is a composite of the unstimulated control value from each of the 5 days.

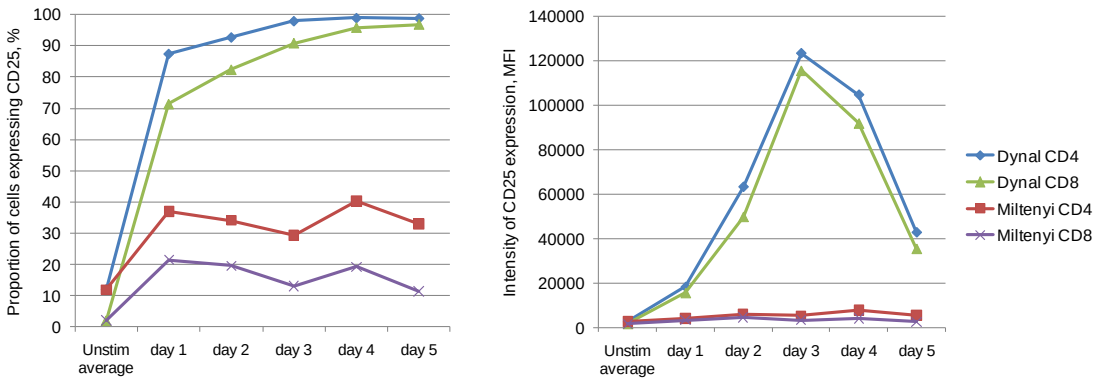

Figure 2-25: Effect of Dynal and Miltenyi anti-CD3/anti-CD28 mAb coated beads on CD25 expression by CD4$^+$ and CD8$^+$ T cells (Volunteer 2).
The proportion (%) of CD4$^+$ and CD8$^+$ T cells expressing CD25 (**left panel**) and the intensity of this expression on the positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with either Dynal or Miltenyi anti-CD3/anti-CD28 mAb coated beads for between 1 and 5 days prior to assessing CD25 expression. The unstimulated (unstim) average is a composite of the unstimulated control value from each of the 5 days.

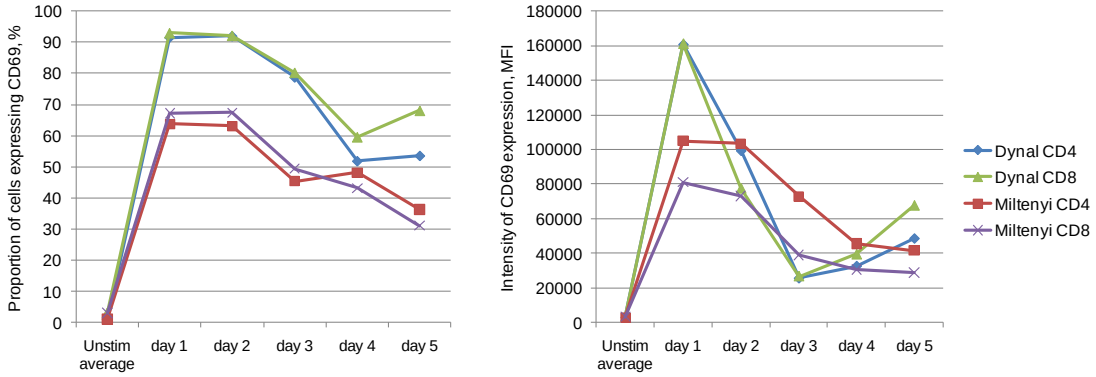

Figure 2-26: Effect of Dynal and Miltenyi anti-CD3/anti-28 mAb coated beads on CD69 expression in CD4$^+$ and CD8$^+$ T cells (Volunteer 2).
The proportion (%) of CD4$^+$ and CD8$^+$ T cells expressing CD69 (**left panel**) and the intensity of this expression on the positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with either Dynal or Miltenyi anti-CD3/anti-CD28 mAb coated beads for between 1 and 5 days prior to assessing CD69 expression. The unstimulated (unstim) average is a composite of the unstimulated control value from each of the 5 days.

A similar pattern of CD4$^+$ and CD8$^+$ T cell activation (measured by cell surface expression of CD25 and CD69) in response to the two different anti-CD3/anti-CD28 mAb coated beads was apparent in both volunteers, although the Dynal beads induced more activation than the Miltenyi beads. CD25 expression was increased after 1 day of stimulation and the proportion of cells expressing this activation marker continued to increase expression over 3 days, after which it plateaued, close to 100% with the Dynal

bead and less than 50% with the Miltenyi bead stimulus. The intensity of CD25 receptor expression increased over the first 3 days before decreasing and was many orders of magnitude greater with the Dynal beads than with the Miltenyi beads. CD69, being an earlier marker of activation, peaked sooner, within 1-2 days, before beginning to decrease over the remainder of the 5 days, although it continued to be expressed at high levels throughout this time.

These initial experiments which also assessed the activation of PBMC subpopulations with different Dynal bead:cell ratios revealed that almost 100% of cells were activated and expressed CD69 after 24 hr at the 1:1 bead:cell ratio. At lower bead ratios, some cells were being activated maximally, with other cells remaining unactivated.

On the basis of these activation kinetics experiments, a 3-day incubation with Miltenyi anti-CD3/anti-CD28 mAb coated beads was selected as an approach to assess the effect of opioids on T cell activation. This gave sub-maximal stimulation and thereby would enable enhancing as well as suppressive effects to be detected. This is also critical as these co-cultures should be incubated for at least 3 days to allow for T cell activation and subsequent cytokine production, which enables a more physiological microenvironment to be replicated *in vitro*.

The effect of Miltenyi anti-CD3/anti-CD28 mAb coated beads on NK cell activation was also assessed in this assay. For this the PBMCs were isolated and activated as described above and the expression of CD69 and CD25 by $CD16^+$ and $CD56^+$ NK cells determined.

2.8 Influence of opioids on cytokine secretion by activated peripheral blood mononuclear cells

The supernatants from the Miltenyi anti-CD3/anti-CD28 mAb coated bead PBMC stimulation assays and IL-2 stimulated PBMCs, as well as unstimulated samples (described above) were harvested and stored at -80°C until they were aliquoted and cytokine levels analysed using BD Bioscience Cytometric Bead Arrays (CBAs) and a BD FACSArray™ flow cytometer. The underlying principle of the assay is that populations of beads that are labelled with different levels of two fluorescent molecules, in order to confer upon them distinctive fluorescent properties, are coated with antibodies to the appropriate cytokines. The beads are then incubated with the sample (or known concentrations of cytokines in order to generate a standard dose-response

curve), and the bound cytokine is detected using a secondary ‘capture’ antibody that is conjugated to a third fluorochrome. The beads are then identified on the basis of their fluorescence and the intensity of capture antibody binding is quantified (Figure 2-27 - Figure 2-28).

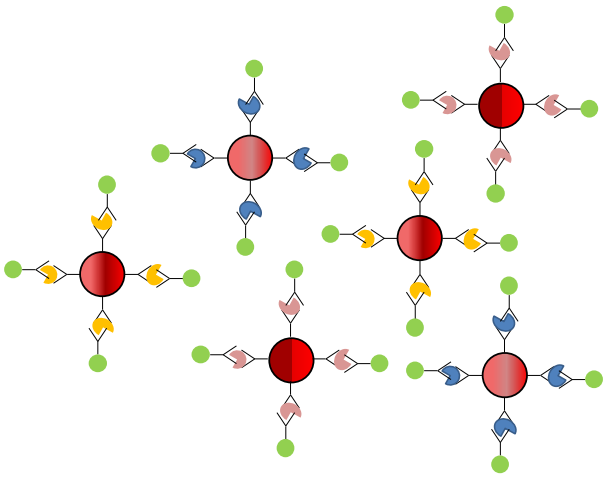

Figure 2-27: Schematic of cytometric bead analysis system.
Each set of beads are labelled with different intensity of two flurochromes (represented by different grades of red) to enable detection by the BD FACSArray™, and specific positioning on a two dimensional acquisition grid (Figure 2-28). The beads are also coated with a specific antibody to a cytokine, enabling binding of that cytokine. There are also labelled capture antibodies (green) which detect a different epitope on the cytokine, the amount of fluorescence, and hence concentration, of the cytokine is determined by the amount of binding of this labelled capture antibody. In this diagram three different bead types are represented, each specifically detecting a cytokine (denoted as blue, yellow and pink).

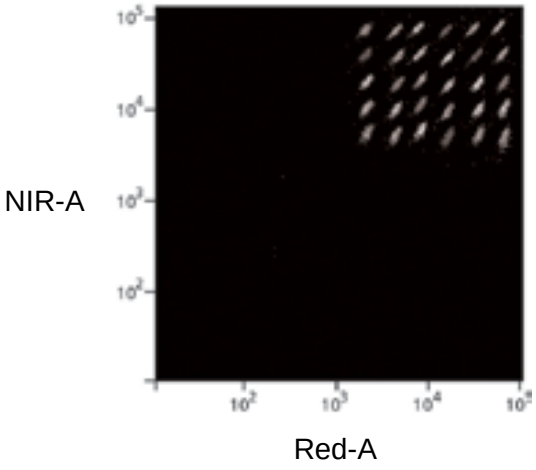

Figure 2-28: Acquisition grid on the BD FACSArray™.
Each bead subset has a specific position determined by the intensity of two flurochromes on the bead (on red and near infrared, NIR). Each of the 30 different potential positions represents an individual cytokine, which can be further analysed to determine its concentration by the intensity of the capture antibody.

Although ELISAs are traditionally the “standard” for the quantification of ligands and especially cytokines, there is a vast literature comparing bead assay detection and ELISAs (Elshal and McCoy, 2006). The majority of these studies have shown good correlations between bead array assays and ELISAs, however the degree of correlation does vary, due to the antibodies used in each of the assays and how these comparisons were made. Both ELISAs and CBAs show similar results, provided the same clones of antibodies are used; and the same assay and antibodies should be used for all tests which are to be compared (Elshal and McCoy, 2006).

Cytokines are cell signalling molecules primarily secreted from immune cells which regulate both humoral and cellular immune responses. The functional classification is used clinically and experimentally, creating two groups. The type 1 T_H cell cytokines, which are pro-inflammatory, enhance the cellular immune response (i.e. IFN-γ, TNF-β), and type 2 T_H cell cytokines (i.e. IL-4, IL-10, IL-13) which promote antibody (humoral) immunity and can suppress cellular immune responses (Figure 2-29) (Cutolo et al., 1998, Logani et al., 2011, Serreze et al., 2001, Teixeira et al., 2005, Yates et al., 2000).

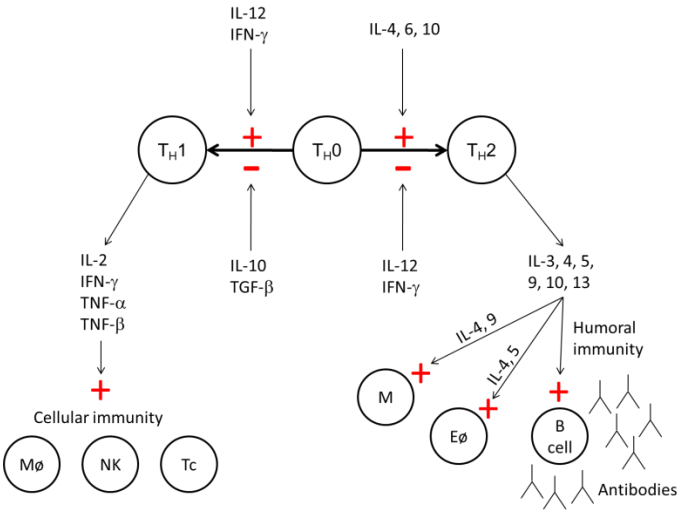

Figure 2-29: Differentiation of T_H cells.
Naïve $CD4^+$ T helper cells (T_H0) under cytokine influence differentiate into T_H1, T_H2 or other T cell sub-populations. IL-12 and IFN-γ promote the differentiation towards T_H1 cells and thereby enhance the cellular immune response. IL-4, IL-6 and IL-10 promote differentiation towards T_H2 cells and enhance antibody (humoral) responses. The cytokines produced by the predominant arm of this response feedback to further enhance differentiation along that arm and inhibit the other arm. The T_H1 response drives cellular immunity, with activation of the monocyte-macrophage lineage (Mø), natural killer cells (NK) and $CD8^+$ cytotoxic T cells (Tc). The T_H2 response drives humoral immunity by activating mast cells (M), eosinophils (Eø) and B cells (B), increasing antibody production (Cutolo et al., 1998, Klein et al., 2004, Logani et al., 2011, Serreze et al., 2001, Teixeira et al., 2005, Yates et al., 2000).

There are several subgroups of T cells having different immunological roles which are in part classified by their pattern of cytokine production. As well as the classical T_H1 and T_H2 cells, these now include T_H9, T_H17, T_H22 and T_{reg} cells.

T_H1 cells are involved in the cellular immune response against intracellular pathogens. They are characterised by the production of pro-inflammatory cytokines (IFN-γ, IL-2, and lymphotoxin-α). These cytokines stimulate the phagocytosis and destruction of microbial pathogens by macrophages and other lymphocytes (Kapsenberg, 2003, Liew, 2002). When T_H1 cells produce IFN-γ, this prompts macrophages to produce TNF and oxygen free radicals which kill microorganisms within phagosomes and lysosomes.

T_H2 cells are involved in the humoral immune response against extracellular pathogens. They are characterised by the production of IL-4, IL-5, IL-6, IL-10, and IL-13 (Kapsenberg, 2003, Liew, 2002). IL-4 stimulates antibody production and IL-5 stimulates an eosinophil response toward large extracellular parasites. On the other hand, when T_H2 cells produce IL-4 and IL-10, these cytokines block the microbial killing that is activated by IFN-γ.

T_H9 cells are characterised by the production of IL-9 and IL-10 (Dardalhon et al., 2008). Their differentiation is promoted by TGF-β in combination with IL-4, this is enhanced by IL-21 (Wong et al., 2010). They are thought to be involved in intestinal response to helminthes and in allergy, however further studies are needed to reveal the function of these cells in pathogen clearance and inflammatory diseases (Ma et al., 2010).

T_H17 cells are involved in the host defence against extracellular pathogens (van de Veerdonk et al., 2009). They do not produce classical T_H1 or T_H2 cytokines such as IFN-γ or IL-4, but produce IL-17A and IL-22 (Fouser et al., 2008). IL-6, IL-23 and TGF-β promote the differentiation of naïve T_H cells towards the T_H17 phenotype. T_H17 cells have an important role in autoimmune tissue injury (Ferraccioli and Zizzo, 2011). IL-17A increases the induction of pro-inflammatory cytokines such as TNF, IL-6, and IL-1β.

T_H22 cells produce IL-22, IL-23 and TNF-α but not IFN-γ, IL-4, or IL-17. They are pro-inflammatory and have a role in epidermal immunity and promote angiogenesis, fibrosis and wound healing (Eyerich et al., 2009).

Regulatory T cells produce IL-4, IL-10, IFN-α and TGF-β, which promote the differentiation of naïve T_H cells toward the T_{reg} phenotype. They tend to inhibit the immune response and maintain immune self-tolerance (Shalev et al., 2011).

Cytokines are induced by specific stimuli, including bacterial products, and can activate and induce differentiation in many cell types as well as modulating the production of other cytokines. This complex regulatory network of cytokines may ultimately result in the eradication of the pathogen or if the cytokine balance is suboptimal then this will be deleterious to the host (either due to overwhelming infection or autoimmunity/allergy). The production of specific cytokines in response to infection or tumour may determine the clinical outcome (Lucey et al., 1996, Watkins et al., 1995).

Preliminary studies analysed the effect of opioids on the concentrations of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN-α, IFN-γ, TNF-α and IP-10 in the supernatants of the cell cultures.

IL-1β is a member of the IL-1 cytokine family, produced as a precursor, Pro-IL-1β, by activated macrophages, monocytes, fibroblasts and DCs, it is subsequently cleaved by caspase 1 to its active form, IL-1β. It enhances the inflammatory response against infection, and is involved in cell proliferation, differentiation, and apoptosis. IL-1β increases the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes to sites of inflammation and can have effects at femtomolar concentrations (Dinarello, 1994). It also increases body temperature by an effect on the hypothalamic thermoregulatory centre. IL-1β is associated with an increased sensitivity to inflammatory pain (Gabay et al., 2010), both peripherally and in the CNS where it induces cyclooxygenase-2. The two forms of IL-1 bind to the same physiologically active receptor, IL-1R1, which is composed of two related subunits that transmit intracellular signals via activation of the MAPK/JNK signalling pathway, leading to activation of the NF-κB complex (Kuno and Matsushima, 1994). This pathway overlaps with the TLR and IL-18 receptor signalling pathways.

IL-2 is important in the inflammatory response against infection. It is a member of the IL-2 subfamily of cytokines, which includes IL-4, IL-7, IL-9, IL-15 and IL-21. It is produced by activated T cells, mainly $CD4^+$ cells, often in response to IL-1β produced by monocytes and DCs (when in PBMC culture). IL-2 produced by activated T cells can have an autocrine effect, inducing further activation, receptor expression and IL-2

production, stimulating the growth, differentiation, proliferation and survival of antigen-selected T and B cells (Curfs et al., 1997). IL-2 also activates NK cells (although their receptors are of lower affinity compared with T and B cells), which promotes defence against tumours (Castriconi et al., 2007). IL-2 signals through a receptor complex consisting of IL-2 receptor α (CD25), IL-2 receptor β (CD122) and a common γ chain (γc), which is shared by this subfamily of cytokine receptors. Binding of IL-2 activates the Ras/MAPK, JAK/STAT and PI 3-kinase/Akt signalling pathways. Antigen binding to the TCR stimulates the secretion of IL-2 and the expression of IL-2 receptors on T cells.

IL-4 induces differentiation of naïve $CD4^+$ T cells to T_H2 cells and when activated by IL-4, T_H2 cells secrete IL-4. It also enhances T_H2 cell proliferation and the proliferation of anti-IgM stimulated murine B cells (Paul, 1991). Parasite and allergen products mediate activation of epithelial cells, basophils and DCs and the production of IL-4, IL-25 and IL-33 in the initiation and amplification of T_H2 immune responses *in vivo* (Paul and Zhu, 2010, Sokol and Medzhitov, 2010). Basophils are central to the initiation of the polarisation of T_H2 immune responses by the production of IL-4 (Paul and Zhu, 2010, Sokol and Medzhitov, 2010). In mice IL-4 induces the production of IgE (Paul, 1991). IL-4 acts as an anti-inflammatory cytokine, modulating the immune response by suppressing the production of IL-1, IL-6, IFN-γ and TNF-α (Lee et al., 1995a). IL-4 and IFN-γ have antagonistic properties, polarising the T cell response towards T_H2 and T_H1 respectively (O'Garra and Arai, 2000). The heterodimeric IL-4 receptor has an α chain with the IL-4 extracellular binding domain and a transactivating chain, either the common γ chain or the α1 chain of the IL-13 receptor (Zhang et al., 2002).

IL-6 can act as both a pro-inflammatory and anti-inflammatory cytokine (Xing et al., 1998). It is secreted mainly by T cells and macrophages (in response to specific pathogen-associated molecular patterns binding to pathogen recognition receptors) and stimulates the immune system in response to tissue damage, especially burns. IL-6 is also produced from muscle, and is elevated by exercise (Pedersen, 2011). Osteoblasts secrete IL-6 to stimulate osteoclasts (Eriksen et al., 2010). Smooth muscle cells in the tunica media of blood vessels also produce IL-6 where it is pro-inflammatory. IL-6 is produced early in inflammation and is important for controlling acute infection as it initiates the acute phase response and increases C-reactive protein (CRP) production from the liver (Heikkila et al., 2007). It also causes fever, as it crosses the blood-brain barrier promoting prostaglandin E_2 synthesis in the

hypothalamus. Its pro-inflammatory properties include maturation and activation of neutrophils and macrophages, differentiation and maintenance of $CD8^+$ T cells and NK cells, and under some circumstances increased expression of IL-1 and TNF-α (Barton, 1996). IL-6 can also act as an anti-inflammatory cytokine as in certain circumstances it can inhibit the pro-inflammatory cytokines TNF-α and IL-1, and activate IL-1 receptor antagonist and IL-10. IL-6 signals by binding to IL-6Rα chain (CD126) and the signal-transducing component gp130 (CD130). Gp130 is expressed in most tissues, whereas the expression of IL-6Rα is more restricted. When IL-6 interacts with its receptor, gp130 and IL-6Rα form a complex, bringing together the intracellular regions of gp130 to initiate a signal transduction cascade through the transcription factors JAK/STAT and MAPK (Heinrich et al., 2003a).

IL-8 is a chemokine which is also known as C-X-C chemokine ligand 8 (CXCL8) (Bacon et al., 2002) and it can be secreted by any cells involved in the innate immune response with TLRs, especially macrophages, epithelial and endothelial cells. It is secreted in response to TLR activation by bacterial products, especially LPS. IL-8 activates neutrophils and is involved in their migration towards sites of inflammation where it can be further produced by neutrophils recruiting monocytes (Curfs et al., 1997). Both monomer and homodimer forms of IL-8 bind to CXCR1 (IL-8RA) and CXCR2 (IL-8RB), with the homodimer being more potent (Bacon et al., 2002). It is important in innate immune system chemotaxis, acting as a chemoattractant, and is also a potent angiogenic factor. The primary function of IL-8 is to recruit neutrophils to phagocytose foreign bodies, the antigens of which trigger TLR's.

IL-10 is a T_H2 cytokine with anti-inflammatory properties (Sabat et al., 2010) which inhibits the synthesis of pro-inflammatory T_H1 cytokines (IL-2, IFN-γ and TNF-α), MHC class II antigens and co-stimulatory molecules on macrophages. Although it down-regulates T cell-mediated immunity, it enhances B cell survival, proliferation and antibody production as well as stimulating NK cell cytotoxicity (Mocellin et al., 2004). IL-10 is produced primarily by monocytes but also by T_H2, T_{reg} and mast cells (Sabat et al., 2010). It is also released by cytotoxic T cells in order to inhibit NK cells during the immune response to viral infection. Tumours might also utilise a similar mechanism to optimise their survival (Szkaradkiewicz et al., 2010). IL-10 is homodimer molecule which binds to the IL-10 receptor (IL-10R) which is expressed on many immune cells including $CD4^+$ T cells, NK cells and macrophages (Grutz, 2005). Binding of IL-10 to the IL-10R activates JAK1 and Try2 tyrosine kinases, phosphorylating STAT1 and

STAT3; and can block NF-κB activity (Mocellin et al., 2004).

IL-12p70 is a heterodimeric cytokine composed of p35 and p40 subunits that are linked by disulphide bonds and the production of the biologically active IL-12p70 requires the genes of both subunits to be expressed (Watford et al., 2003). It is primarily produced by activated monocytes and B cells and it enhances the proliferation and cytotoxicity of T cells and NK cells (Adorini, 2003, Trinchieri, 1994). IL-12p70 promotes the differentiation of naïve $CD4^+$ T cell towards the T_H1 phenotype and, along with NK cell and macrophages, induces T cells to produce IFN-γ (Watford et al., 2003). IL-12p70 production early in infection is vital in the control of intracellular pathogens (Biron and Gazzinelli, 1995). IL-10 inhibits IL-12p70 production and thereby suppresses the development of T_H1 responses (Adorini, 2003). The IL-12 receptor is composed of two chains IL-12Rß1 and IL-12Rß2, binding of IL-12p70 activates the JAK-STAT4 signalling pathway (Trinchieri, 2003).

IL-17A along with IL-17B – F comprises the IL-17 family. IL-17A is primarily produced by T_H17 cells, a recently reported lineage of $CD4^+$ T cells that are characterised by the production of this cytokine (Fouser et al., 2008). IL-17A is involved in inducing and mediating pro-inflammatory responses and has an important role in regulating granulocytes. It is also important for host defence and is commonly associated with allergic and autoimmune diseases (Gaffen, 2009). IL-17 induces the production of many other cytokines, including IL-6, IL-1β, TGF-β, TNF-α and IL-8, as well as prostaglandins (e.g., prostaglandin E_2) from many cell types including fibroblasts, endothelial cells, epithelial cells, keratinocytes, and macrophages. IL-17 mobilises neutrophils from the bone marrow and promotes production of IL-1. In contrast to the restricted expression of IL-17A, the IL-17 receptor A is ubiquitously expressed, and thus most cells are potential physiological targets of IL-17A (Gaffen et al., 2006).

IFN-γ is a homodimer which belongs to the group of interferons. The term interferon was originally coined as these cytokines "interfere" with viral replication within host cells. There are seven different interferons in humans and IFN-γ is the only member of the type II group (the type I group includes IFN-α and β). Interferons activate NK cells and cells of the monocyte/macrophages lineage; increase T cell recognition of infection and tumour cells by up-regulating antigen presentation (increasing MHC class I and II expression, and immunoproteasome activity), and increase the ability of uninfected host cells to resist viral infection (Bach et al., 1997). IFN-γ is produced by T_H1 and NK

cells. It induces naïve T cell differentiation towards the T_H1 polarity, inhibiting T_H2 responses. IFN-γ antagonises the effect of IL-4 and is itself antagonised by IL-4 (O'Garra and Arai, 2000). It binds to the IFN-γ receptor and modulates nearly all phases of the immune and inflammatory responses (Farrar and Schreiber, 1993). IFN-γ regulates the production of a variety of other pro-inflammatory cytokines including IL-12 and TNF-α (Bach et al., 1997). IFN-γ activates the JAK-STAT signalling pathway (Platanias, 2005). JAKs associate with IFN receptors and upon binding IFN-γ, phosphorylate STAT1 and STAT2. This results in IFN-stimulated gene factor 3 complex (STAT1, STAT2 and IRF9) which translocates into the cell nucleus inducing gene transcription (Platanias, 2005). Interferons can activate several other signalling cascades including CrkL, a nuclear adaptor for STAT5 that regulates signalling through the C3G/Rap1 pathway, as well as the phosphatidylinositol 3-kinase signalling pathway, increasing protein synthesis and cell proliferation (Alsayed et al., 2000).

The homotrimer, **TNF-α**, is a pro-inflammatory mediator which promotes systemic inflammation, stimulates the acute phase reaction and induces pyrexia. TNF-α is important for controlling acute infection. It has a plethora of effects including the regulation of immune cells, being a chemoattractant for neutrophils and increasing their adhesion and migration. In macrophages it stimulates phagocytosis, and production of IL-1. TNF-α induces apoptotic cell death and inhibits tumorigenesis and viral replication. It increases production of corticotrophin-releasing factor which stimulates the HPA axis. TNF-α is mainly produced by macrophages, but also by lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neuronal tissue. TNF-α is primarily secreted in response to LPS, whereas IL-1, IL-6 and IL-10 inhibit its secretion (Curfs et al., 1997). TNF-α can bind to the TNF-R1 and TNF-R2 receptors; the former is ubiquitous whereas the latter is only expressed on immune cells. Upon binding to TNF-α, the receptors form trimers inducing a conformational change, leading to dissociation of the inhibitory protein Silencer of Death Domain (SODD) from the intracellular death domain. This dissociation enables TNF-R1-associated death domain protein (TRADD) to activate; NF-κB, MAPK and induction of death signalling pathways (Ihnatko and Kubes, 2007, Wullaert et al., 2006).

IFN-γ induced protein 10 kDa (**IP-10**), also known as C-X-C chemokine ligand 10 (CXCL10), is secreted by monocytes and endothelial cells in response to IFN-γ (Luster et al., 1985). IP-10 has a role in chemoattraction of monocytes/macrophages, T cells, NK cells and DCs, promotion of T cell adhesion to endothelial cells, antitumor activity,

and inhibition of bone marrow colony formation and angiogenesis (Angiolillo et al., 1995, Dufour et al., 2002, Rabquer et al., 2011). IP-10 binds to the cell surface chemokine receptor CXCR3 (Booth et al., 2002).

Preliminary experiments showed that IL-4, IL-12p70 and IFN-α were below the detection threshold and these cytokines were not assessed in the main cytokine analysis experiments. IL-17A and IP-10 were detected by the same laser and would need to be analysed independently, further investigation of these cytokines showed that IP-10 was below the level of detection in some of the samples and it would therefore be more appropriate to analyse the effect of opioids on IL-17A. For reasons that remain unclear, the assay failed to detect IL-2 in any of the samples that were analysed and no data for these analyses are therefore available. IL-6 and IL-8 were above the highest standard in the standard curve and needed dilution to ensure accurate quantification. The final array of cytokines analysed were IL-1β, IL-6, IL-8, IL-10, IL-17A, IFN-γ and TNF-α.

2.9 Statistical Analysis

Statistical analysis was undertaken in collaboration with Kathleen Baster (Senior Consultant, Statistical Services Unit, University of Sheffield). Several options exploring different analyses methods were undertaken and it was decided that the optimal way to analyse the data was with a paired 2-way Student *t*-test. This test was chosen as each assay assessing the effect of an opioid on immune function had a ‘no opioid’ control and the paired Student *t*-test allowed a direct comparison between the effects that are induced with each concentration of opioid and it’s no opioid equivalent (baseline). The potential of both stimulatory and inhibitory changes induced by opioids could be interrogated using the 2-way test.

Initially the Univariate General Linear Model in Statistical package for the Social Sciences (SPSS) v15 was used. Homogeneity of variances were checked using the one way ANOVA and if these were non-significant (> 0.05) the data were then analysed using the Univariate General Linear Model, including subject as a 'random' factor. These tests were performed to check for variation between differences in the responsiveness of untreated and opioid-treated cells, as well as to check if there was a dose-dependent effect from the opioids using the Scheffe Post hoc test. If the homogeneity of variance was significant (< 0.05) the data were then log-transformed

and then analysed using the Univariate General Linear Model as described above. However, on further discussion with the statistician, using this approach meant that all of the baselines were treated equally and an average baseline value was generated in SPSS and compared with the average of each of the different opioid concentration values. As each experiment had its own baseline and not all concentrations of an opioid were tested against the same control, thus it is incorrect to combine the baselines for comparison. Using the paired 2-way Student *t*-test each value was compared to its appropriate control value.

The data were considered as being normally distributed as the means and medians were approximately the same, as a consequence of which the data were analysed as such.

Data are presented as mean ± standard error of the mean (SEM), or as individual experiments, as indicated.

**P* value < 0.05
***P* value < 0.01
****P* value < 0.005

Type I and type II errors

When testing a statistical hypothesis, two types of incorrect conclusions can be drawn. A type I error is when the null hypothesis is inappropriately rejected (false positive), indicating a test of poor specificity. A type II error is when the null hypothesis is falsely accepted (false negative), indicating a test of poor sensitivity.

As a parametric test, the *t*-test assumes normal sample distributions and large sample sizes are required to make this assumption. However, it has been shown that the use of a *t*-test with small sample sizes and none bell shaped sample distributions does not result in an increase of type I errors (Heeren and D'Agostino, 1987).

Although type I errors are considered to be more serious than type II errors, they are also important and can be large due to small sample sizes and small differences in the means between samples, and large variabilities (Bosshart, 2009).

CHAPTER 3

RESULTS

3.1 Effect of opioids on natural killer cells

NK cells are central to innate immune protection against cancer and viral infections. The effect of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on IL-2 stimulated NK cell cytotoxicity against K562 human erythroleukaemic target cells was assessed at different E:T cell ratios. The effect of these opioids on NK cell activation (as determined on the basis of the induction of CD69 expression) in response to both IL-2, and also as a bystander effect of Miltenyi anti-CD3/anti-CD28 mAb bead stimulation of PBMCs was also explored. Although CD69 expression is the most reliable and sensitive marker for $CD16^{+}56^{+}$ NK cell activation, CD25 expression might also be a marker of NK cell responsiveness and was also measured. Subsequent experiments evaluated the effect of morphine, tramadol and fentanyl on the cytotoxic potential of unstimulated NK cells at an E:T ratio of 100:1, and also on their activation status (CD69 expression).

3.1.1 Effect of opioids on IL-2 stimulated natural killer cell cytotoxicity

Platelet-free PBMCs were isolated from whole blood, freshly taken from healthy volunteers, by density gradient centrifugation and incubated for 3 days with IL-2 (100 U/ml) in the presence or absence of two clinically relevant concentrations of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine or codeine. K562 target cell killing at the different E:T ratios were determined, the amount of background (non-specific) target cell death subtracted and the AUCC was calculated. The proportion of NK cells in the PBMCs was determined by flow cytometry and these data were used to calculate the adjusted AUCC. The data presented below show the cytotoxicity of NK cells against K562 target cells (Y axis) for the different E:T ratios (X axis) for the opioids (Figure 3-1 - Figure 3-8). Values for the adjusted AUCC means (± SEM) for each of the experimental conditions are indicated in the figure legends.

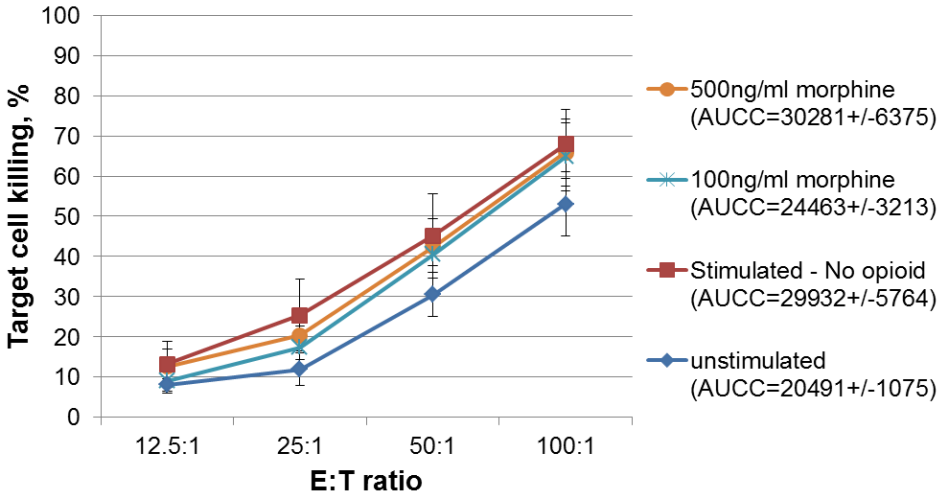

Figure 3-1: Effect of morphine on IL-2 induced NK cell cytotoxicity of K562 target cells. Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and morphine or no opioid control (there was also an unstimulated control, without IL-2 or morphine). The PBMCs (effector cells) were then incubated for 3 hr with K562 (target cells) at the effector:target (E:T) ratios shown on the graph and the proportion (%) of killed K562 cells at the different E:T ratios was measured using flow cytometry. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods (section 2.5.3.1 and Figure 2-3). Data are means ± SEM of 3 independent experiments. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and morphine-treated PBMCs was observed (paired Student *t*-test).

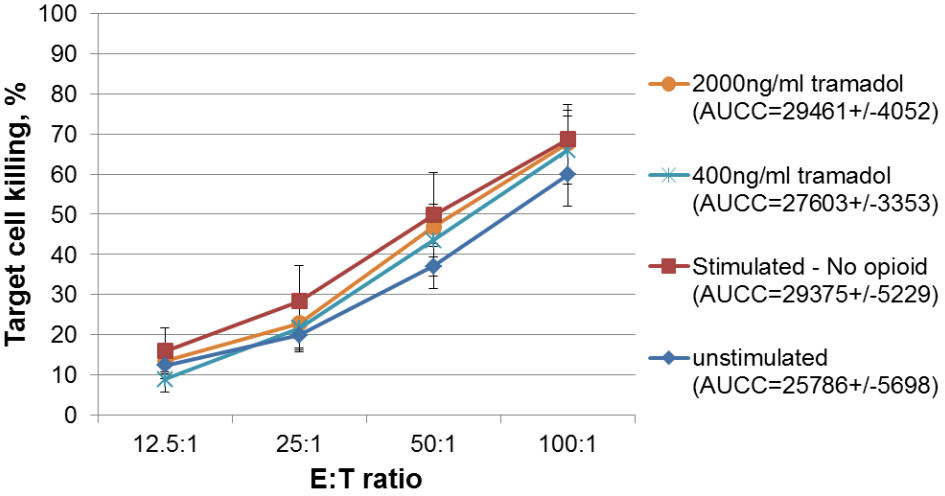

Figure 3-2: Effect of tramadol on IL-2 induced NK cell cytotoxicity of K562 target cells. Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and tramadol or no opioid control (there was also an unstimulated control, without IL-2 or tramadol). The PBMCs (effector cells) were then incubated for 3 hr with K562 (target cells) at the effector:target (E:T) ratios shown on the graph and the proportion (%) of killed K562 cells at the different E:T ratios was measured using flow cytometry. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods (section 2.5.3.1 and Figure 2-3). Data are means ± SEM of 3 independent experiments. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and tramadol-treated PBMCs was observed (paired Student *t*-test).

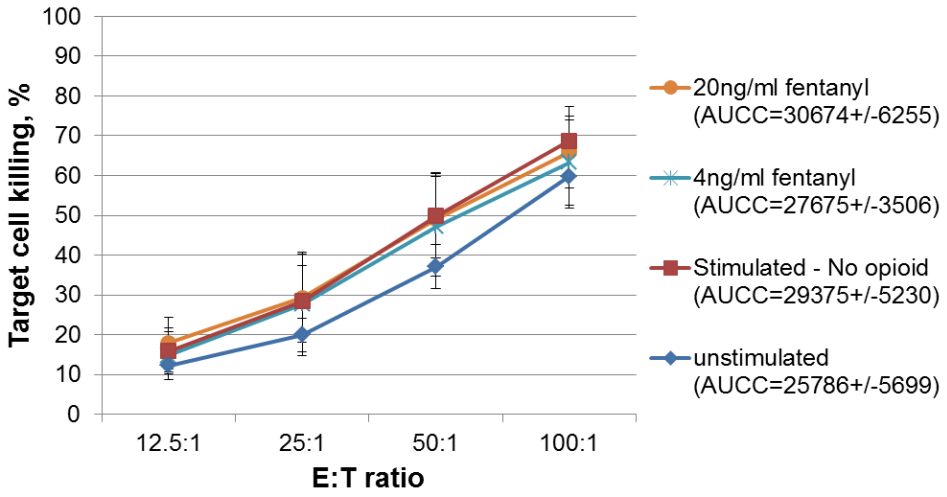

Figure 3-3: Effect of fentanyl on IL-2 induced NK cell cytotoxicity of K562 target cells.
Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and fentanyl or no opioid control (there was also an unstimulated control, without IL-2 or fentanyl). The PBMCs (effector cells) were then incubated for 3 hr with K562 (target cells) at the effector:target (E:T) ratios shown on the graph and the proportion (%) of killed K562 cells at the different E:T ratios was measured using flow cytometry. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods (section 2.5.3.1 and Figure 2-3). Data are means ± SEM of 3 independent experiments. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and fentanyl-treated PBMCs was observed (paired Student *t*-test).

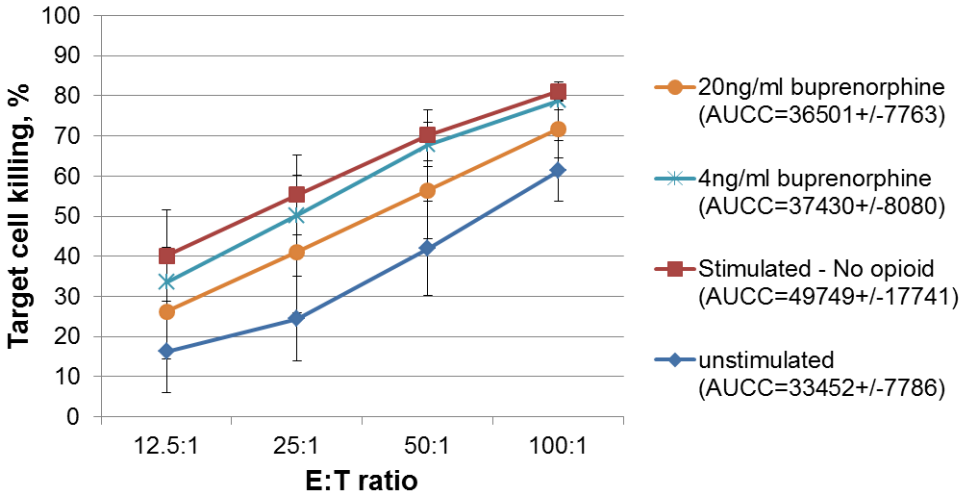

Figure 3-4: Effect of buprenorphine on IL-2 induced NK cell cytotoxicity of K562 cells.
Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and buprenorphine or no opioid control (there was also an unstimulated control, without IL-2 or buprenorphine). The PBMCs (effector cells) were then incubated for 3 hr with K562 (target cells) at the effector:target (E:T) ratios shown on the graph and the proportion (%) of killed K562 cells at the different E:T ratios was measured using flow cytometry. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods (section 2.5.3.1 and Figure 2-3). Data are means ± SEM of 3 independent experiments. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and buprenorphine-treated PBMCs was observed (paired Student *t*-test).

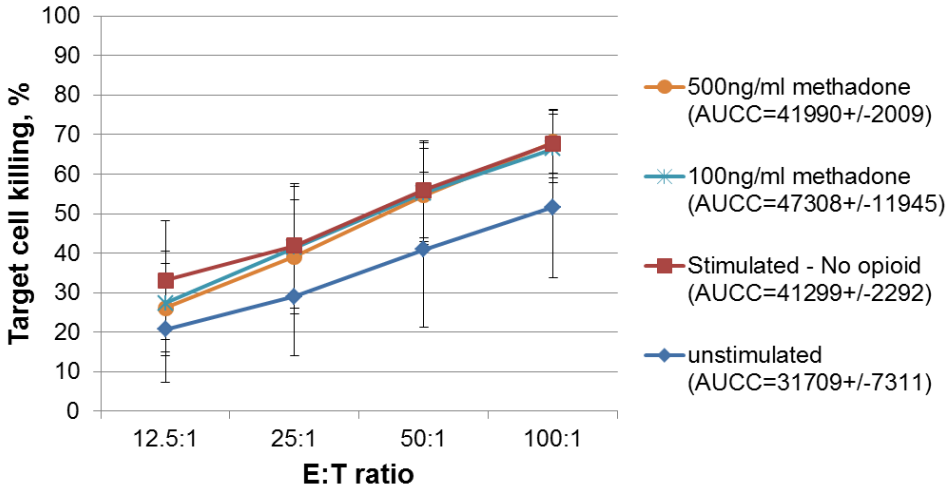

Figure 3-5: Effect of methadone on IL-2 induced NK cell cytotoxicity of K562 target cells. Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and methadone or no opioid control (there was also an unstimulated control, without IL-2 or methadone). The PBMCs (effector cells) were then incubated for 3 hr with K562 (target cells) at the effector:target (E:T) ratios shown on the graph and the proportion (%) of killed K562 cells at the different E:T ratios was measured using flow cytometry. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods (section 2.5.3.1 and Figure 2-3). Data are means ± SEM of 3 independent experiments. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and methadone-treated PBMCs was observed (paired Student *t*-test).

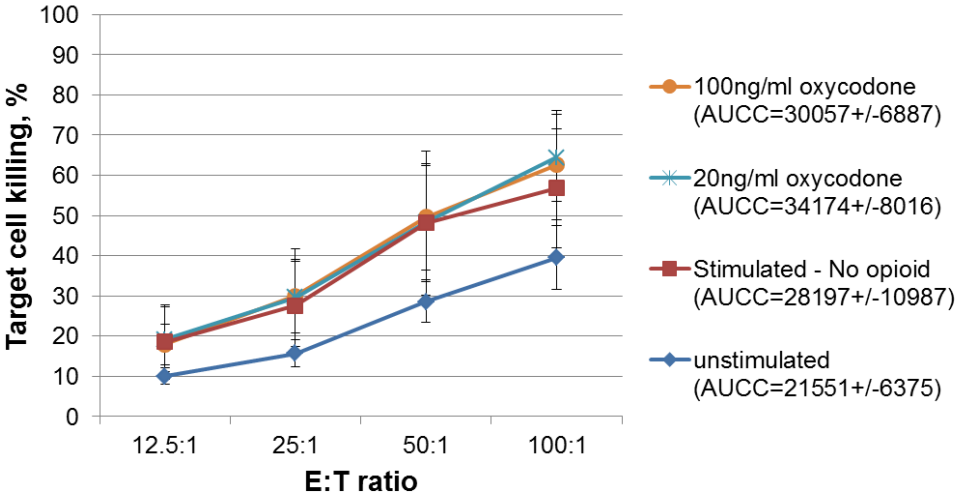

Figure 3-6: Effect of oxycodone on IL-2 induced NK cell cytotoxicity of K562 target cells. Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and oxycodone or no opioid control (there was also an unstimulated control, without IL-2 or oxycodone). The PBMCs (effector cells) were then incubated for 3 hr with K562 (target cells) at the effector:target (E:T) ratios shown on the graph and the proportion (%) of killed K562 cells at the different E:T ratios was measured using flow cytometry. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods (section 2.5.3.1 and Figure 2-3). Data are means ± SEM of 3 independent experiments. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and oxycodone-treated PBMCs was observed (paired Student *t*-test).

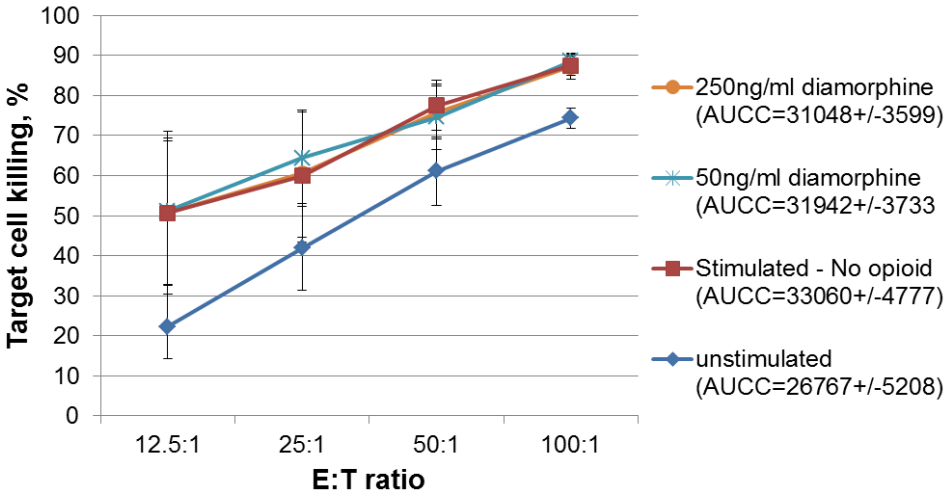

Figure 3-7: Effect of diamorphine on IL-2 induced NK cell cytotoxicity of K562 cells.
Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and diamorphine or no opioid control (there was also an unstimulated control, without IL-2 or diamorphine). The PBMCs (effector cells) were then incubated for 3 hr with K562 (target cells) at the effector:target (E:T) ratios shown on the graph and the proportion (%) of killed K562 cells at the different E:T ratios was measured using flow cytometry. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods (section 2.5.3.1 and Figure 2-3). Data are means ± SEM of 3 independent experiments. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and diamorphine-treated PBMCs was observed (paired Student *t*-test).

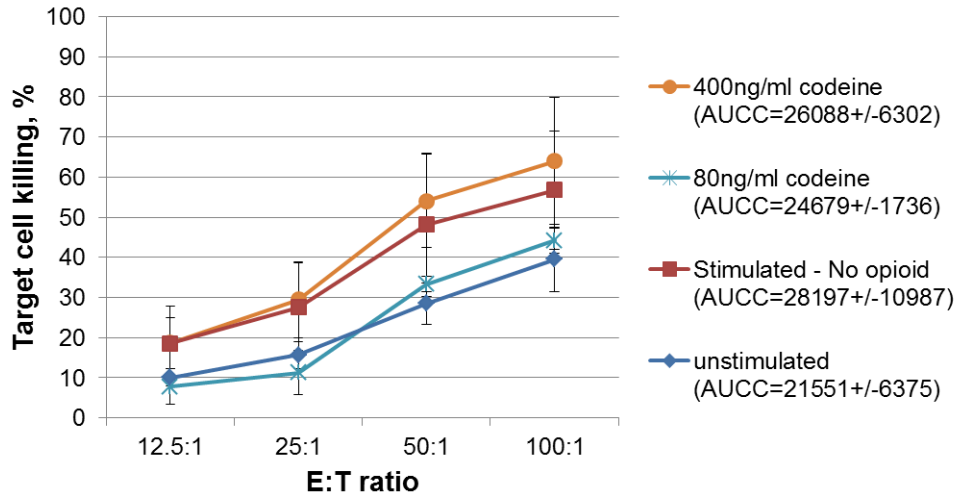

Figure 3-8: Effect of codeine on IL-2 induced NK cell cytotoxicity of K562 target cells.
Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and codeine or no opioid control (there was also an unstimulated control, without IL-2 or codeine). The PBMCs (effector cells) were then incubated for 3 hr with K562 (target cells) at the effector:target (E:T) ratios shown on the graph and the proportion (%) of killed K562 cells at the different E:T ratios was measured using flow cytometry. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods (section 2.5.3.1 and Figure 2-3). Data are means ± SEM of 3 independent experiments. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and codeine-treated PBMCs was observed (paired Student *t*-test).

IL-2 (100 U/ml) enhanced the ability of NK cells to kill K562 target cells. In all experiments the higher E:T ratios led to an increased killing of target cells. None of the opioids at the concentrations tested had a significant effect on the ability of IL-2 stimulated cells to kill K562 target cells (two tailed paired Student *t*-test, Figure 3-1 to 3.8). This might be because IL-2 potently stimulated the NK cells and the opioids were unable to inhibit this, or noticeably enhance it further. The influence of opioids on the activation status of unstimulated and IL-2 stimulated NK cells (CD69 expression) was also measured, as this might be more sensitive as a measure of effects on NK cell functional potential than cytotoxicity.

A relatively fixed cohort of volunteers was used throughout these studies in order to minimise the inter- and intra-individual variability in the NK cell cytotoxicity assay which has been observed previously (Hopkinson et al., 2007), as this might limit the sensitivity of the assay to the effects of opioids. Although some of the potential variability in the NK cell cytotoxicity assay was minimised by removing platelets from the PBMC preparations, other factors such as genetic, gender, menstrual cycle, exercise and diet were not compensated for (Campa et al., 2008, Chandra, 1999, Lawson et al., 2010, Manson, 2010). A previous study has shown that when NK cell cytotoxicity was assessed weekly in five donors over 3 weeks, variability was up to 51% in individual subjects and up to 32% for the means of all subjects (Hopkinson et al., 2007).

3.1.2 Effect of opioids on the cytotoxic potential of unstimulated natural killer cells

None of the opioids had an effect on the cytotoxicity of IL-2 stimulated NK cells. This might be because the immunoregulatory effects of opioids were not capable of tempering or enhancing the stimulatory capacity of IL-2. As a consequence, experiments assessing the effects of morphine, tramadol and fentanyl on the cytotoxic capacity of unstimulated NK cells were performed. For these experiments, isolated PBMCs (unstimulated) were incubated for 3 days with two clinically relevant concentrations of morphine, tramadol and fentanyl. Using flow cytometry, K562 target cell killing at the maximum E:T ratio of 100:1 was determined, as this is the most likely ratio to show any effect. The amount of background (non-specific) target cell death was subtracted.

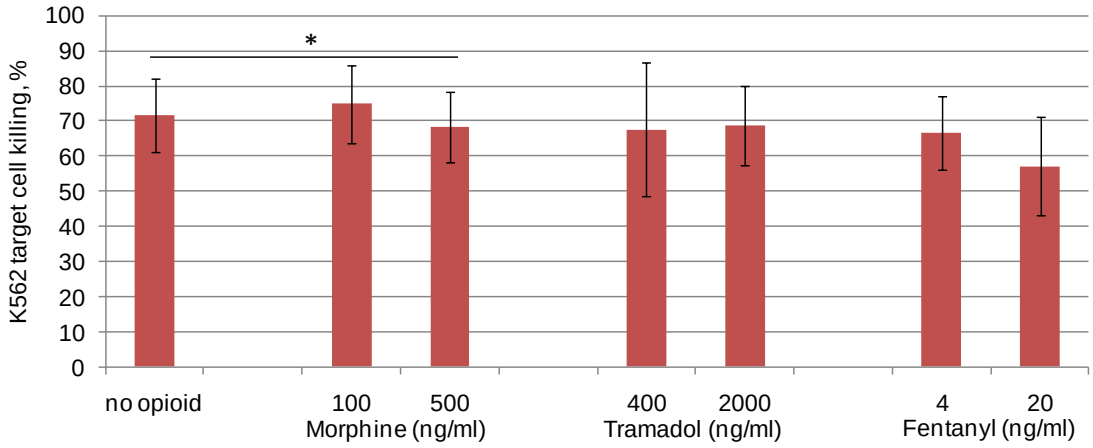

Figure 3-9: Effect of morphine, tramadol and fentanyl on the cytotoxic capacity of unstimulated NK cells (3-day incubation).
Unstimulated PBMCs were incubated with the indicated concentrations of morphine, tramadol and fentanyl for 3 days before assessing their ability to kill K562 cells. This is presented as % cytotoxicity using a PBMC to K562 (E:T) cell ratio of 100:1. Data are means ± SEM of 3 independent experiments (except 400 ng/ml of tramadol and 4 ng/ml of fentanyl, where n=2). 500 ng/ml of morphine for 3 days inhibited unstimulated NK cell cytotoxicity compared to untreated PBMCs, * $p<0.05$ (paired Student *t*-test).

Although 500 ng/ml of morphine significantly inhibited NK cell cytotoxicity, this effect was quantitatively very small (Figure 3-9) and unlikely to be of clinical importance. As there were no clear effects from the opioids tested, no further opioids were assessed using this experimental approach.

Opioid agonists might have a rapid effect on immune function and might induce receptor desensitisation, endocytosis and down regulation over time. Thus, the immediate effect of opioids on immune cells could be very different from those that are observed at later time points (Bidlack et al., 2006). If this remains the case in patients, then it is less likely to be a clinically significant problem in those on regular opioids. However, these short term effects might be significant for patients administered opioids in the acute situation, i.e. for trauma or minor surgery.

Rodent-derived data suggest that the immune effects of single opioid doses can dissipate within hours. For example, it has been shown in rats that a single subcutaneous injection of morphine suppresses NK cell cytotoxicity within hours, but that this suppression lasts less than a day (Nelson et al., 1997). This study indicates that the *in vivo* effects of morphine are dependent on its continuing presence and decline following metabolism and excretion.

The findings of Nelson's study might not necessarily be applicable to the current study, as it is expected that the concentrations of the opioids are relatively stable during culture. Nonetheless, in order to investigate the potential for such effects, some short incubation time experiments were performed. As this approach was aimed at screening for any potential effect, it was performed with only the higher doses of morphine, tramadol and fentanyl used in the NK assays, and only at the 100:1 E:T cell ratio. The shorter time point experiments only used unstimulated NK cells.

For these, unstimulated PBMCs were incubated with morphine, tramadol and fentanyl for 1, 3 and 24 hr prior to assessing NK cell cytotoxicity against K562 cells. None of the opioids had an effect on the cytotoxic capacity of unstimulated NK cells at any time point (Figure 3-10), data not shown for the 24-hr incubation.

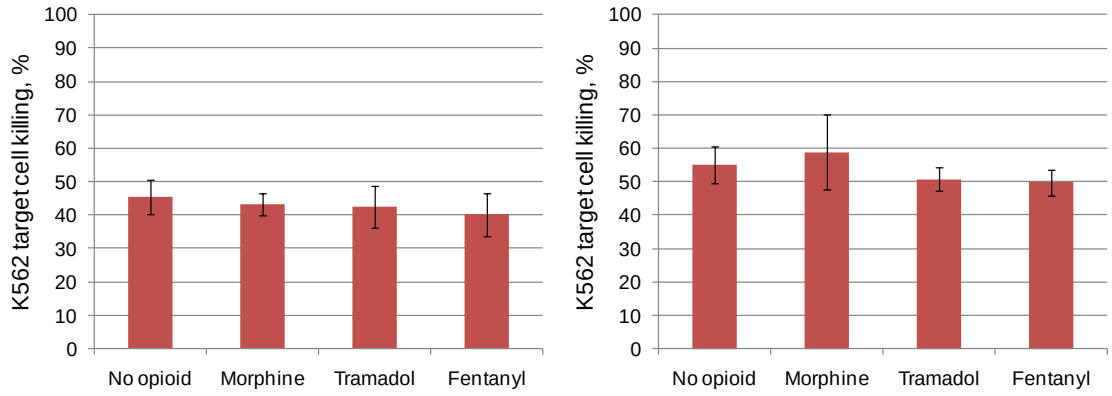

Figure 3-10: Effect of morphine, tramadol and fentanyl on the cytotoxic capacity of unstimulated NK cells (1- and 3-hr incubation).
Unstimulated PBMCs were incubated with 500 ng/ml of morphine, 2000 ng/ml of tramadol and 20 ng/ml of fentanyl for 1 hr (**left panel**) or 3 hr (**right panel**), before cytotoxicity was measured against K562 target cells, using an E:T ratio of 100:1. Data are means ± SEM of 2 independent experiments.

The unstimulated NK cell cytotoxicity assay was performed in order to ensure that IL-2 had not rendered NK cells resistant to opioid-mediated suppression. Although this was a limited, screening experiment, only 500 ng/ml of morphine for 3 days inhibited unstimulated NK cell cytotoxicity compared to untreated unstimulated PBMCs. Although statistically significant, this effect was quantitatively very small. Tramadol and fentanyl, like morphine at shorter time points had no effect on the cytotoxic capacity of unstimulated $CD16^{+}56^{+}$ NK cells. As a consequence, buprenorphine, methadone, oxycodone, diamorphine and codeine were not investigated. Overall, these findings indicate that the opioids tested have no effect on the cytotoxic capacity of NK cells against K562 target cells.

3.1.3 Effect of opioids on IL-2 stimulated natural killer cell activation

In addition to evaluating the effects of opioids on NK cell cytotoxicity, their effect on NK cell activation was also assessed. CD69 is an early activation marker and has been demonstrated to be a good reflection of NK cell activation status (Gerosa et al., 1993).

For these experiments, platelet-free PBMCs were isolated from whole blood by density gradient centrifugation and incubated for 3 days with IL-2 (100 U/ml) in the presence or absence of two clinically relevant concentrations of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine. At the end of the culture period, cells were harvested and the proportion (%) of $CD16^{+}CD56^{+}$ NK cells expressing CD69 and the intensity of CD69 expression (MFI) in those positive cells were determined using flow cytometry (Figure 3-11 - Figure 3-18). Auto-fluorescence and non-specific mAb binding was excluded using unstained and non-reactive isotype controls respectively. Although CD69 expression is a sensitive marker for $CD16^{+}56^{+}$ NK cell activation, CD25 expression might also indicate the activation status of these cells. However, IL-2 stimulation had no effect on CD25 expression on $CD16^{+}CD56^{+}$ NK cells and this was not influenced by any of the opioids (data not shown).

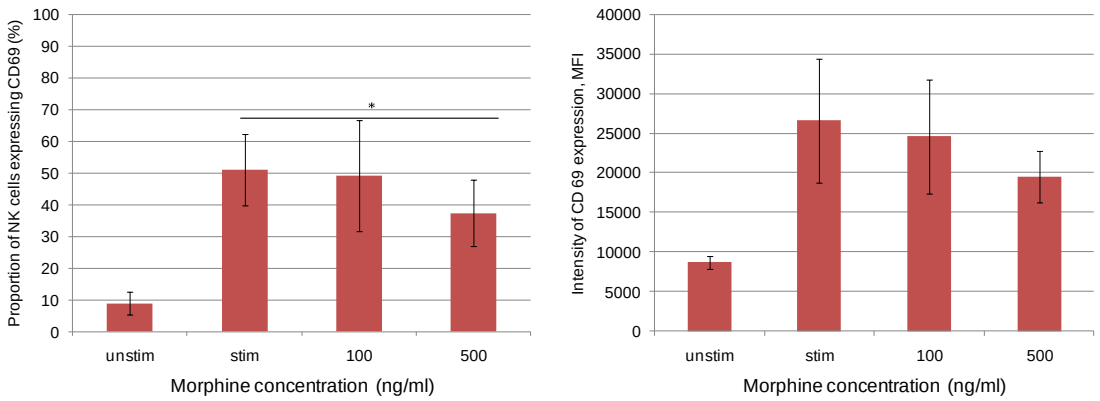

Figure 3-11: Effect of morphine on CD69 expression by IL-2 stimulated NK cells.
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**) was determined by multi-colour flow cytometry. PBMCs were incubated with the indicated concentrations of morphine for 3 days, prior to assessing CD69 expression; the stimulated control had no opioid (stim). All cells, other than the unstimulated control (unstim), were activated with IL-2 (100 U/ml) for 3 days, added at the same time as the opioid. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM of 3 independent experiments (n=4 for 100 ng/ml). 500 ng/ml of morphine significantly inhibited the proportion of IL-2 stimulated NK cells that expressed CD69 expression, *p<0.05 (paired Student *t*-test).

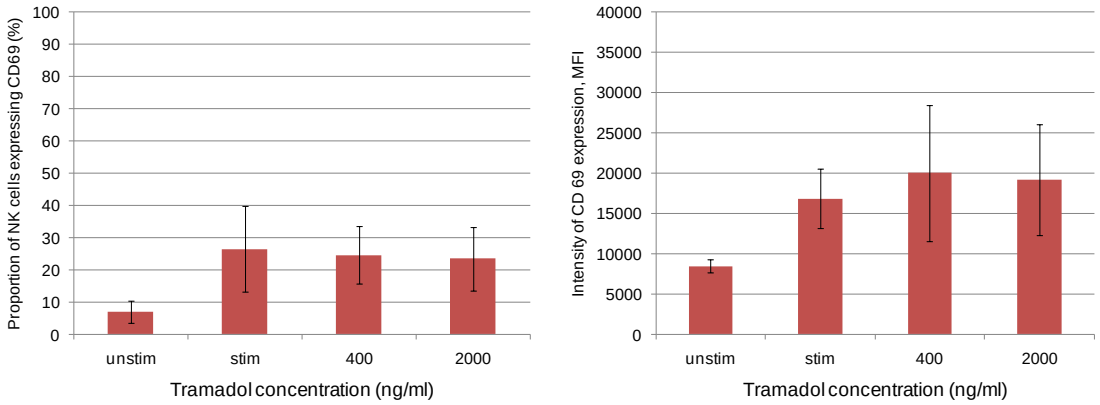

Figure 3-12: Effect of tramadol on CD69 expression by IL-2 stimulated NK cells.
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**) was determined by multi-colour flow cytometry. PBMCs were incubated with the indicated concentrations of tramadol for 3 days, prior to assessing CD69 expression; the stimulated control had no opioid (stim). All cells, other than the unstimulated control (unstim), were activated with IL-2 (100 U/ml) for 3 days, added at the same time as the opioid. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM of 3 independent experiments. No statistical difference in the proportion of NK cells expressing CD69 or the intensity of CD69 expression was observed between no opioid and tramadol-treated IL-2 stimulated NK cells (paired Student *t*-test).

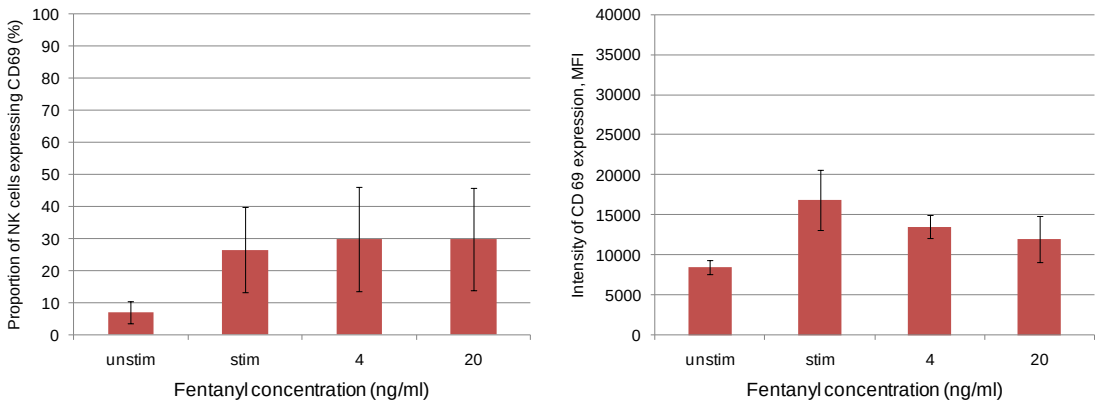

Figure 3-13: Effect of fentanyl on CD69 expression by IL-2 stimulated NK cells.
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**) was determined by multi-colour flow cytometry. PBMCs were incubated with the indicated concentrations of fentanyl for 3 days, prior to assessing CD69 expression; the stimulated control had no opioid (stim). All cells, other than the unstimulated control (unstim), were activated with IL-2 (100 U/ml) for 3 days, added at the same time as the opioid. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM of 3 independent experiments (n=2 for 20 ng/ml for % CD69 expression). No statistical difference in the proportion of NK cells expressing CD69 or the intensity of CD69 expression was observed between no opioid and fentanyl-treated IL-2 stimulated NK cells (paired Student *t*-test).

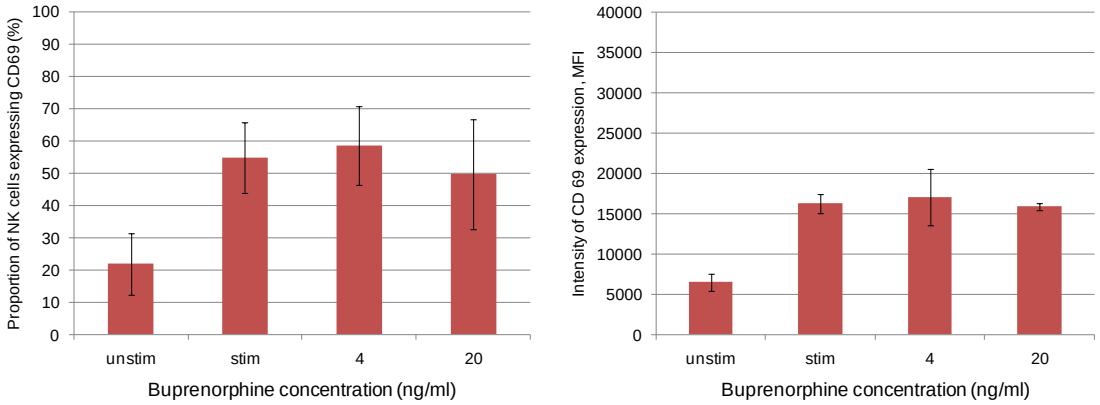

Figure 3-14: Effect of buprenorphine on CD69 expression by IL-2 stimulated NK cells.
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**) was determined by multi-colour flow cytometry. PBMCs were incubated with the indicated concentrations of buprenorphine for 3 days, prior to assessing CD69 expression; the stimulated control had no opioid (stim). All cells, other than the unstimulated control (unstim), were activated with IL-2 (100 U/ml) for 3 days, added at the same time as the opioid. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM of 3 independent experiments. No statistical difference in the proportion of NK cells expressing CD69 or the intensity of CD69 expression was observed between no opioid and buprenorphine-treated IL-2 stimulated NK cells (paired Student *t*-test).

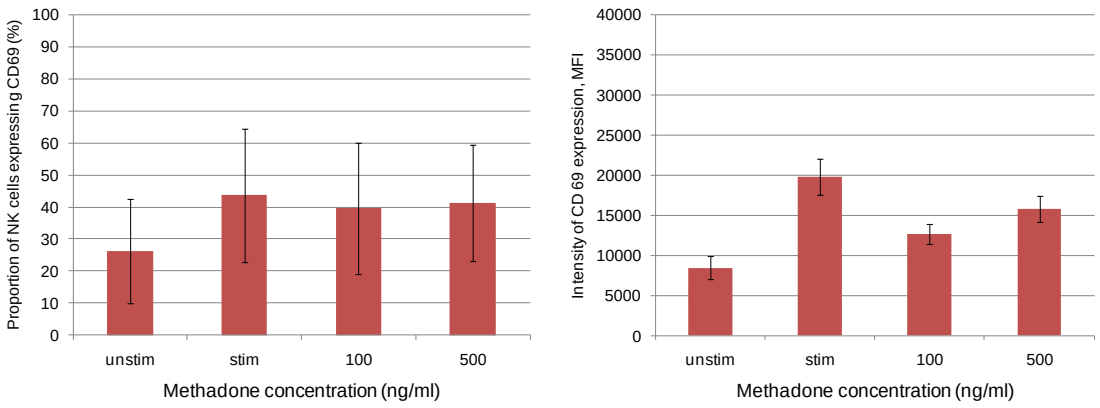

Figure 3-15: Effect of methadone on CD69 expression by IL-2 stimulated NK cells.
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**) was determined by multi-colour flow cytometry. PBMCs were incubated with the indicated concentrations of methadone for 3 days, prior to assessing CD69 expression; the stimulated control had no opioid (stim). All cells, other than the unstimulated control (unstim), were activated with IL-2 (100 U/ml) for 3 days, added at the same time as the opioid. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM of 3 independent experiments. No statistical difference in the proportion of NK cells expressing CD69 or the intensity of CD69 expression was observed between no opioid and methadone-treated IL-2 stimulated NK cells (paired Student *t*-test).

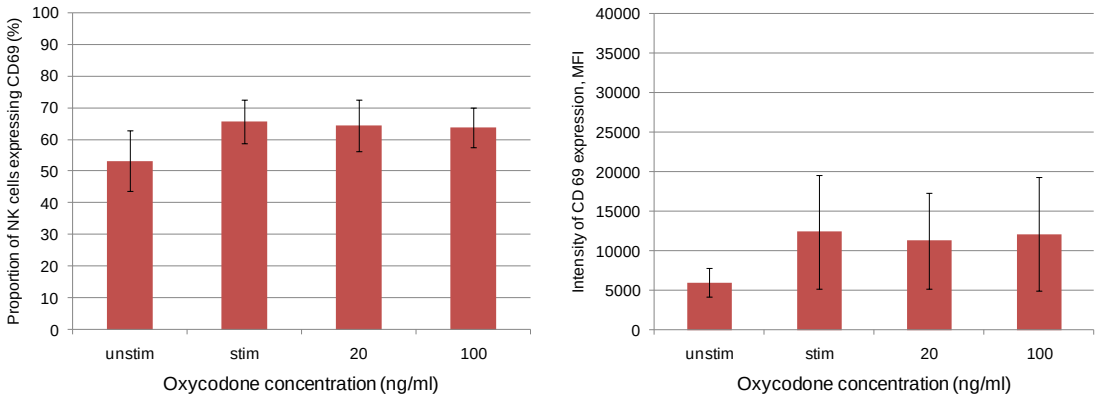

Figure 3-16: Effect of oxycodone on CD69 expression by IL-2 stimulated NK cells.
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**) was determined by multi-colour flow cytometry. PBMCs were incubated with the indicated concentrations of oxycodone for 3 days, prior to assessing CD69 expression; the stimulated control had no opioid (stim). All cells, other than the unstimulated control (unstim), were activated with IL-2 (100 U/ml) for 3 days, added at the same time as the opioid. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM of 3 independent experiments. No statistical difference in the proportion of NK cells expressing CD69 or the intensity of CD69 expression was observed between no opioid and oxycodone-treated IL-2 stimulated NK cells (paired Student *t*-test).

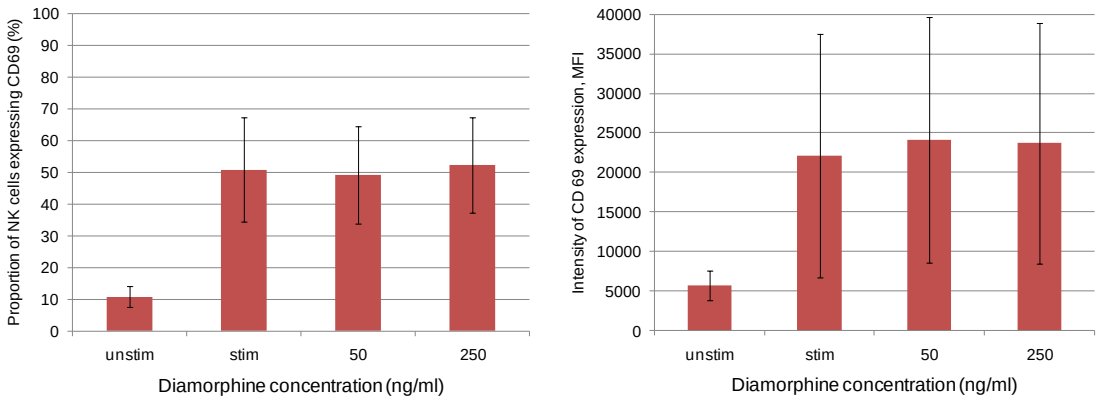

Figure 3-17: Effect of diamorphine on CD69 expression by IL-2 stimulated NK cells.
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**) was determined by multi-colour flow cytometry. PBMCs were incubated with the indicated concentrations of diamorphine for 3 days, prior to assessing CD69 expression; the stimulated control had no opioid (stim). All cells, other than the unstimulated control (unstim), were activated with IL-2 (100 U/ml) for 3 days, added at the same time as the opioid. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM of 3 independent experiments. No statistical difference in the proportion of NK cells expressing CD69 or the intensity of CD69 expression was observed between no opioid and diamorphine-treated IL-2 stimulated NK cells (paired Student *t*-test).

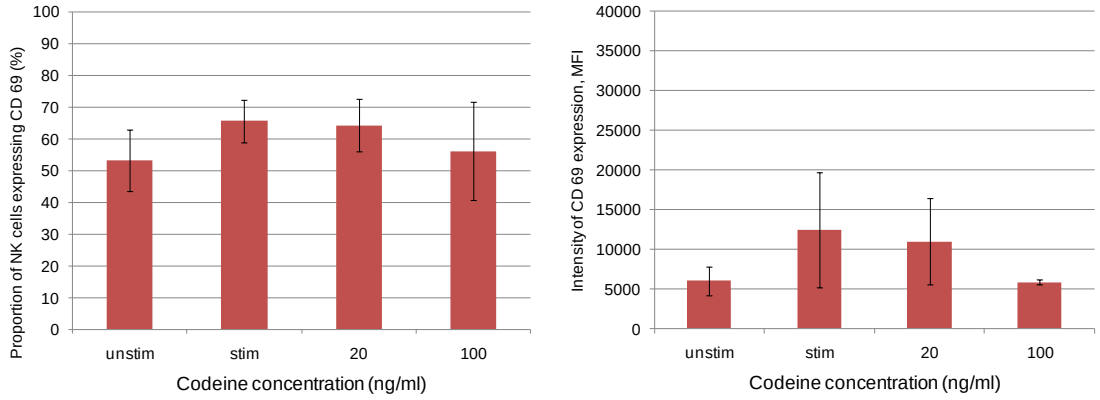

Figure 3-18: Effect of codeine on CD69 expression by IL-2 stimulated NK cells.
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**) was determined by multi-colour flow cytometry. PBMCs were incubated with the indicated concentrations of codeine for 3 days, prior to assessing CD69 expression; the stimulated control had no opioid (stim). All cells, other than the unstimulated control (unstim), were activated with IL-2 (100 U/ml) for 3 days, added at the same time as the opioid. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM of 3 independent experiments. No statistical difference in the proportion of NK cells expressing CD69 or the intensity of CD69 expression was observed between no opioid and codeine-treated IL-2 stimulated NK cells (paired Student *t*-test).

$CD16^+56^+$ NK cells were responsive to IL-2 stimulation, as IL-2 increased the proportion and intensity of CD69 expression on NK cells. 500 ng/ml of morphine decreased the proportion of $CD16^+56^+$ cells expressing CD69 and there was also a trend towards morphine decreasing the intensity of this expression, however, none of the other opioids had an effect on CD69 expression on IL-2 stimulated $CD16^+56^+$ cells. It might be that opioids do not have a direct effect on NK cells or because IL-2 stimulated the NK cells to such an extent that this could not be overcome or enhanced by the other opioids. The effects of opioids on unstimulated NK cells were subsequently assessed, as were their effects on the expression of activation antigens on $CD16^+56^+$ NK cells in PBMC cultures that had been activated with Miltenyi anti-CD3/anti-CD28 mAb coated beads.

3.1.4 Effect of opioids on the activation status of natural killer cells in activated peripheral blood mononuclear cells preparations

The effect of the opioids on the activation status (CD69 and CD25 expression) of $CD16^+56^+$ NK cells in PBMC cultures that had been stimulated with Miltenyi anti-CD3/anti-CD28 mAb coated beads for 3 days was assessed. Although these beads do not stimulate the NK cells directly, over the 3 days they activate T cells, resulting in a *milieu* which might simulate that which is found during *in vivo* inflammatory events. These beads polyclonally activate T cells via a physiological mechanism which involves TCR triggering via interactions with cell surface CD3 and the induction of essential co-stimulatory signals via binding to CD28. This results in the activation of intracellular cascades, including PKC and the transcription of mRNA for several cytokines, including IL-2 and IL-6. The aim of these studies was to assess the influence of opioids on NK cells that have been activated by a physiologically-relevant combination of cytokines.

Incubation of PBMCs with Miltenyi anti-CD3/anti-CD28 mAb coated beads for 3 days increased the proportion of $CD16^+56^+$ NK cells expressing CD69 and, to a greater extent, the intensity of CD69 expression. Anti-CD3/anti-CD28 mAb coated beads did not have an effect on CD25 expression (either the proportion or the intensity of response) on $CD16^+56^+$ NK cells in PBMC cultures (data not shown).

The data presented below (Figure 3-19 - Figure 3-26) illustrate the effect of the opioids on the proportion (%) of $CD16^+56^+$ NK cells expressing CD69 and the intensity of this expression (MFI) in those positive cells.

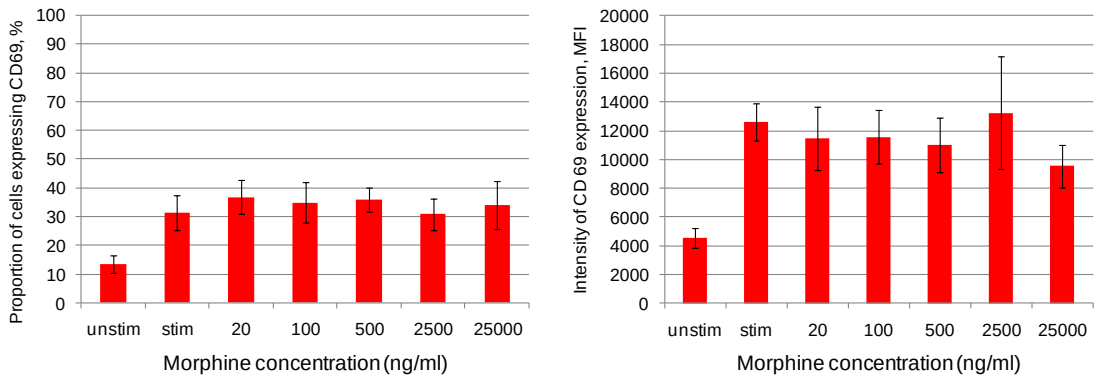

Figure 3-19: Effect of morphine on CD69 expression by NK cells in anti-CD3/28 mAb stimulated PBMC cultures.

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of CD69 expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of morphine for 3 days prior to assessing CD69 expression; the stimulated control group had no morphine (stim). The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. All cells, other than the unstimulated control (unstim), were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the morphine. Data are means ± SEM from 5 independent experiments (highest concentration n=4). No statistically significant differences between the responsiveness of morphine-treated and untreated anti-CD3/28 mAb stimulated NK cells were observed (paired Student *t*-test).

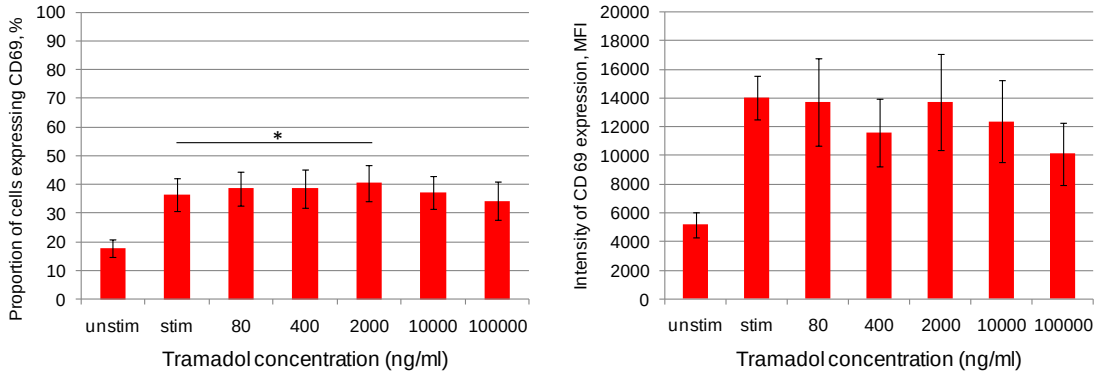

Figure 3-20: Effect of tramadol on CD69 expression by NK cells in anti-CD3/28 mAb stimulated PBMC cultures.

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of CD69 expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of tramadol for 3 days prior to assessing CD69 expression; the stimulated control group had no tramadol (stim). The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. All cells, other than the unstimulated control (unstim), were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the tramadol. Data are means ± SEM from 6 independent experiments (highest concentration n=3). 2000 ng/ml of tramadol significantly increased the percentage of anti-CD3/28 mAb stimulated NK cells expressing CD69 *$p<0.05$ (paired Student *t*-test).

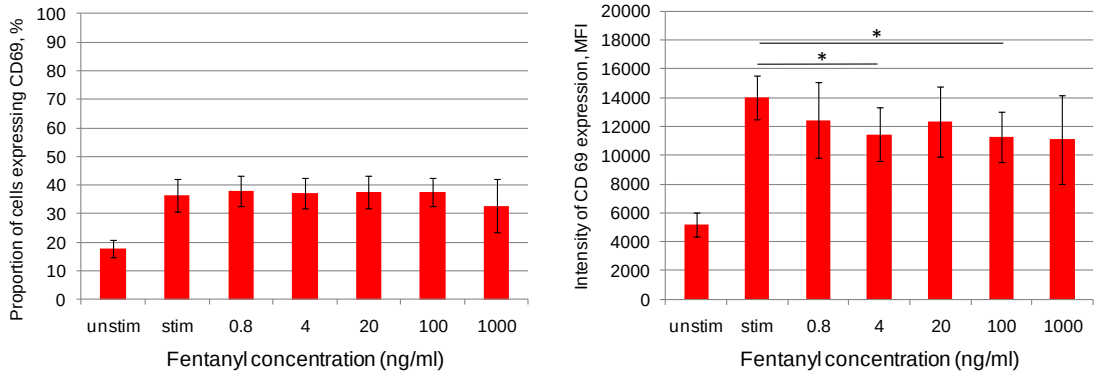

Figure 3-21: Effect of fentanyl on CD69 expression by NK cells in anti-CD3/28 mAb stimulated PBMC cultures.

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of CD69 expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of fentanyl for 3 days prior to assessing CD69 expression; the stimulated control group had no fentanyl (stim). The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. All cells, other than the unstimulated control (unstim), were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the fentanyl. Data are means ± SEM from 6 independent experiments (highest concentration n=3). 4 and 100 ng/ml of fentanyl significantly inhibited the intensity of CD69 expression on anti-CD3/28 mAb stimulated NK cells, *p<0.05 (paired Student *t*-test).

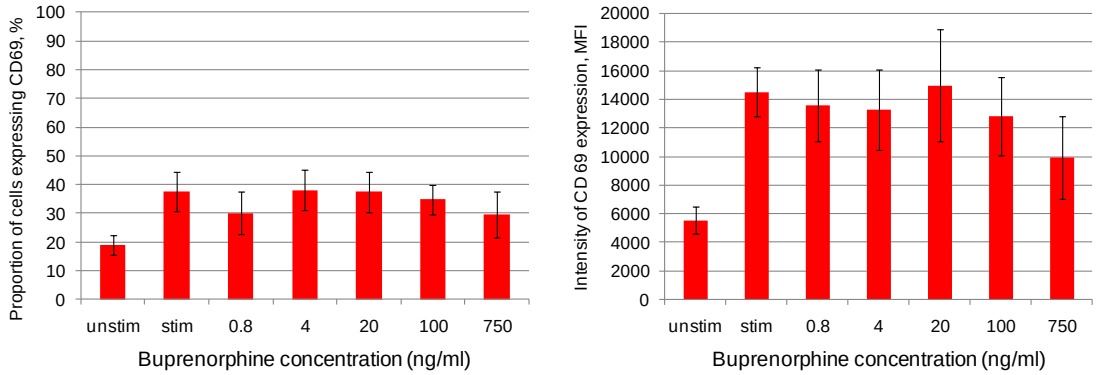

Figure 3-22: Effect of buprenorphine on CD69 expression by NK cells in anti-CD3/28 mAb stimulated PBMC cultures.

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of CD69 expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of buprenorphine for 3 days prior to assessing CD69 expression; the stimulated control group had no buprenorphine (stim). The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. All cells, other than the unstimulated control (unstim), were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the buprenorphine. Data are means ± SEM from 5 independent experiments (except the top concentration which is n=3). No statistically significant differences between the responsiveness of buprenorphine-treated and untreated anti-CD3/28 mAb stimulated NK cells were observed (paired Student *t*-test).

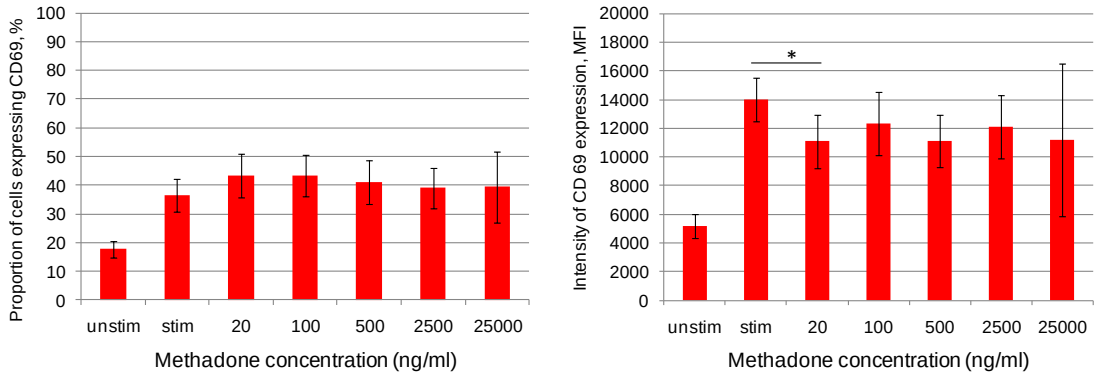

Figure 3-23: Effect of methadone on CD69 expression by NK cells in anti-CD3/28 mAb stimulated PBMC cultures.

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**upper panel**) and the intensity of CD69 expression on these positive cells (median channel of fluorescent intensity, MFI; **lower panel**). PBMCs were incubated with the indicated concentrations of methadone for 3 days prior to assessing CD69 expression; the stimulated control group had no methadone (stim). The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. All cells, other than the unstimulated control (unstim), were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the methadone. Data are means ± SEM from 6 independent experiments (highest concentration n=3). 20 ng/ml of methadone significantly inhibited the intensity of CD69 expression on anti-CD3/28 mAb stimulated NK cells, *p<0.05 (paired Student *t*-test).

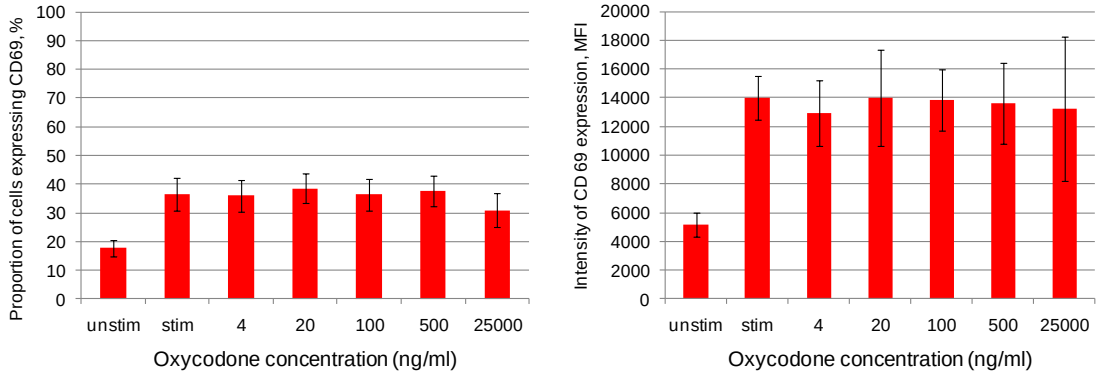

Figure 3-24: Effect of oxycodone on CD69 expression by NK cells in anti-CD3/28 mAb stimulated PBMC cultures.

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of CD69 expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of oxycodone for 3 days prior to assessing CD69 expression; the stimulated control group had no oxycodone (stim). The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. All cells, other than the unstimulated control (unstim), were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the oxycodone. Data are means ± SEM from 6 independent experiments (highest concentration n=3). No statistically significant differences between the responsiveness of oxycodone-treated and untreated anti-CD3/28 mAb stimulated NK cells were observed (paired Student *t*-test).

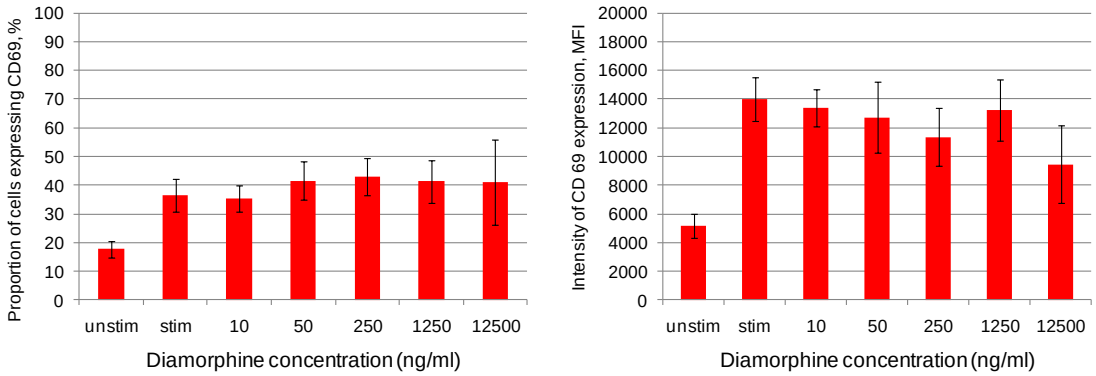

Figure 3-25: Effect of diamorphine on CD69 expression by NK cells in anti-CD3/28 mAb stimulated PBMC cultures.

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of CD69 expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of diamorphine for 3 days prior to assessing CD69 expression; the stimulated control group had no diamorphine (stim). The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. All cells, other than the unstimulated control (unstim), were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the diamorphine. Data are means ± SEM from 6 independent experiments (lowest concentration n=5; highest concentration n=3). No statistically significant differences between the responsiveness of diamorphine-treated and untreated anti-CD3/28 mAb stimulated NK cells were observed (paired Student *t*-test).

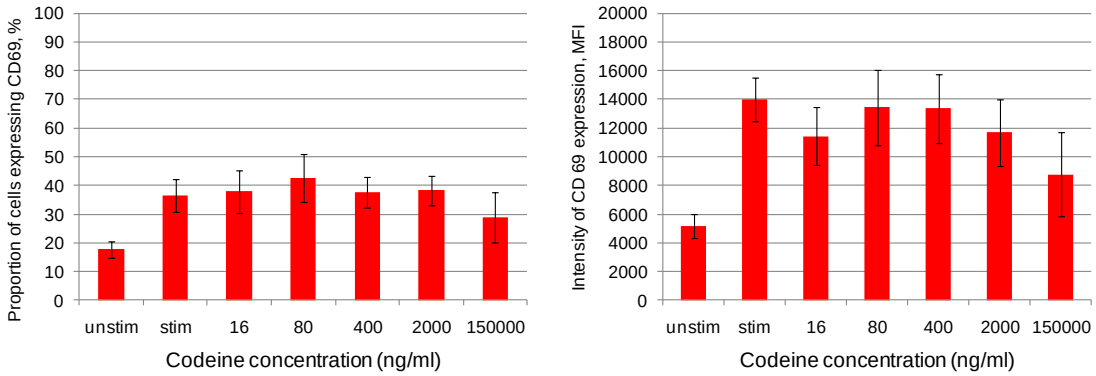

Figure 3-26: Effect of codeine on CD69 expression by NK cells in anti-CD3/28 mAb stimulated PBMC cultures.

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of CD69 expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of codeine for 3 days prior to assessing CD69 expression; the stimulated control group had no codeine (stim). The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. All cells, other than the unstimulated control (unstim), were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the codeine. Data are means ± SEM from 6 independent experiments (highest concentration n=3). No statistically significant differences between the responsiveness of codeine-treated and untreated anti-CD3/28 mAb stimulated NK cells were observed (paired Student *t*-test).

Although 4 and 100 ng/ml of fentanyl and 20 ng/ml of methadone in these cultures inhibited the intensity of CD69 expression, and 2000 ng/ml of tramadol increased the proportion of NK cells expressing CD69, these were quantitatively small and none of the opioids had any consistent effect on NK cell activation in anti-CD3/anti-CD28 stimulated PBMC cultures.

3.1.5 Effect of opioids on the activation status of unstimulated natural killer cells

The observation that none of the opioids had any effect on the activation status of IL-2 or anti-CD3/anti-CD28 stimulated NK cells might be because these opioids cannot overcome the potent stimulatory consequences of IL-2 or anti-CD3/anti-CD28 mAb or, if the opioid was to be stimulatory, it might not be potent enough to further enhance IL-2 or anti-CD3/anti-CD28 mAb induced stimulation. The influence of morphine, tramadol and fentanyl on CD69 and CD25 expression by unstimulated $CD16^{+}56^{+}$ NK cells was therefore evaluated. CD25 expression was unaffected by stimulation or opioids (data not shown).

For these studies, platelet-free PBMCs were isolated from whole blood by density gradient centrifugation and incubated for 3-days with clinically relevant concentrations of morphine, tramadol and fentanyl. At the end of the culture period, cells were harvested and the proportion of $CD16^{+}CD56^{+}$ NK cells expressing CD69 and CD25, and the intensity of this expression (MFI) were determined using multi-colour flow cytometry. Auto-fluorescence and non-specific mAb binding was excluded using unstained and non-reactive isotype-matched controls respectively. These experiments demonstrated that morphine, tramadol and fentanyl had no effect on the activation status (CD69 and CD25) of unstimulated NK cells (Figure 3-27 - Figure 3-29).

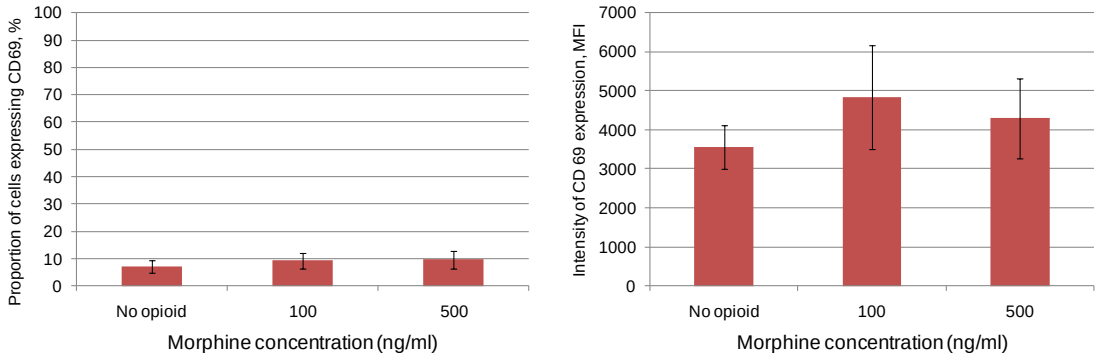

Figure 3-27: Effect of morphine on CD69 expression by unstimulated NK cells (3-day incubation).

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentration of morphine for 3 days prior to assessing CD69 expression. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM from 3 independent experiments. No statistically significant differences between the responsiveness of morphine-treated and untreated unstimulated NK cells (paired Student *t*-test).

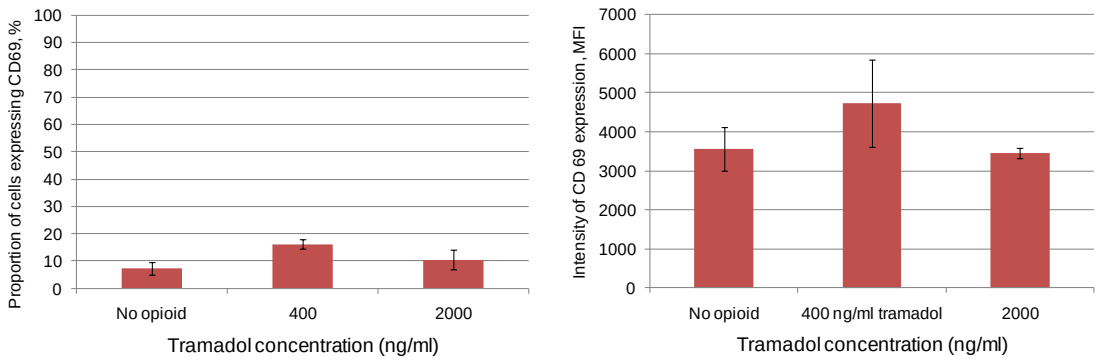

Figure 3-28: Effect of tramadol on CD69 expression by unstimulated NK cells (3-day incubation).
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentration of tramadol for 3 days prior to assessing CD69 expression. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM from 3 independent experiments (n=2 for 400 ng/ml of tramadol). No statistically significant differences between the responsiveness of tramadol-treated and untreated unstimulated NK cells (paired Student *t*-test).

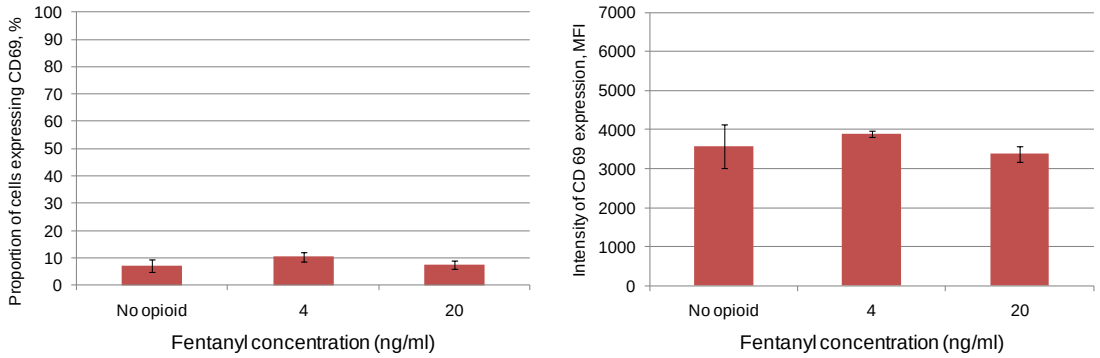

Figure 3-29: Effect of fentanyl on CD69 expression by unstimulated NK cells (3-day incubation).
The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentration of fentanyl for 3 days prior to assessing CD69 expression. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM from 3 independent experiments (n=2 for 4 ng/ml of fentanyl). No statistically significant differences between the responsiveness of fentanyl-treated and untreated unstimulated NK cells (paired Student *t*-test).

As there is some evidence to suggest that morphine can suppress NK cell cytotoxicity within hours, and that tolerance to this can occur within a day (Nelson et al., 1997), additional short-term experiments (1, 3 and 24 hr) which used only the higher doses of morphine, tramadol and fentanyl were performed in order to ensure that this effect was not missed (Figure 3-30 - Figure 3-32). As IL-2 takes 3 days to elicit its stimulatory effect on NK cells, all assays with shorter time points used unstimulated NK cells, as adding IL-2 for less than 3 days would not lead to a meaningful activation.

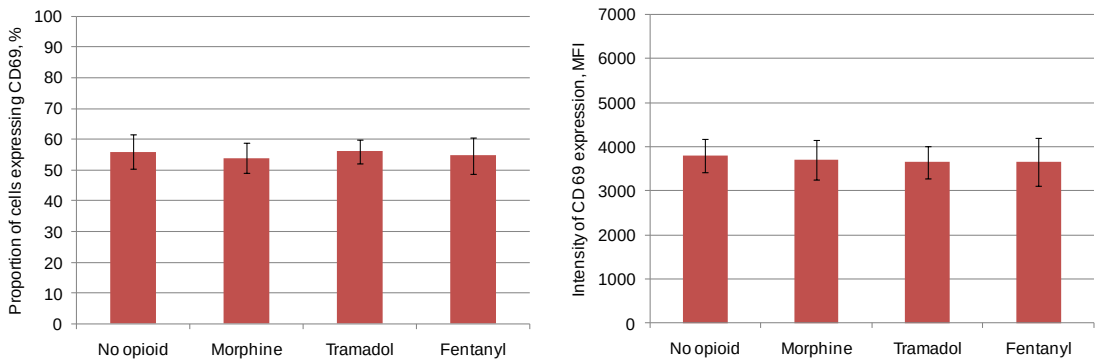

Figure 3-30: Effect morphine, tramadol and fentanyl on CD69 expression by unstimulated NK cells (1-hr incubation).

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). Unstimulated PBMCs were incubated with the indicated opioid (morphine 500 ng/ml, tramadol 2000 ng/ml and fentanyl 20 ng/ml) for 1 hr prior to assessing CD69 expression. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM from 2 independent experiments.

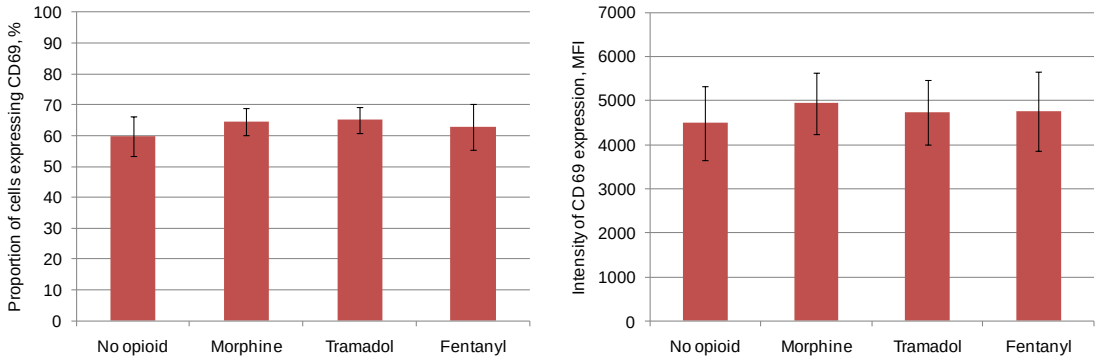

Figure 3-31: Effect of morphine, tramadol and fentanyl on CD69 expression by unstimulated NK cells (3-hr incubation).

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). Unstimulated PBMCs were incubated with the indicated opioid (morphine 500 ng/ml, tramadol 2000 ng/ml and fentanyl 20 ng/ml) for 3 hr prior to assessing CD69 expression. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM from 2 independent experiments.

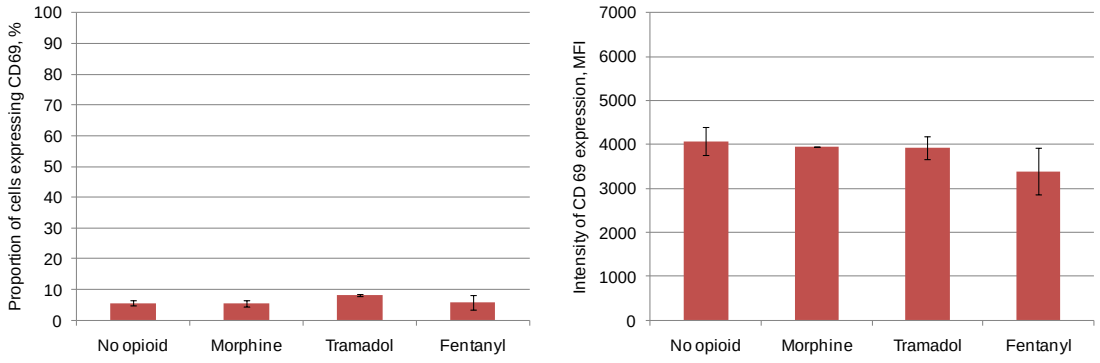

Figure 3-32: Effect of morphine, tramadol and fentanyl on CD69 expression in unstimulated NK cells (24-hr incubation).

The proportion (%) of $CD16^{+}56^{+}$ NK cells expressing CD69 (**left panel**) and the intensity of this expression on these positive cells (median channel of fluorescent intensity, MFI; **right panel**). Unstimulated PBMCs were incubated with the indicated opioid (morphine 500 ng/ml, tramadol 2000 ng/ml and fentanyl 20 ng/ml) for 24 hr prior to assessing CD69 expression. The NK cells are identified as being CD4 and CD8 negative, and $CD16^{+}$ or $CD56^{+}$. Data are means ± SEM from 2 independent experiments.

This limited, screening experiment, assessed the effects of clinically relevant concentrations of morphine, tramadol and fentanyl on the activation (CD69 and CD25 expression) of unstimulated $CD16^{+}56^{+}$ NK cells at 1, 3, 24 and 72 hr. It showed that none of the opioids had an effect on the expression of CD69 and CD25 on unstimulated $CD16^{+}56^{+}$ NK cells. No additional opioids were therefore assessed.

3.1.6 Effects of opioids on natural killer cells in individual subjects

None of the opioids tested had a consistent, statistically significant effect on NK cell cytotoxicity in IL-2 stimulated or unstimulated PBMCs. They also had no consistent effect on the activation status of NK cells in IL-2 or Miltenyi anti-CD3/anti-CD28 mAb stimulated PBMCs or unstimulated PBMC cultures. There did however seem to be an effect of certain opioids on the NK cells of some individual subjects and there was interindividual variability in the observed responses. The genetic and molecular basis to interindividual variability to opioids will be discussed in section 4.7.

Most of the opioids had no consistent effect on NK cells in most of the subjects tested. Subject 3 was most sensitive to the effect of opioids, with NK cell cytotoxicity and intensity of CD69 expression being inhibited by morphine, tramadol and buprenorphine. Buprenorphine also reduced NK cell cytotoxicity in subject 1 and 5, but did not influence CD69 expression. Fentanyl inhibited NK cell cytotoxicity and CD69 expression in subject 1. (NB: subject numbers are constant throughout all the experiments).

3.1.7 Discussion

Flow cytometry has been used to assess the effects of eight commonly used opioids at two concentrations, approximating those found clinically, on the *in vitro* ability of resting and IL-2 activated human NK cells to kill K562 cells. The effect of these opioids on the expression of CD69 and CD25 by resting NK cells, IL-2 activated NK cells and also NK cells in anti-CD3/anti-CD28 mAb activated PBMC cultures were also assessed.

None of the opioids at the concentrations tested had any effect on the killing of K562 cells by NK cells in unstimulated and IL-2 stimulated PBMC preparations. Several experimental paradigms were explored; both with IL-2 stimulated or unstimulated PBMCs over 3 days, and in a series of screening experiments with a shorter incubation time with unstimulated PBMCs. As there were small numbers of subjects, this could lead to a type II error, but no consistent effect of any of the opioids was detected, thus

the number of subjects was not increased. If effects were seen, but they could not be demonstrated to be of statistical significance, then there would have been an advantage to increase the number of subjects to potentially gain significance.

The activation status, as measured by CD69 and CD25 expression, on unstimulated and IL-2 stimulated NK cells, and NK cells in anti-CD3/anti-CD28 mAb stimulated PBMC preparations was also determined. There were occasional statistically significant effects on CD69 expression at some of the concentrations of the opioids tested in these three different experimental paradigms. However, in view of the numerous tests performed and that the significant results generally occurred at just one concentration of the opioid on one of the measures of NK cell function in one of the experiments, it is likely that this occurred by chance, rather than being a real result at low levels of significance. Furthermore, these effects tended to be quantitatively small, bringing doubt to their potential clinical significance.

The previous *in vitro* studies assessing the effects of opioids on NK cells are reviewed below and then compared to the findings of the current study. It should be noted that the previous studies only assessed NK cell cytotoxicity (using unstimulated NK cells), not NK cell activation status as was also undertaken herein.

Yeager and colleagues investigated the *in vitro* effect of morphine on NK cell cytotoxicity in 10 healthy volunteers (Yeager et al., 1992). PBMCs were isolated from whole blood using density gradient centrifugation, without the removal of platelets. These cells were incubated with 10^{-12} – 10^{-4} M of morphine (equivalent to $3x10^{-4}$ – $3x10^{4}$ ng/ml) for 18 hr. NK cell cytotoxicity was measured using a 4-hr incubation of PBMCs with ^{51}Cr labelled K562 cells. Only 10^{-4} M of morphine (≈ 28,500 ng/ml), over 50x the maximum concentration used in the current study, and far in excess of that found clinically, had any significant inhibition on NK cell cytotoxicity, decreasing it by 18%, individual variability was not discussed (Yeager et al., 1992).

Although Yeager used the same effector cells (healthy human PBMCs) as the current study, they were unstimulated and incubated with morphine for a shorter period (18 vs. 72 hr). However, shorter incubation times with unstimulated PBMCs were also assessed in the current study. They also used a 4-hr (as opposed to 3-hr) incubation with K562 and a radiolabelled assay rather than flow cytometry. However, as in the current study, morphine at the concentrations detected clinically, had no effect on NK cell cytotoxicity.

Condevaux and colleagues assessed the effect of *in vitro* morphine on the cytotoxicity of splenic NK cells in the Sprague-Dawley rat and PBMCs from the cynomolgus monkey (Condevaux et al., 2001). For the rat experiments, NK cells were incubated with 0.01, 1 and 1000 nM of morphine (equivalent to 0.003, 0.3 and 285 ng/ml) for 2-hr, before being incubated with ^{51}Cr labelled YAC-1 target cells for 4 hr (E:T ratios; 200:1, 100:1 and 50:1). ^{51}Cr labelled K562 target cells were used in the monkey experiments (E:T ratios; 100:1, 50:1 and 25:1). Only 1000 nM of morphine (≈ 285 ng/ml) inhibited NK cell cytotoxicity in these experiments, decreasing it by up to 35% in the rat and 160% in the monkey (Condevaux et al., 2001).

Compared with the current study, Condevaux used effector cells from different species which were unstimulated (although the current study also screened for effects from unstimulated cells) and had a shorter incubation period with morphine (2 vs. 72 hr), although comparable incubation times were also evaluated in the current study. They also used a 4-hr (as opposed to 3-hr) incubation with the target cells (which differed in the rat experiments YAC-1 vs. K562), and a radiolabelled assay rather than flow cytometry. 1000 nM (≈ 285 ng/ml) is comparable to concentrations found clinically and overlaps with the current study, where NK cell cytotoxicity was not inhibited, this might be due to the different species used.

The *in vitro* effect of fentanyl was assessed in four healthy volunteers (Jacobs et al., 1999). PBMCs were isolated and incubated for 45 min with 20 and 40 μM of fentanyl (equivalent to 5,700 and 11,400 ng/ml). NK cell cytotoxicity was assessed using a 4-hr incubation with ^{51}Cr labelled K562 target cells (fentanyl was not removed before this). NK cell cytotoxicity was not affected, variability was not reported (Jacobs et al., 1999). Although using much higher concentrations of fentanyl with a shorter incubation time than the current study, Jacobs also showed fentanyl did not affect NK cell cytotoxicity.

Molitor and colleagues investigated the *in vitro* effects of methadone on PBMCs isolated from five pigs (Molitor et al., 1992). PBMCs were incubated for 48 hr with 10^{-18} – 10^{-6} M of methadone (equivalent to $3x10^{-10}$ – 309 ng/ml) and NK cell cytotoxicity was measured following a 4-hr incubation with ^{51}Cr-labeled K562 target cells. Higher concentrations of methadone (10^{-9} – 10^{-6} M; equivalent to 0.3 – 309 ng/ml) decreased the ability of NK cells to kill K562 cells by up to four fold (Molitor et al., 1992).

Compared with the current study, Molitor used effector cells from different species which were unstimulated, but used a comparable incubation period with methadone (2

vs. 3 days). They used a 4-hr (as opposed to 3-hr) incubation with the target cells and a radiolabelled assay rather than flow cytometry. 10^{-9} – 10^{-6} M is comparable to clinical concentrations and overlaps with the current study, in which NK cell cytotoxicity was not inhibited, this might be due to the species used or NK cell activation status.

The previous literature assessing the *in vitro* effect of opioids on NK cell cytotoxicity is sparse, with only morphine, fentanyl and methadone having been studied on unstimulated NK cells. These studies were in differing species, with different concentrations and incubation times with opioids, as well as utilising differing target cells. Many previous studies have used ^{51}Cr release detected in the assay supernatant by a γ counter as a measure of NK cell cytotoxicity; the current study used flow cytometry which accurately determined the number of live/dead target cells (as well as enabling cell surface activation marker expression to be evaluated). These two techniques have been shown to closely correlate (Kane et al., 1996, Shi et al., 1987).

The previous studies all used unstimulated NK cells and the current study screened for the effects of morphine, tramadol and fentanyl on unstimulated NK cells. Being part of the innate immune system the response of unactivated NK cell might be of some importance in the very early stages of disease. However, unstimulated NK cells are less likely to be clinically relevant in patients with establishing cancer or infection in whom cells are likely to be activated. However, in patients with established cancer, the cancer-immune interaction can induce up-regulation of inhibitory NK cell receptors (i.e. killer immunoglobulin-like receptor) and down-regulation of stimulatory NK cell receptors (i.e. NKG2D), overall inhibiting NK cells in patients with cancer (Al Omar et al., 2011, Saito et al., 2011). Even in these patients the continual recruitment of activated NK cells into the tumour microenvironment might still be important.

No previous studies have assessed the effects of any opioids on the activation status (either CD69 or CD25) expression of NK cells. In the current study, there is no clear relationship between any of the opioids and effect on NK cell activation. This may in part be due to small numbers of subjects and type II error, however, no effect of any of the opioids was seen on any of the individuals and there was variability between the individuals, which more likely leads to the lack of statistical effect.

3.1.8 Conclusion

NK cells have a vital role in tumour immunosurveillance and in the control of viral infection. Previous *in vivo* studies have shown that NK cell cytotoxicity can be affected by opioids, although there are different effects from the different opioids and between the models used (section 1.12.1). From the little previous *in vitro* evidence available, morphine and methadone have been shown to inhibit NK cell cytotoxicity at high concentrations. In the current study, eight opioids, at concentrations overlapping those detected clinically, were incubated for 3 days (and shorter time points) with unstimulated or stimulated (IL-2 or anti-CD3/anti-CD28 mAb) PBMCs before assessing NK cell cytotoxicity against K562 cells and NK cell activation using flow cytometry. None of the opioids at the concentrations tested had any effect on the cytotoxicity of unstimulated or IL-2 stimulated NK cells against the K562 erythroleukaemic cell line. They also had no consistent effect on CD69 or CD25 expression on unstimulated or stimulated (IL-2 or anti-CD3/anti-CD28 mAb) NK cells over 3 days or, in the screening experiments, at shorter time points. These findings indicate that none of the commonly used opioids directly inhibit the function of NK cells under these *in vitro* experimental conditions and suggest that any effects that opioids might have *in vivo* and clinically are likely to arise as a consequence of indirect actions, possibly via effects on the SNS and HPA axis.

3.2 Effect of opioids on neutrophil and monocyte phagocytosis and oxidative burst responses

Neutrophils and monocytes are part of the innate immune system and have a role in the defence against bacteria, fungi and foreign particles. Their main functional mechanisms in this defence are phagocytosis and the oxidative burst reaction.

Initial experiments investigated the influence of pre-incubating whole blood with a single intermediate concentration of opioid (approximating to the clinically relevant concentrations that are found in human studies), namely 100 ng/ml of morphine, 400 ng/ml of tramadol or 4 ng/ml of fentanyl for 0, 30, 60 or 120 min, on neutrophil and monocyte phagocytosis and oxidative burst responses. These preliminary experiments indicated that opioid-mediated effects were best studied following a 60-min pre-incubation. On the basis of these findings, all subsequent experiments in which the effects of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on phagocytosis and oxidative burst responses were evaluated used a pre-incubation time of 60 min at 37°C of whole blood with the opioids (see Table 2.12 for the opioid concentrations used for these assays).

The data from the initial set of experiments which explored the effect of time were combined with the data that were obtained in subsequent experiments which examined the dose-dependent effects of opioids. As different individuals exhibit a range of phagocytosis and oxidative burst activity, the no opioid controls for these experiments were variable. As a consequence, data were expressed as change from control by subtracting the effect of the opioid from the no opioid control. In view of this and the variability among the individual subjects tested, further experiments were performed in order to ensure that the opioids had been tested on a similar profile of volunteers. This was especially important as the initial results demonstrated a difference between the intermediate concentration and the other concentrations which were only partially corrected for by subtracting the effect of the opioid from the no opioid control. This was most likely due to different volunteers being used for the experiments.

3.2.1 Phagocytosis

The effect of the opioids (60 min incubation) on the ability of neutrophils and monocytes to phagocytose FITC-labelled *E.coli* over 10 min was assessed and the data are presented below as change from the no opioid controls. The absolute baseline values, without opioid, are given at the top of the graphs (Figure 3-33 - Figure 3-40).

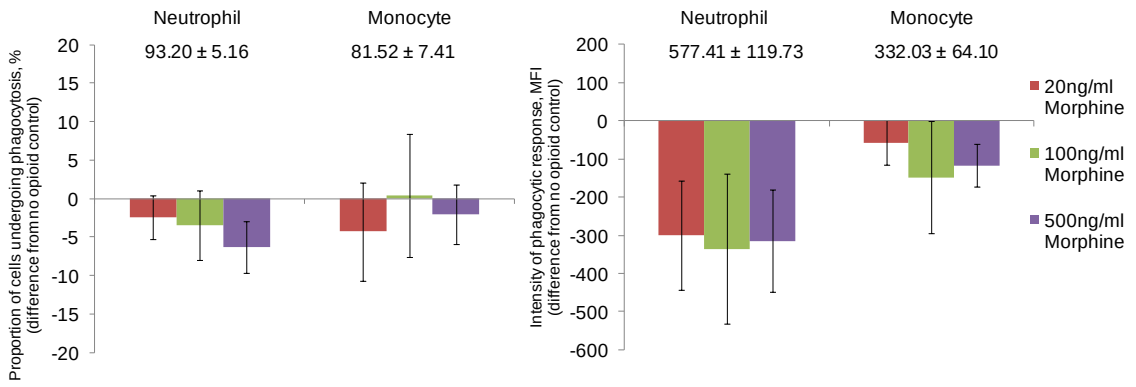

Figure 3-33: **Effect of morphine on neutrophil and monocyte phagocytosis.**
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are stated at the top of the graphs. Whole blood was incubated with the indicated concentrations of morphine for 60 min prior to assessing the phagocytosis of FITC-*E.coli*. Data are means ± SEM from 5 independent experiments (n=4 for monocytes at 20 and 100 ng/ml of morphine). No statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

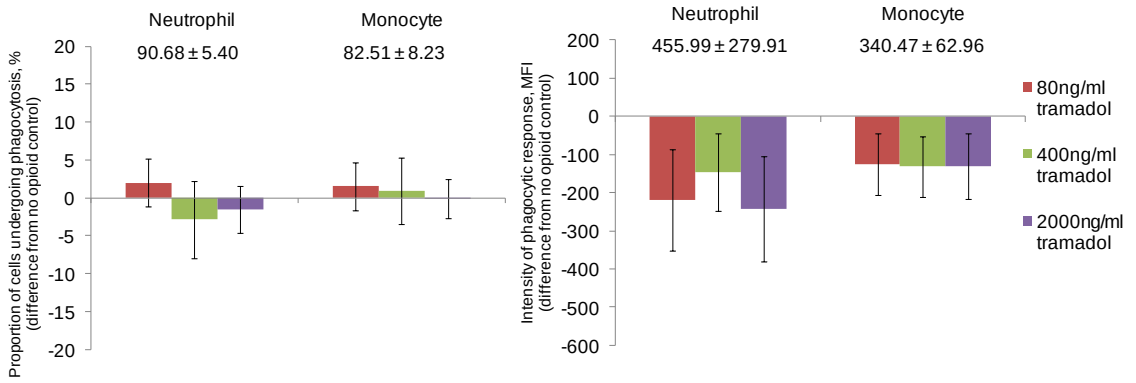

Figure 3-34: Effect of tramadol on neutrophil and monocyte phagocytosis.
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are stated at the top of the graphs. Whole blood was incubated with the indicated concentrations of tramadol for 60 min prior to assessing the phagocytosis of FITC-*E.coli*. Data are means ± SEM from 5 independent experiments and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

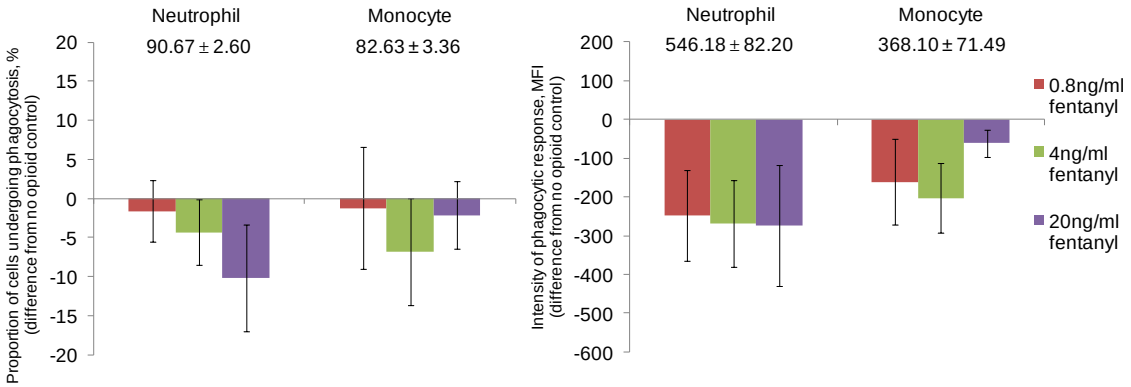

Figure 3-35: Effect of fentanyl on neutrophil and monocyte phagocytosis.
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are stated at the top of the graphs Whole blood was incubated with the indicated concentrations of fentanyl for 60 min prior to assessing the phagocytosis of FITC-*E.coli*. Data are means ± SEM from 5 independent experiments (n=4 for neutrophils at 20ng/ml of fentanyl, n=4 for monocytes at 0.8ng/ml of fentanyl, and n=3 for monocytes at 20ng/ml of fentanyl) and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

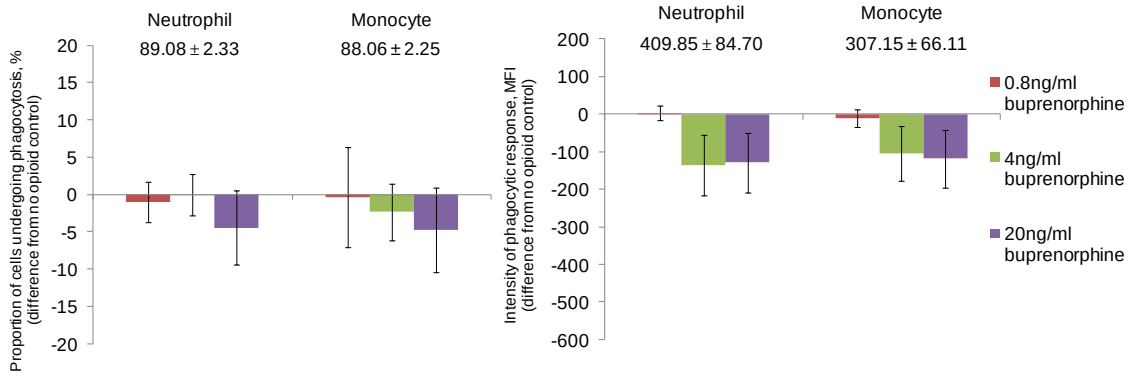

Figure 3-36: Effect of buprenorphine on neutrophil and monocyte phagocytosis.
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are stated at the top of the graphs. Whole blood was incubated with the indicated concentrations of buprenorphine for 60 min prior to assessing the phagocytosis of FITC-*E.coli*. Data are means ± SEM from 5 independent experiments (n=4 for 0.8ng/ml of buprenorphine, and n=6 and 7 for neutrophils and monocytes at 4ng/ml of fentanyl respectively). No statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

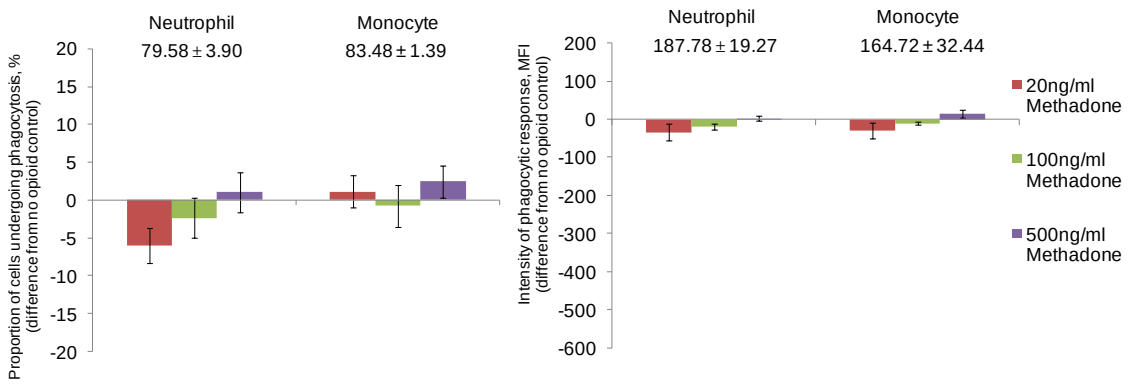

Figure 3-37: Effect of methadone on neutrophil and monocyte phagocytosis.
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are stated at the top of the graphs. Whole blood was incubated with the indicated concentrations of methadone for 60 min prior to assessing the phagocytosis of FITC-*E.coli*. Data are means ± SEM from 3 independent experiments and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

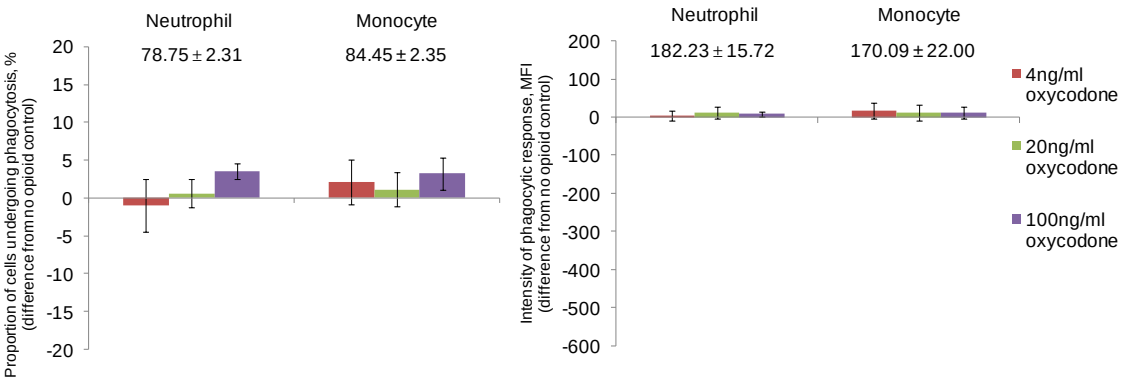

Figure 3-38: Effect of oxycodone on neutrophil and monocyte phagocytosis.
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are stated at the top of the graphs. Whole blood was incubated with the indicated concentrations of oxycodone for 60 min prior to assessing the phagocytosis of FITC-*E.coli*. Data are means ± SEM from 3 independent experiments and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

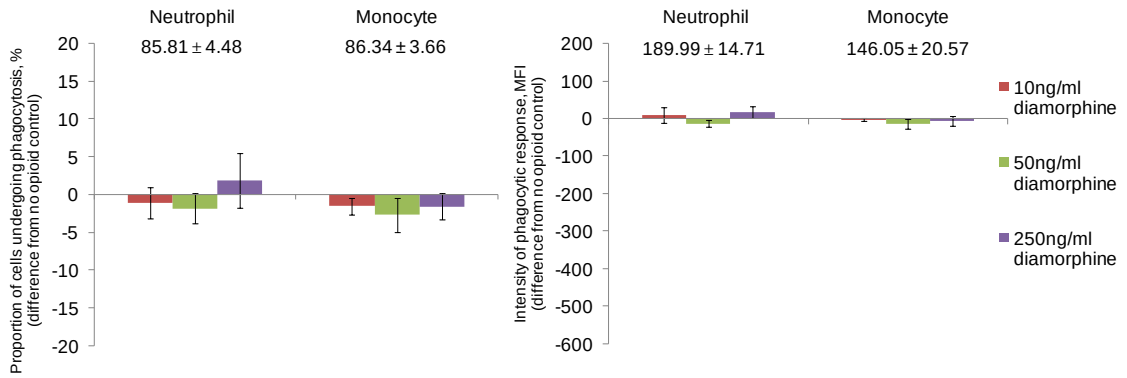

Figure 3-39: Effect of diamorphine on neutrophil and monocyte phagocytosis.
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are stated at the top of the graphs. Whole blood was incubated with the indicated concentrations of diamorphine for 60 min prior to assessing the phagocytosis of FITC-*E.coli*. Data are means ± SEM from 3 independent experiments and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

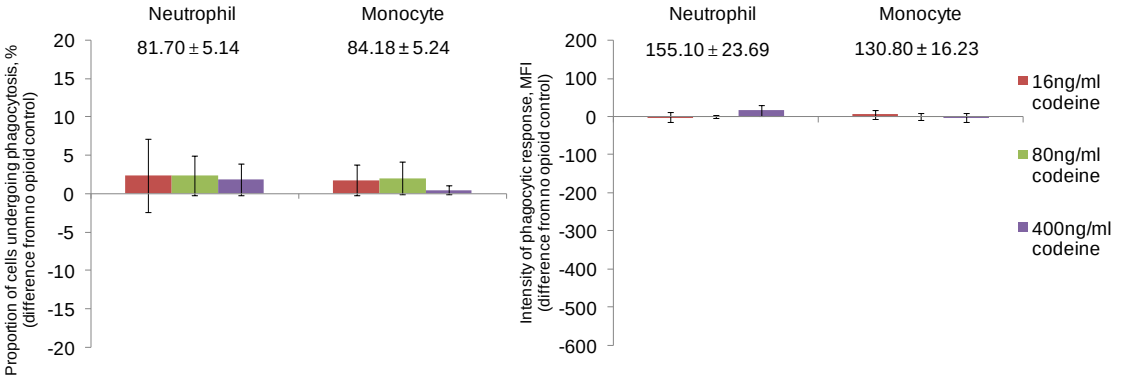

Figure 3-40: Effect of codeine on neutrophil and monocyte phagocytosis.
The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are stated at the top of the graphs. Whole blood was incubated with the indicated concentrations of codeine for 60 min prior to assessing the phagocytosis of FITC-*E.coli*. Data are means ± SEM from 3 independent experiments and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

Trends that opioids influenced phagocytosis were apparent, however interindividual variability and the limited sample size makes type II errors likely. However, the relevance of the relationship between statistical and clinical significance must also be considered. The clinical significance of any effect might be more dependent on individual rather than group effects and this will be explored in the next section.

3.2.2 Effects of opioids on phagocytosis in individual subjects

No consistent, statistically significant effect of any of the opioids tested on neutrophil and monocyte phagocytosis was detected in the current study. Although there does appear to be an effect of certain opioids on phagocytosis for individual subjects, there was a large interindividual variability in the observed responses. Baseline phagocytic activity was also variable. Although the initial analysis of the data, after three subjects had been assessed, demonstrated that morphine, tramadol and fentanyl statistically significantly inhibited phagocytosis, this statistical significance was lost when the group size was increased to five. For some subjects, some of the opioids markedly inhibited or in some cases activated the phagocytic response, whereas no effects were seen in others. The genetic and molecular basis to interindividual variability to opioids will be discussed in section 4.7.

Below are graphs showing the variability in the response of each individual subject to the intermediate concentrations of morphine, tramadol, fentanyl and buprenorphine which approximates to those that are found clinically (Figure 3-41 - Figure 3-48). The other opioids had no influence on phagocytosis and there was minimal interindividual variability. Each graph shows the effect of opioid vs. no opioid for each subject plotted separately. To enable comparisons for each subject across the range of opioids for which they were tested, they have been allocated a subject number, which remains consistent across tests. Not all subjects were tested for each opioid.

The phagocytosis assays have been shown to be reproducible with Coefficients of Variation of 0.2% for the proportion of neutrophils phagocytosing *E.coli* and 1.5% for the MFI of this response, and 1.1% for the proportion of monocytes phagocytosing *E.coli* and 3.6% for the MFI of this response (PHAGOBURST® test kit data sheet, Orpegen Pharma). It is thus likely that it is biological rather than experimental variations leading to this interindividual effect.

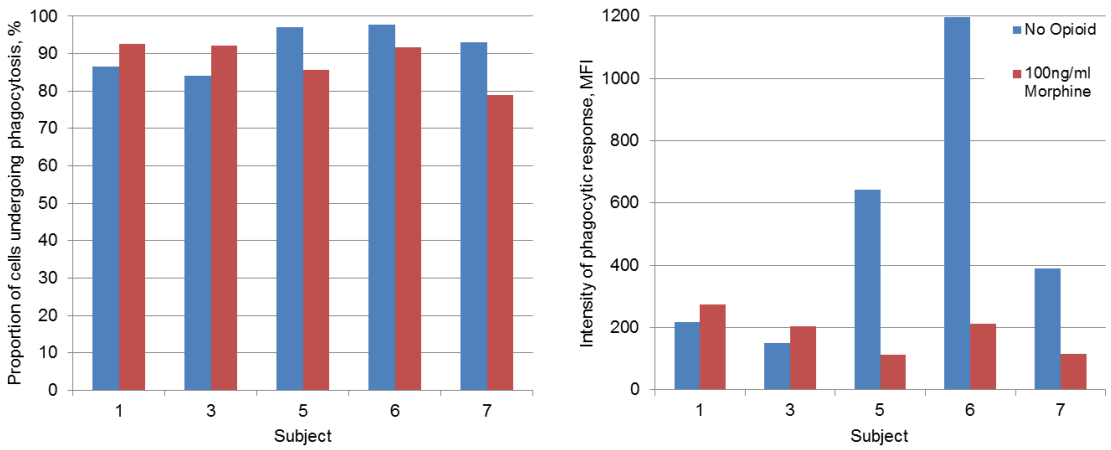

Figure 3-41: Effect of morphine on neutrophil phagocytosis for individuals.
The proportion of neutrophils phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 100ng/ml of morphine. Whole blood was incubated with either no opioid or 100ng/ml of morphine for 60 min prior to assessing the phagocytosis of FITC-*E.coli*.

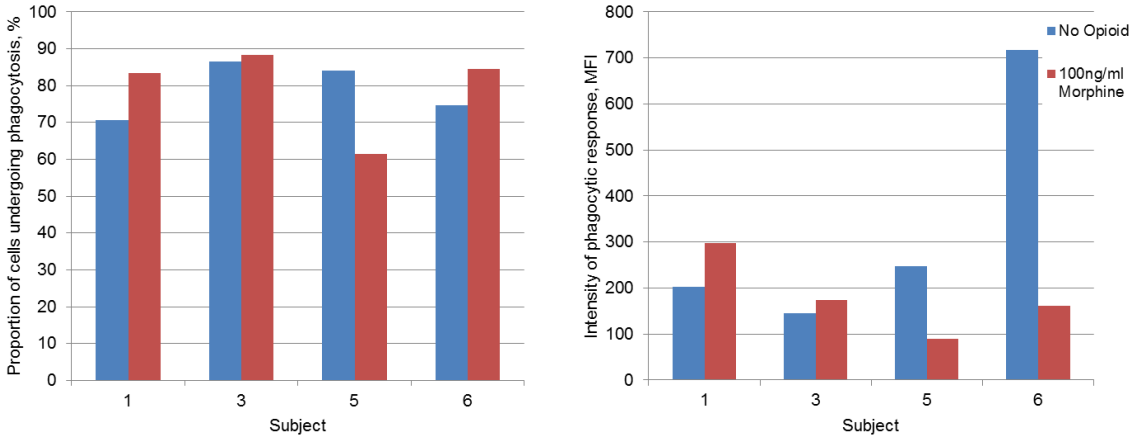

Figure 3-42: Effect of morphine on monocyte phagocytosis for individuals.
The proportion of monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 100ng/ml of morphine. Whole blood was incubated with either no opioid or 100ng/ml of morphine for 60 min prior to assessing the phagocytosis of FITC-*E.coli*.

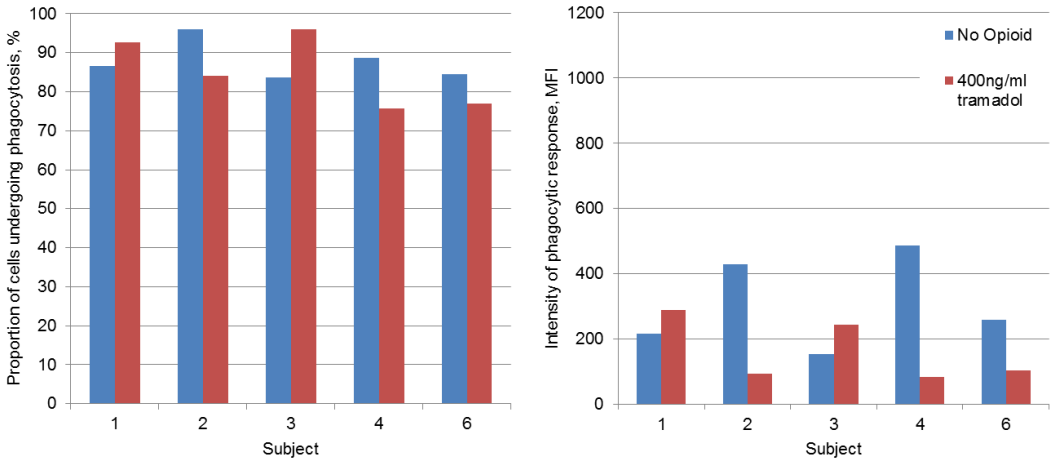

Figure 3-43: Effect of tramadol on neutrophil phagocytosis for individuals.
The proportion of neutrophils phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 400ng/ml of tramadol. Whole blood was incubated with either no opioid or 400ng/ml of tramadol for 60 min prior to assessing the phagocytosis of FITC-*E.coli*.

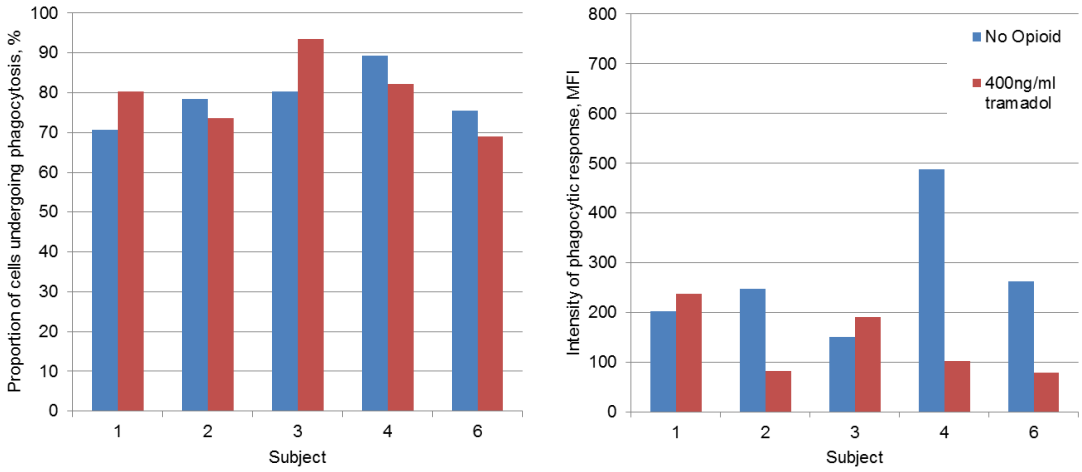

Figure 3-44: Effect of tramadol on monocyte phagocytosis for individuals.
The proportion of monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 400ng/ml of tramadol. Whole blood was incubated with either no opioid or 400ng/ml of tramadol for 60 min prior to assessing the phagocytosis of FITC-*E.coli*.

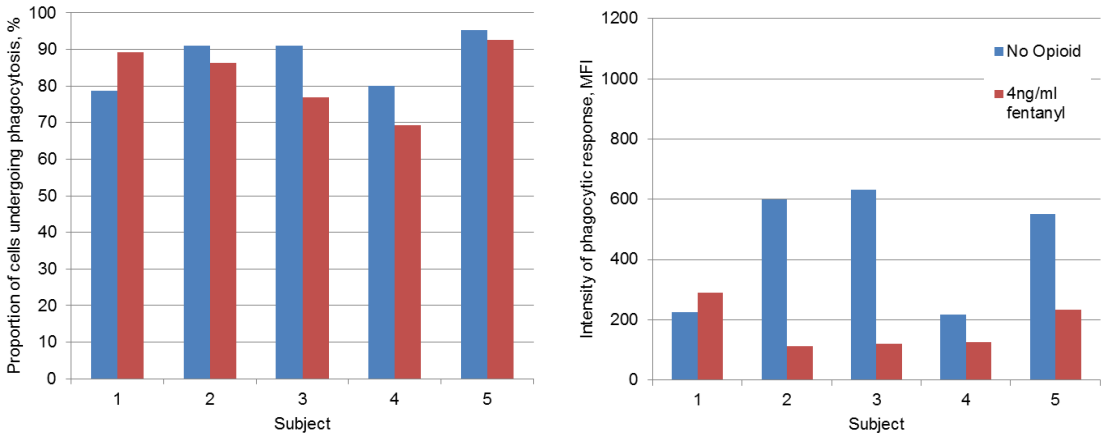

Figure 3-45: Effect of fentanyl on neutrophil phagocytosis for individuals.
The proportion of neutrophils phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 4ng/ml of fentanyl. Whole blood was incubated with either no opioid or 4ng/ml of fentanyl for 60 min prior to assessing the phagocytosis of FITC-*E.coli*.

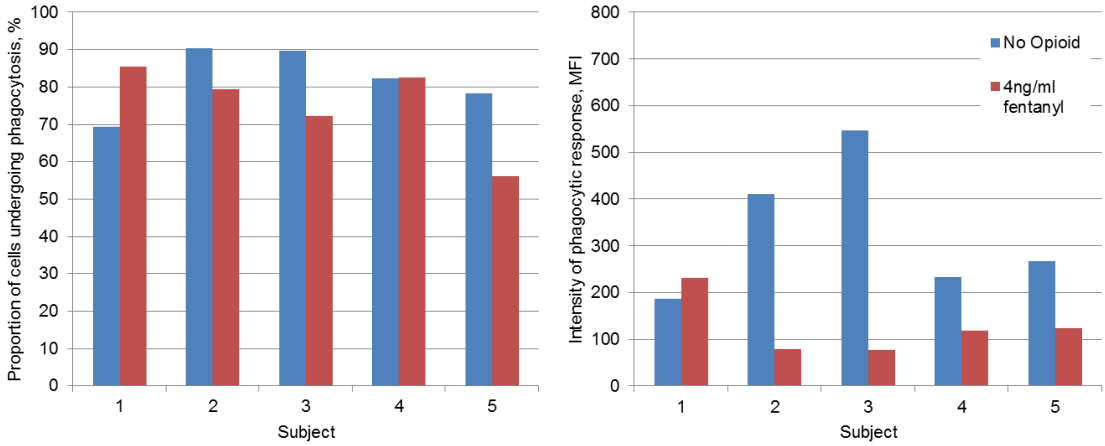

Figure 3-46: Effect of fentanyl on monocyte phagocytosis for individuals.
The proportion of monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 4ng/ml of fentanyl. Whole blood was incubated with either no opioid or 4ng/ml of fentanyl for 60 min prior to assessing the phagocytosis of FITC-*E.coli*.

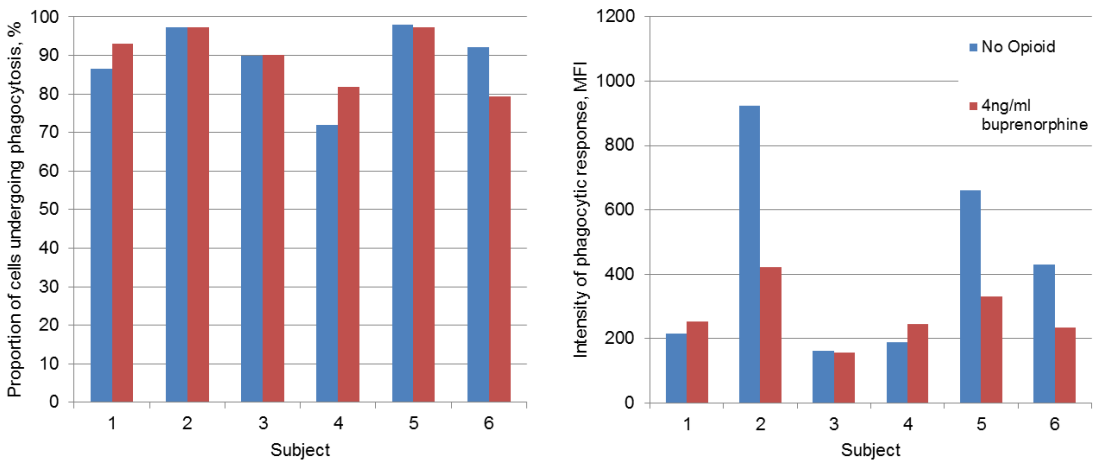

Figure 3-47: Effect of buprenorphine on neutrophil phagocytosis for individuals.
The proportion of neutrophils phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 4ng/ml of buprenorphine. Whole blood was incubated with either no opioid or 4ng/ml of buprenorphine for 60 min prior to assessing the phagocytosis of FITC-*E.coli*.

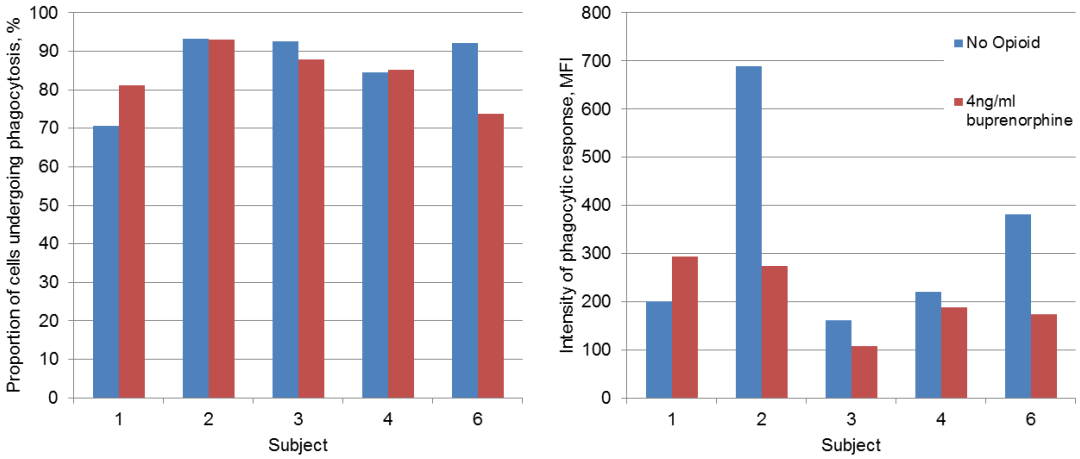

Figure 3-48: Effect of buprenorphine on monocyte phagocytosis for individuals.
The proportion of monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 4ng/ml of buprenorphine. Whole blood was incubated with either no opioid or 4ng/ml of buprenorphine for 60 min prior to assessing the phagocytosis of FITC-*E.coli*.

The tables below summarise the effect of the opioids at all concentrations tested on phagocytosis of neutrophils (Table 3.1) and monocytes (Table 3.2) for the individual subjects tested. When assessing the effect of individuals across the opioids at a range of concentrations for all the different tests of immune function, many potential trends could be extrapolated (i.e. only effects at specific concentrations, or u or n shaped curves). However, due to the number of permutations, only when an opioid increased or inhibited the immune response by at least 20% at most of the concentrations tested was this response considered to be a trend.

Table 3.1: Effect of opioids on phagocytosis of neutrophils for individuals.

Opioid / Subject	Morphine	Tramadol	Fentanyl	Buprenorphine	Methadone, oxycodone, diamorphine, codeine
1	Increase	Increase	Increase	Increase	No effect
2		Decrease	Decrease	Decrease	No effect
3	No effect	Increase	Decrease	No effect	No effect
4		Decrease	Decrease	No effect	No effect
5	Decrease		Decrease	Decrease	No effect
6	Decrease	Decrease		Decrease	No effect
7	Decrease				

The effect of the opioids on total phagocytosis (MFI) is summarised. Red indicates an inhibitory effect, green a stimulatory effect and yellow no effect (boxes are uncoloured if not tested).

Table 3.2: Effect of opioids on phagocytosis of monocytes for individuals.

Opioid / Subject	Morphine	Tramadol	Fentanyl	Buprenorphine	Methadone, oxycodone, diamorphine, codeine
1	Increase	Increase	Increase	Increase	No effect
2		Decrease	Decrease	Decrease	No effect
3	No effect	Increase	Decrease	No effect	No effect
4		Decrease	Decrease	Decrease	No effect
5	Decrease		Decrease	Decrease	
6	Decrease	Decrease		Decrease	

The effect of the opioids on total phagocytosis (MFI) is summarised. Red indicates an inhibitory effect, green a stimulatory effect and yellow no effect (boxes are uncoloured if not tested).

For the opioids that did influence phagocytosis (morphine, tramadol, fentanyl and buprenorphine), there were clear differences between individuals, with some opioids increasing phagocytosis in some subjects, decreasing it in others, or not having any effect. Even though some opioids appeared to have stimulatory properties in some subjects, the magnitude of this effect tended to be less than their suppressive effects, when these occurred. Subject 1 was most likely to have an enhancing effect from the opioids, whereas all of the opioids tended to inhibit responses in the other subjects (except tramadol in subject 3). From the opioids that influenced phagocytosis, there was no clear pattern in the direction of this effect from the different opioids as any effect was more dependent on the subject than the opioid. In general, there was a

greater effect on the total amount of phagocytosis (i.e. total number of *E.coli* ingested, denoted as MFI), compared with the number of neutrophils ingesting the bacteria (proportion of neutrophils phagocytosing FITC-*E.coli*). Morphine, tramadol, fentanyl and buprenorphine might therefore be detrimental in certain individuals with, or at risk of a bacterial infection. Although methadone, oxycodone, diamorphine and codeine did not affect phagocytosis, diamorphine and codeine are pro-drugs and *in vivo* are converted to morphine and thus would be expected to have similar immune effects to morphine. Methadone and oxycodone might thus be safer, although this hypothesis would need confirmation in clinical studies.

3.2.3 Discussion

The current study used whole blood flow cytometry to assess the effects of a 1-hr incubation with eight commonly used opioids at three clinically relevant concentrations on the ability of human neutrophils and monocytes to phagocytose *E.coli*. None of the opioids had a statistically significant effect on the ability of neutrophils and monocytes to phagocytose *E.coli*.

Flow cytometry allows both the proportion of neutrophils and monocytes phagocytosing the *E.coli* (i.e. number of cells phagocytosing the bacteria) and intensity of this response (i.e. the total fluorescent signal from each cell, which correlates to the number of *E.coli* phagocytosed) to be measured. This enabled the impact of opioids on the ability of individual neutrophils and monocytes to be assessed, and the phagocytic capacity of the cell population as a whole, as if either fewer cells phagocytose or the total number of bacteria phagocytosed is decreased this could impact on host immunity. From these results, the intensity of the phagocytic response was a more sensitive test than the proportion of cells phagocytosing *E.coli* to the effects of opioids.

Retrospective power and sample size calculations were performed using DSS research programmes as morphine, tramadol, fentanyl and buprenorphine inhibited the total amount of phagocytosis (MFI) but these did not reach statistical significance. The power calculations used a two-tail, two sample test, using average values and a 95% confidence interval, with an appropriate power being 80% or above. The sample size calculations used a two samples using average values test with a 95% confidence interval. The sample size calculations for neutrophil phagocytosis were around five to seven samples for morphine, tramadol and buprenorphine, and around 20 for fentanyl. These studies were therefore underpowered (power less than 80%). For the total amount of monocyte phagocytosis (MFI) the sample size calculations were around five

to seven samples for morphine, fentanyl and buprenorphine, and several fold higher for tramadol. These studies were also generally underpowered, with only some concentrations of morphine being above 80%.

Several previous *in vitro* studies have evaluated the influence of opioids on phagocytosis, these are reviewed below and compared to the current study.

Szabo and colleagues investigated the *in vitro* effect of morphine on the ability of murine peritoneal macrophages to phagocytose *Candida albicans* (Szabo et al., 1993). For this study, non-elicited resident peritoneal macrophages were harvested from female C^3HeB/FeJ mice. Cultured macrophage monolayers were used to assess the phagocytosis of *Candida albicans* (with a yeast to macrophage ratio of 10:1) over 30 min at 37°C. The proportion of macrophages that had ingested three or more Candida cells and the average number of these ingested per macrophage (phagocytic index) were counted microscopically. The cultured macrophages were treated with between 10^{-10} – 10^{-6} M of morphine (equivalent to 0.03 – 285 ng/ml). There was a concentration-dependent decrease in both the percentage of macrophages phagocytosing the Candida and the phagocytic index, with a maximum effect of 36% and 40% respectively, at the highest concentration of morphine. The same results were obtained if cells were pre-treated with morphine for periods up to 2 hr. The authors speculated that the opioids could act directly on the living Candida and this might also influence phagocytosis (Szabo et al., 1993).

Using concentrations that overlapped with the current study, Szabo and colleagues showed that morphine was inhibitory. Although the current study showed that morphine inhibited phagocytosis in some individuals, this effect was not statistically significant. Szabo used peritoneal macrophages from inbred mice against yeast cells, whereas whole blood was used from healthy humans against *E.coli* in the current study.

Lazaro and colleagues investigated the reversibility of the effects of *in vitro* morphine on murine macrophage phagocytosis (Lazaro et al., 2000). Female C3HeB/FeJ mice received intraperitoneal injections of thioglycollate, 5 days after which they were killed and their peritoneal macrophages isolated. Thioglycollate initiates macrophage recruitment and activation. For the acute exposure experiments, the macrophages were incubated with 100 and 500 nM of morphine (equivalent to 28 and 143 ng/ml) for between 0 and 30 min before phagocytosis was assessed. To assess the influence of chronic exposure, macrophages were incubated for 8 and 17 hr with 100 nM of

morphine. Drug withdrawal was assessed by washing the cells to remove the morphine or by adding the non-selective opioid receptor antagonist, naloxone. Phagocytosis was measured by the ability of the macrophages to ingest opsonised SRBCs over 20 min, as measured by microscopic examination. Only a 30 min incubation with 100 nM of morphine (≈ 28 ng/ml) inhibited murine macrophage phagocytosis of SRBCs, this was by about 18%. Exposure to morphine for >8 hr had no effect on macrophage phagocytosis. Opioid withdrawal from tolerant cells resulted in an inhibition of phagocytosis which was maximal 1 hr after drug withdrawal. 500 nM morphine had no effect at the time points assessed (Lazaro et al., 2000). Although this might be due to rapid desensitisation of opioid receptors, it would have had to occur within 5 min (Tomei and Renaud, 1997). To assess desensitisation, the 500 nM of morphine was removed after 30 min and 100 nM of morphine was added after different times. If added within 1 hr, 100 nM of morphine had no effect, but if added after 2 hr, phagocytosis was inhibited, as in opioid naïve macrophages, indicating that desensitisation had occurred (Lazaro et al., 2000). The effects of *in vitro* morphine on phagocytosis by murine macrophages in this model were dependent on the conditions to which the cells are exposed, with short incubation periods inhibiting phagocytosis, and longer incubation periods as well as higher doses inducing tolerance.

Lazaro and colleagues demonstrated that morphine inhibited murine macrophage phagocytosis of SRBCs, although the effect was quantitatively modest. The current study did not have a consistent effect with similar concentrations of morphine, but did show that phagocytosis could be inhibited in some individuals. The more consistent effect in the study by Lazaro is likely to be due to inbred mice being used. Other variations between the studies which could account for the differences include the effector and target cell types, incubation times and the analysis method.

In several studies by the same group, the effect morphine and methadone have been assessed in various models, using different methodologies (Lipovsky et al., 1998, Molitor et al., 1992, Peterson et al., 1995, Sowa et al., 1997). Molitor and colleagues investigated the *in vitro* effects of methadone on swine PBMCs (Molitor et al., 1992). Cells were incubated for 48 hr with 10^{-18} – 10^{-6} M of methadone (equivalent to $3x10^{-10}$ – 309 ng/ml). Antibody-mediated phagocytosis was measured using ^{51}Cr-labeled opsonised SRBCs after a 1-hr incubation. Methadone had no effect on monocyte antibody-mediated phagocytosis of opsonised SRBCs (Molitor et al., 1992).

Peterson and colleagues investigated the effect of morphine on the phagocytosis of *Mycobacterium tuberculosis* by human fetal microglial cells (Peterson et al., 1995). The microglial cells were incubated with 10^{-14} – 10^{-6} M of morphine (equivalent to $3x10^{-6}$ – 285 ng/ml) for 24 hr, before being incubated at 37°C for 18 hr with non-opsonised *M. tuberculosis* (with a bacteria to microglia ratio of 10:1). The proportion of microglial cells that had phagocytosed any bacilli (% phagocytosis) and the total number of bacteria ingested by 100 microglial cells were recorded. At 10^{-8} M (≈ 2.9 ng/ml)**,** morphine stimulated the phagocytosis of *M. tuberculosis*, increasing the proportion of microglia phagocytosing any bacteria from 45 to 75% and the number of bacteria ingested per 100 microglia from 70 to 120***.*** At all other concentrations tested, morphine had no effect on phagocytosis. There was no effect when 10^{-8} M of morphine (≈ 2.9 ng/ml) was added at the same time as the *M. tuberculosis*, but there was activation with a 6-hr incubation, although less than the 24-hr incubation. This was blocked by naloxone, β-funaltrexamine (a MOR alkylating agent) and pertussis toxin, suggesting that a MOR G_i protein coupled receptor is involved (Peterson et al., 1995).

Sowa and colleagues investigated the effect of morphine on the phagocytosis of non-opsonised, heat killed *Cryptococcus neoformans* by neonatal swine microglia (Sowa et al., 1997). The microglial cells were incubated with 10^{-20} – 10^{-6} M of morphine (equivalent to $3x10^{-10}$ – 285 ng/ml) for 24 hr, before being incubated with the cryptococci (with a yeast to microglia ratio of 50:1) for 2 hr at 37°C. The proportion of microglial cells which had phagocytosed any cryptococci (% phagocytosis) and the total number of cryptococci ingested by 100 microglial cells (yeast/100 microglia) were assessed. Morphine dose-dependently inhibited the proportion of microglia phagocytosing the cryptococci and the total number of organisms that were ingested, with a maximal inhibition of 93% at 10^{-6} M (≈ 285 ng/ml). Morphine had no effect when added 3 hr before or at the time of the phagocytosis assay (Sowa et al., 1997).

The same group also investigated the effect of morphine on the phagocytosis of *Cryptococcus neoformans* by human fetal microglial cells (Lipovsky et al., 1998). Microglial cells were treated for 18 hr with 10^{-16} – 10^{-6} M of morphine (equivalent to $3x10^{-8}$ – 285 ng/ml) before being incubated with *Cryptococcus neoformans* for 1 hr at 37°C, Experiments were performed at a yeast to microglia ratio of 10:1. The proportion of microglial cells that had phagocytosed the cryptococci were counted using fluorescent microscopy. Only opsonised yeast were phagocytosed by human microglia, and this was enhanced when yeast were coated with complement. Morphine stimulated microglial phagocytosis, with a bell-shaped dose response curve and a maximal

increase of 50% at 10^{-8} M (≈ 2.9 ng/ml). This was reversed by naloxone and β-funaltrexamine. Blocking the complement receptor also blocked the effect of morphine (and decreased phagocytosis in both no opioid and opioid groups). Overall this study showed that morphine enhanced the ability of human microglia to phagocytose *Cryptococcus neoformans* by a complement receptor-mediated mechanism (Lipovsky et al., 1998). As cryptococci survive and replicate inside human microglia after being phagocytosed, it is difficult to know how relevant this model is to host defence mechanisms (Lee et al., 1995b).

These studies from the same group reported conflicting results that are likely due to methodological differences (Lipovsky et al., 1998, Sowa et al., 1997). Three of the studies assessed the effect of morphine, in doses overlapping with the current study and also much lower concentrations. Although the very low concentrations might be less relevant clinically, they might help to establish the dose-response curve. Incubation time with morphine and target cells also varied between the above studies from between 0 and 30 min up to 24 hr. As demonstrated by some of these studies, this dramatically influences the effect of morphine on phagocytosis (Peterson et al., 1995, Sowa et al., 1997). The current study, after preliminary kinetics experiments, incubated whole blood with the opioids for 60 min and then with *E.coli* for 10 min (the manufacturer's recommended period). Methadone showed a similar lack of effect in both the current study and that by Molitor, despite a very different methodology, using swine cells and ^{51}Cr-labeled SRBC targets (Molitor et al., 1992). The above studies used optical or fluorescent microscopy to count the number of cells phagocytosed, whereas the current study used flow cytometry which enabled the fluorescent characteristics of thousands of cells to be accurately detected (Kampen et al., 2004).

The effect of morphine on neutrophil phagocytosis was evaluated in 16 male opioid naïve rhesus monkeys (Liu et al., 1992). Neutrophils were isolated from the peripheral blood of these monkeys and incubated with 50 fM and 50 pM of morphine (equivalent to 1.4 $x10^{-5}$ and 0.014 ng/ml) for 4 hr. Phagocytosis of *Candida albicans* blastospores after a 45-min incubation was measured using fluorescent microscopy. The proportion (%) of neutrophils which phagocytosed three or more *Candida albicans* blastospores was not affected by either concentration of morphine (Liu et al., 1992). Despite a very different approach, the results of this study correlate with current study, although individual variability is nor reported.

The effect of morphine and tramadol on the phagocytosis of latex beads by neutrophils

and monocytes isolated from the peripheral blood of 30 healthy volunteers has been compared (Beilin et al., 2005). For these studies, cells were incubated with either morphine (20, 40 and 80 ng/ml) or tramadol (5,000, 10,000 and 20,000 ng/ml) for 60 min with no opioid as a control. The proportion of cells phagocytosing the latex particles and the number of latex particles phagocytosed by each cell (phagocytic index) in 60 min at 37°C (100% humidity, 5% v/v CO_2), were counted using a microscope. Morphine dose-dependently decreased the proportion of monocytes undergoing phagocytosis (by up to 19%) and the phagocytic index (by up to 35%), but only decreased the proportion of neutrophils undergoing phagocytosis (by up to 24%). Tramadol had no effect on these parameters in neutrophils and monocytes. Despite having 30 subjects and showing SEM on the bar charts, there were still errors of up to ± 15% for baseline, morphine and tramadol. Effects of the opioids on the individuals tested were not discussed (Beilin et al., 2005).

The study by Beilin showed a significant inhibition by morphine, which was not replicated in the current study, although the effect sizes were comparable, this may be due to the number of subjects studied. Although neither this nor the current study demonstrated a significant effect of tramadol, there was a trend in the current study for tramadol to inhibit phagocytosis. The differential effect of drugs on phagocytosis might depend on the phagocytic target (Galley et al., 2000), which might explain some of the discrepancy with the effect of tramadol on phagocytosis. Although a major role of phagocytes is to kill and remove bacteria, they are also involved in the removal of inert particles (Djaldetti et al., 2002). However, in terms of the role of phagocytosis in the protection against bacterial infection, *E.coli* is a more physiologically relevant target.

Although this is the most comparable experiment to the current study, there are certain key differences. Beilin and colleagues concentrated and extracted the leukocytes from the peripheral venous blood from 30 volunteers, used latex particles as the target for phagocytosis and manually counted the proportion of cells undergoing phagocytosis and the number of latex particles that had been engulfed by each individual cell. The data were averaged from the 100 cells that were counted. The current study used whole blood from five volunteers, FITC-labelled *E.coli* as the phagocytic target and at least 5,000 events from the flow cytometric analysis to determine the proportion of cells phagocytosing *E.coli* and the intensity of this response, for each type of phagocyte. Although the concentrations of morphine used by Beilin overlapped with the current study, they used higher concentrations of tramadol (5,000 – 20,000 ng/ml compared with 80 – 2,000 ng/ml). The incubation time in both studies was 60 min at 37°C,

however Beilin used a humidified atmosphere containing 5% v/v CO_2, whereas incubations for the current study were performed in a water bath (Beilin et al., 2005). This may have led to differences as a heated atmosphere produces a less direct heat and the sample would heat slower; the additional CO_2 could generate acidity in the sample, although this is more likely to occur during longer incubations.

Although there have been several previous *in vitro* studies assessing the effect of morphine and to a lesser extent tramadol and methadone on phagocytosis, these have often been performed in different species (i.e. murine, swine and human) and using different effector (phagocytic) cells at different stages of development (i.e. adult human peripheral blood vs. neonatal swine vs. fetal human microglia), with different methods of cell isolation which modify the responsiveness of cells by pre-activating, priming or over-activating cells. (i.e. elicited peritoneal macrophages). The current study used whole blood from adult humans which is likely to be more applicable to the clinical setting. A range of target cells were also used in the previous studies, these included fungi (*Candida albicans* and *Cryptococcus neoformans*), bacteria (*Mycobacterium tuberculosis* - with a lipid rich cell wall) and foreign material (SRBCs). In some studies these cells were opsonised or killed before phagocytosis. The current study used opsonised *E.coli* as the phagocytic target, which is likely to be more relevant in terms of acute sepsis, although anti-fungal immunity is also important.

There were also large differences in the concentrations of morphine used. The current study used 20 – 500 ng/ml, which approximate to those found clinically. There were different methods of measuring phagocytosis and different target cells leading to different results from these studies and difficulty in comparing them. Microscopy counts far fewer cells and it is difficult to determine differences in intensity, unless the fluorescence is quantitated using image analysis. Although the use of a whole blood approach in the current study might also influence the results, whole blood is more physiological than isolating or culturing individual cell types as used in some studies.

Although there are many potential methodologies, all with advantages and disadvantages (such as short incubation time might be more relevant in patients with acute pain and longer incubations in those on chronic opioids), it is very difficult to compare the previous studies. Owing to these differences morphine was inhibitory in some of the experimental scenarios in some previous studies (Sowa et al., 1997, Szabo et al., 1993), it had no effect in others (Liu et al., 1992) and was stimulatory in two studies (Lipovsky et al., 1998, Peterson et al., 1995).

3.2.4 Conclusion

The *in vitro* effect of eight opioids at three clinically relevant concentrations on neutrophil and monocyte phagocytosis was assessed in the whole blood of healthy volunteers. None of the opioids had a statistically significant effect on phagocytosis. However, by evaluating each subject individually it appears that some subjects may be more susceptible to the effects of morphine, tramadol, fentanyl and buprenorphine (section 3.2.2). The majority of these effects are on the basis of the total phagocytosis, not the proportion of cells phagocytosing. In the cells taken from animals for the previous *in vitro* studies, the animals in each study are genetically very similar, being from the same inbred strains. If the response to opioids is, in part at least, driven by genetic difference, then any opioid-mediated effects are likely to be more consistent if inbred animal strains are used. In the current study in which blood was taken from different individuals, there is variability in the responses, leading to large standard deviation errors and negating any potential statistical significance. Although it would need confirmation in patient studies, these results could mean that some individuals exposed to morphine, tramadol, fentanyl or buprenorphine might be more susceptible to bacterial and fungal infections and that methadone and oxycodone might be safer options.

3.2.5 Oxidative burst reaction

The production of reactive oxygen species (superoxide radical and hydrogen peroxide) as part of the oxidative burst response is vital for the killing and degradation of phagocytosed bacteria, fungi and particles. The effects of three concentrations of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on the proportion of neutrophils and monocytes undergoing the oxidative burst response and the intensity of this response, as represented by the MFI for the positive cells are shown in the graphs below (Figure 3-49 - Figure 3-64).

Peripheral whole blood was incubated at 37°C with the designated concentration of opioid for 60 min before being stimulated with fMLP (a synthetic, chemotactic peptide which mimics the activity of bacterially-derived peptides), PMA (an activator of PKC) and *E.coli* for 10 min. As the subjects had differing baseline values for the oxidative burst response, the graphs are presented below as absolute differences from the no opioid control. The absolute baseline values, without opioid, are given at the top of the graphs (Figure 3-49 - Figure 3-64).

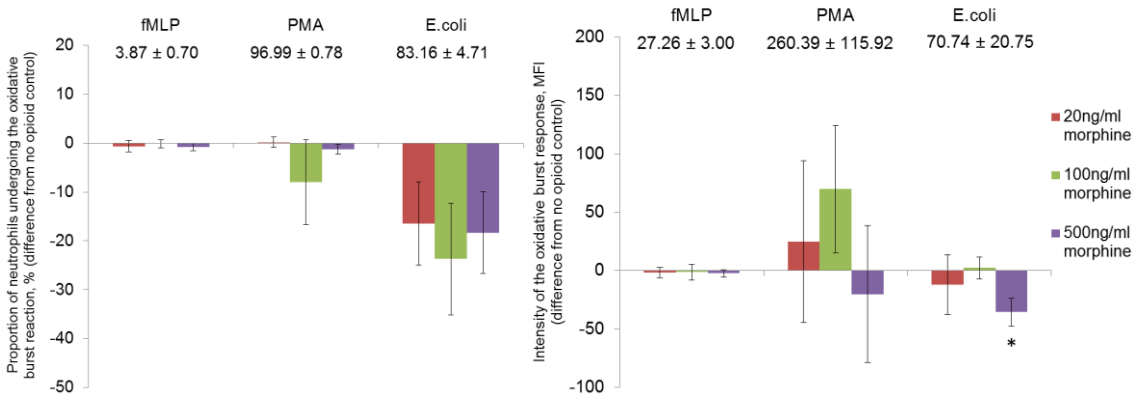

Figure 3-49: Effect of morphine on neutrophil oxidative burst responses.
The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of morphine for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 5 independent experiments and statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells is indicated (* = P<0.05).

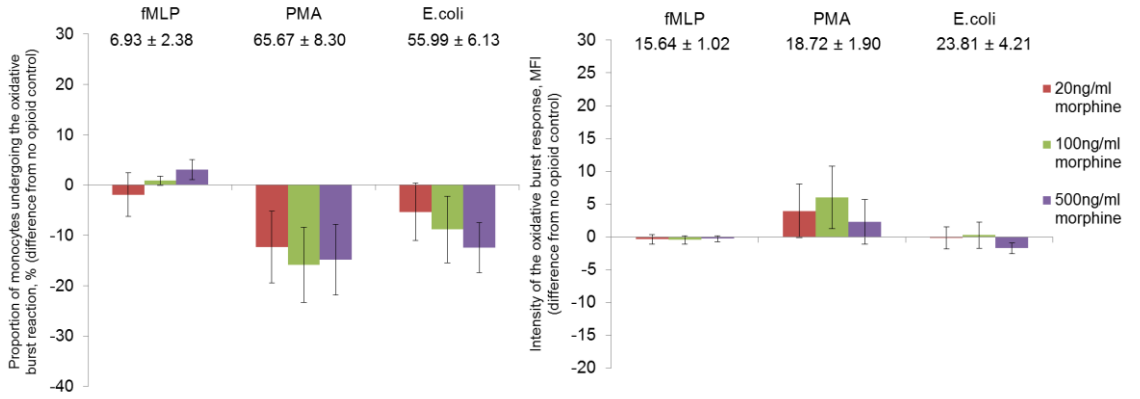

Figure 3-50: Effect of morphine on monocyte oxidative burst responses.
The proportion of monocytes (undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells (median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included in the graph. Whole blood was incubated with the indicated concentrations of morphine for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 5 independent experiments (n=4 for MFI with fMLP at 100ng/ml of morphine) and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

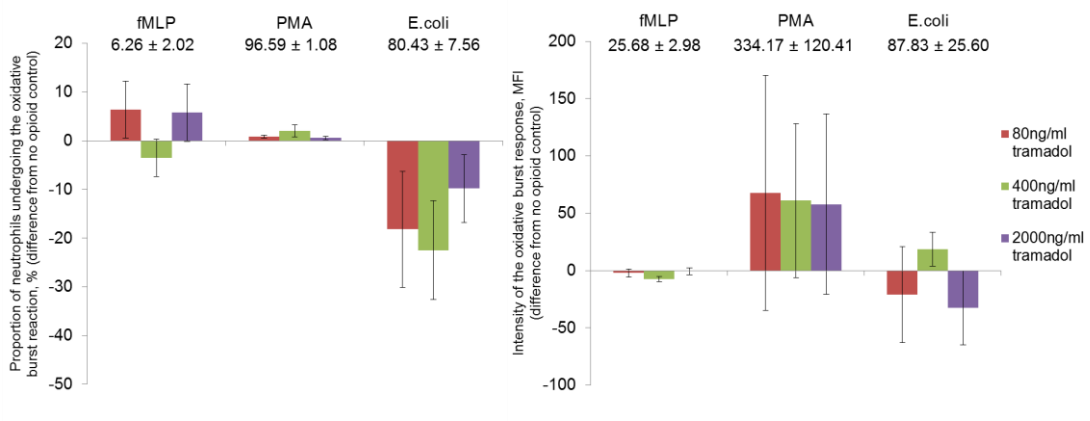

Figure 3-51: Effect of tramadol on neutrophil oxidative burst responses.
The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of tramadol for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 5 independent experiments, no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

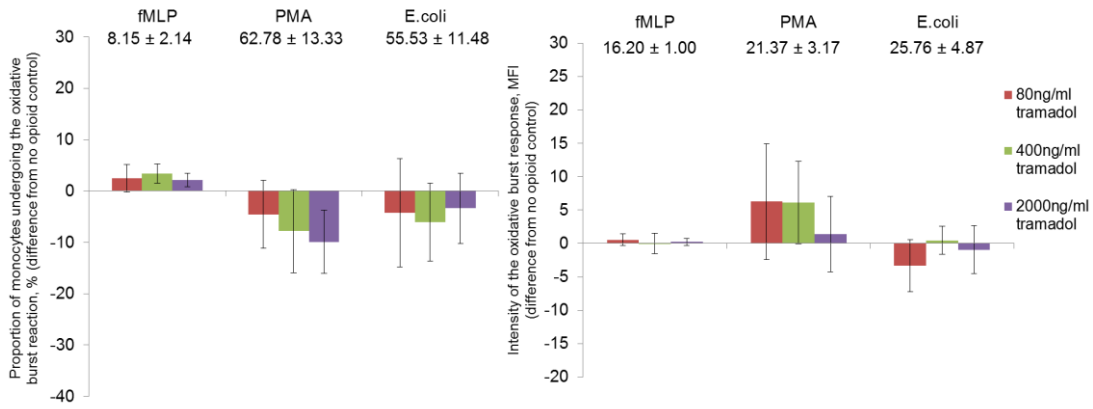

Figure 3-52: Effect of tramadol on monocyte oxidative burst responses.
The proportion of monocytes (undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells (median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of tramadol for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 5 independent experiments (n=4 for % and MFI with fMLP at 400ng/ml of tramadol) and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

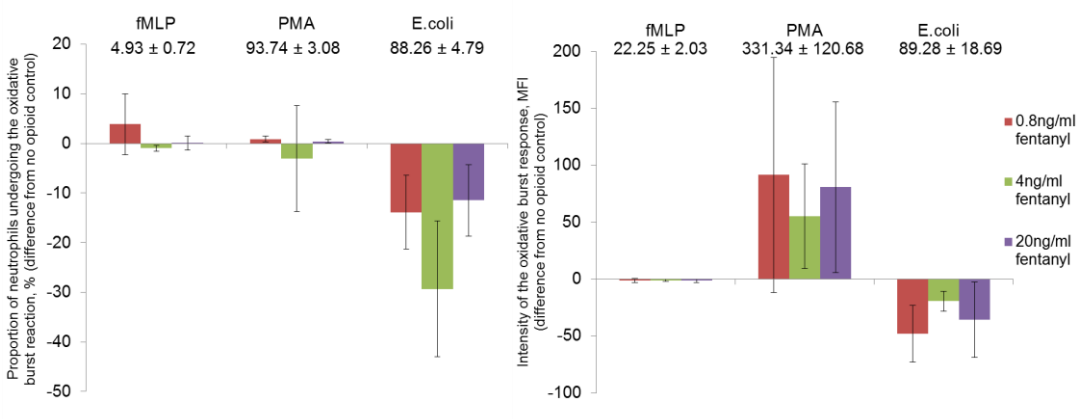

Figure 3-53: Effect of fentanyl on neutrophil oxidative burst responses.
The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of fentanyl for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 5 independent experiments, no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

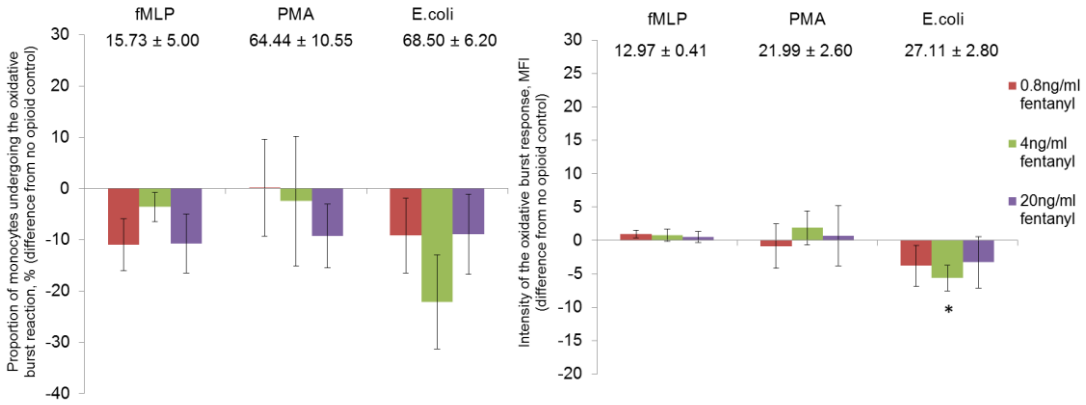

Figure 3-54: Effect of fentanyl on monocyte oxidative burst responses.
The proportion of monocytes (undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells (median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of fentanyl for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 5 independent experiments and statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells is indicated (* = P<0.05).

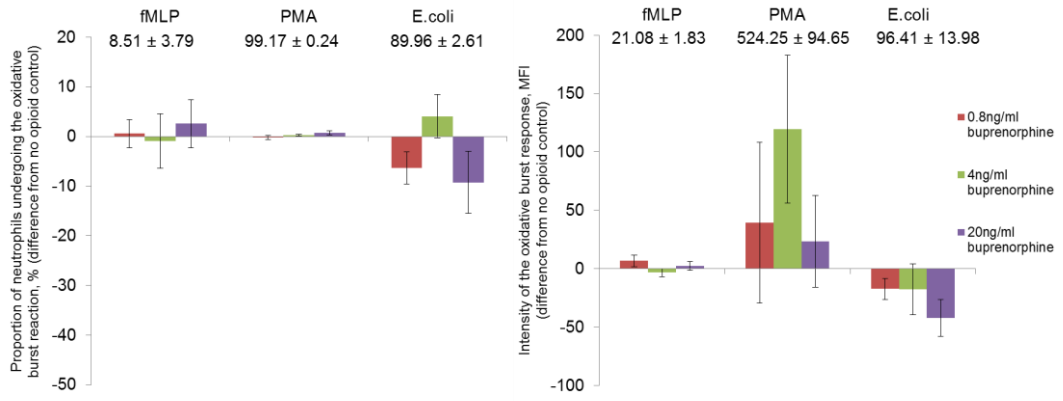

Figure 3-55: Effect of buprenorphine on neutrophil oxidative burst responses.
The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of buprenorphine for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 5 independent experiments (n=4 for 20 ng/ml and n=3 for 0.8 ng/ml of buprenorphine), no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

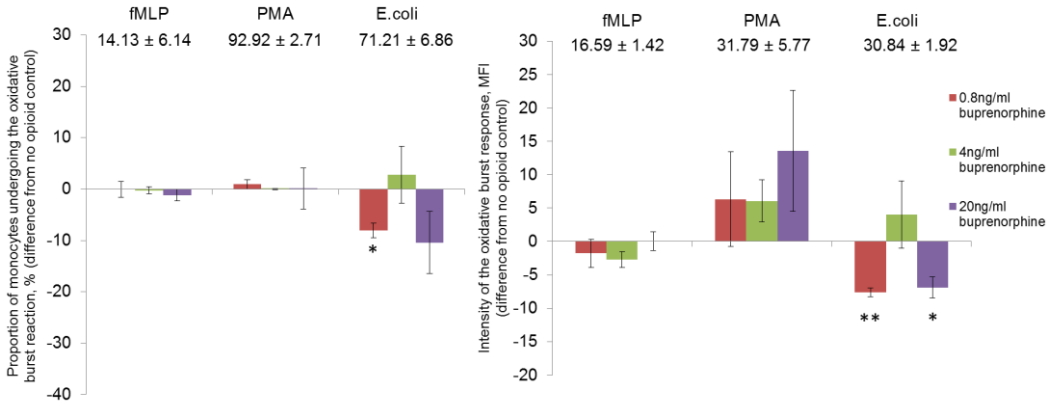

Figure 3-56: Effect of buprenorphine on monocyte oxidative burst responses.
The proportion of monocytes (undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells (median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of buprenorphine for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 4 independent experiments (n=3 for 0.8 ng/ml of buprenorphine) and statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells is indicated (* = P<0.05; ** = P<0.01).

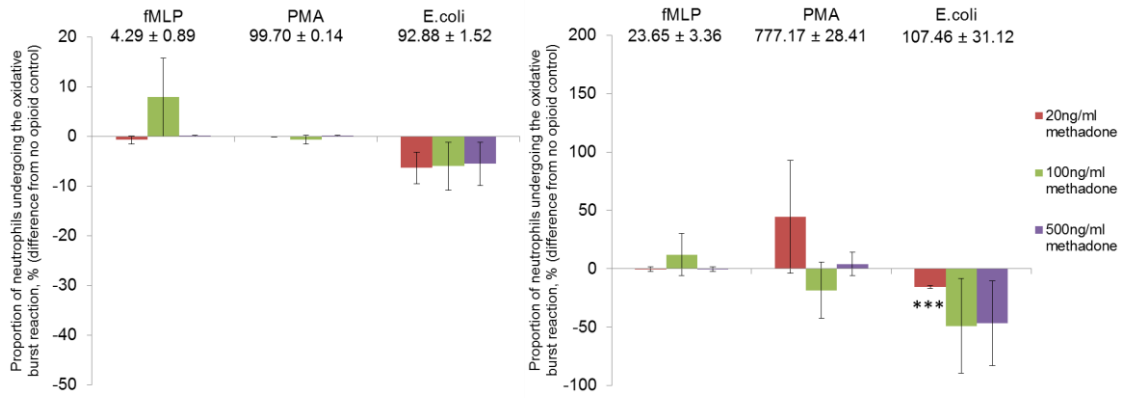

Figure 3-57: Effect of methadone on neutrophil oxidative burst responses.
The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of methadone for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 3 independent experiments and statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells is indicated (*** = P<0.001).

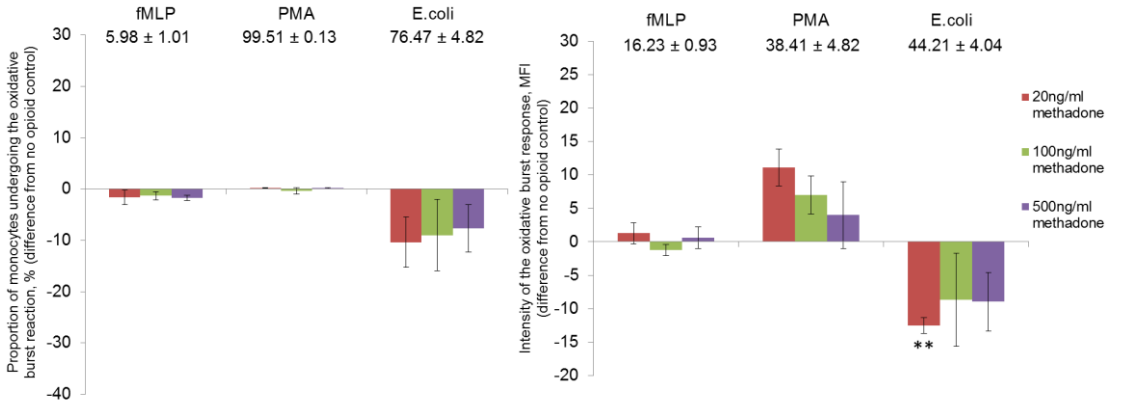

Figure 3-58: Effect of methadone on monocyte oxidative burst responses.
The proportion of monocytes (undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells (median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of methadone for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 3 independent experiments and statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells is indicated (** = P<0.01).

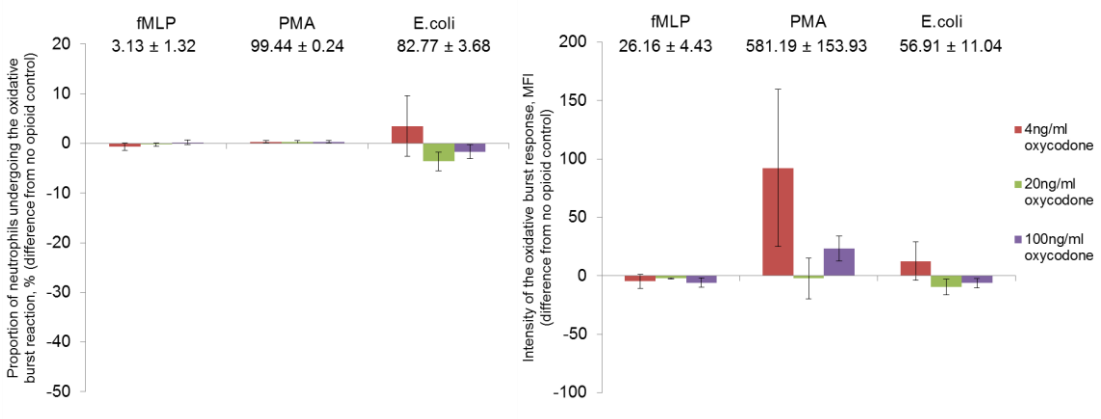

Figure 3-59: Effect of oxycodone on neutrophil oxidative burst responses.
The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of oxycodone for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 3 independent experiments, no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

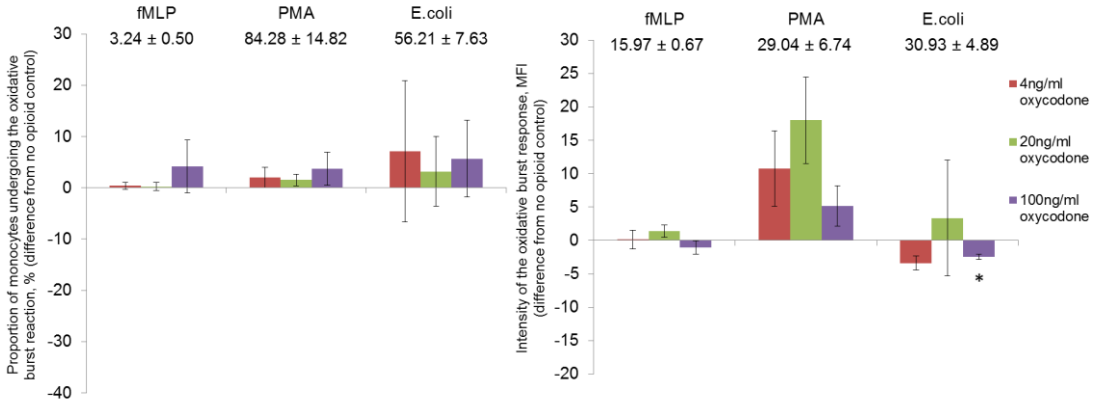

Figure 3-60: Effect of oxycodone on monocyte oxidative burst responses.
The proportion of monocytes (undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells (median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of oxycodone for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 3 independent experiments and statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells is indicated (* = P<0.05).

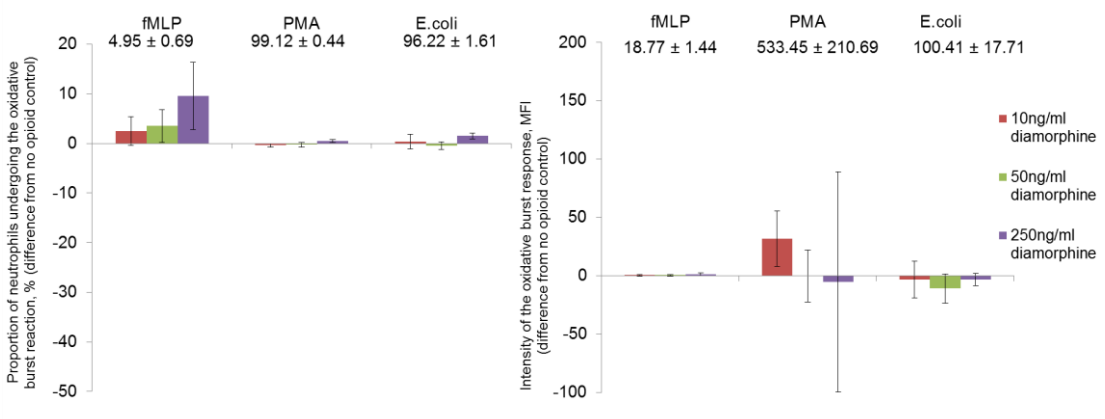

Figure 3-61: Effect of diamorphine on neutrophil oxidative burst responses.
The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of diamorphine for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 3 independent experiments, no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

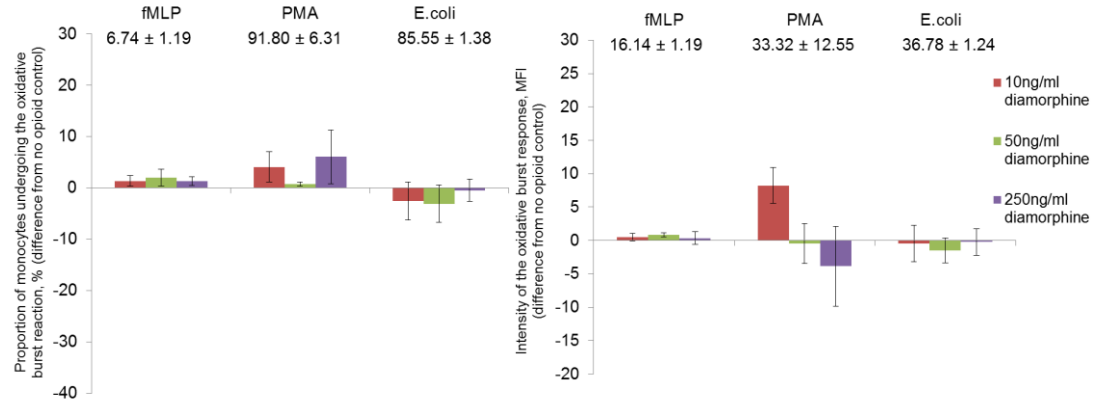

Figure 3-62: Effect of diamorphine on monocyte oxidative burst responses.
The proportion of monocytes (undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells (median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of diamorphine for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 3 independent experiments and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

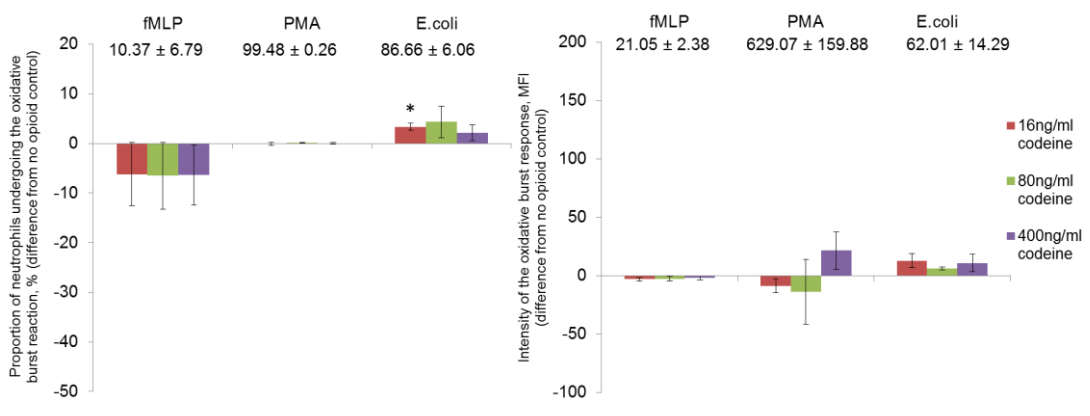

Figure 3-63: Effect of codeine on neutrophil oxidative burst responses.
The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of codeine for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 3 independent experiments and statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells is indicated (* = P<0.05).

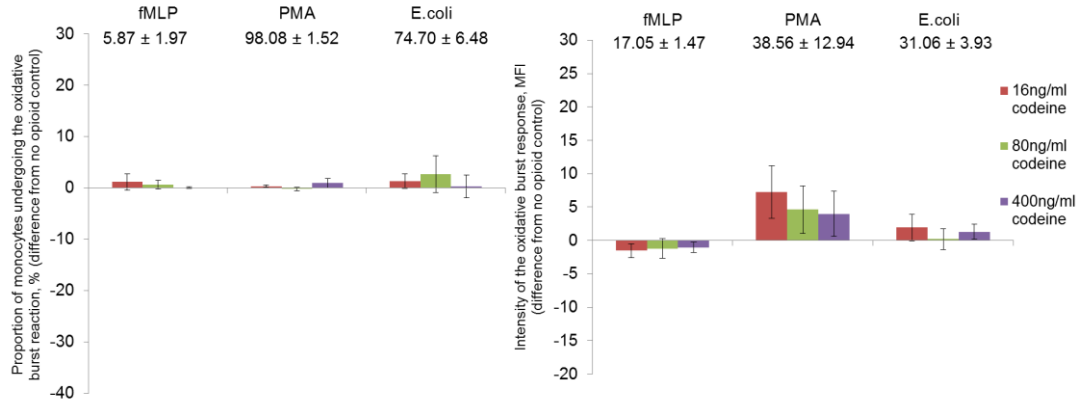

Figure 3-64: Effect of codeine on monocyte oxidative burst responses.
The proportion of monocytes (undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells (median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. These are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Whole blood was incubated with the indicated concentrations of codeine for 60 min prior to assessing the oxidative burst response. Data are means ± SEM from 3 independent experiments and no statistically significant differences, using a paired Student *t*-test, between the responsiveness of opioid-treated and untreated cells was detected.

The data presented above show the absolute change from the no opioid control samples in order to compensate for the differing control values for the oxidative burst response between subjects. The unstimulated controls all have very low levels of activation, comparable to fMLP (the weak stimulus). None of the opioids had an effect on unstimulated neutrophils or monocytes (data not shown). They also had no effect on fMLP stimulated neutrophils or monocytes, which was principally used to look for activating properties of the opioids and/or their effects on 'primed' cells. This indicates that none of the assessed opioids enhanced the oxidative burst capacity of neutrophils or monocytes in this assay. The potent PMA stimulus, which is principally used to evaluate inhibiting properties of the opioids, caused virtually all of the neutrophils and monocytes to be stimulated. The magnitude of this activation was high in the neutrophils, but comparatively low in the monocytes. This activation (either proportion of cells activated or intensity of activation) was unaffected by any of the opioids. This could, at least in part, be due to the potent capacity of PMA to activate the oxidative burst response via PKC induction. The physiological bacterial stimulus, *E.coli*, induced an intermediate activation and there were occasional effects from some of the opioids on the oxidative burst response induced by *E.coli*:

However, as these statistically significant results do not have a clear pattern to them it might be that they occurred by chance rather than the opioid having an effect at that concentration. However, some of the opioids seemed to non-significantly influence the oxidative burst response, but due to interindividual variability and the limited sample size, type II errors were possible. A trend for an effect across the range of doses for some of the opioids (morphine, tramadol, fentanyl, buprenorphine and methadone) was apparent and although the significance of these results should not be overstated, it could be that a larger sample size might have made this trend statistically significant. There may also be a difference between statistical significance and what might be clinically significant. For example 20 ng/ml of methadone significantly decreased the total neutrophil oxidative burst reaction to *E.coli* and 100 ng/ml of oxycodone significantly inhibited the response of monocytes to *E.coli*, but the effect size was very small and is unlikely to be clinically significant. The clinical significance may also be dependent on the individual and this will be explored in the following section.

3.2.6 Effects of opioids on the oxidative burst reaction in individual subjects

There is no consistent statistically significant effect of any of the opioids tested on neutrophil and monocyte oxidative burst response. For some individuals there appears to be an effect of morphine, tramadol, fentanyl, buprenorphine and methadone on the oxidative burst reaction, although any statistical effect is removed when the results are grouped and analysed together due to interindividual variability. There is not only variability in the response to the opioids, but also in the individuals' baseline phagocytic activity. The genetic and molecular basis to interindividual variability to opioids will be discussed in section 4.7. The influence of morphine, tramadol, fentanyl and buprenorphine on the oxidative burst response for neutrophils and monocytes for the individual subjects tested is illustrated below (Figure 3-65 - Figure 3-72). The subject numbers are consistent across experiments.

The oxidative burst response assays have been shown to be reproducible with Coefficients of Variation of 0.1% for the proportion of neutrophils undergoing the oxidative burst reaction to *E.coli* and 4.8% for the MFI of this response, and 1.1% for the proportion of monocytes undergoing the oxidative burst reaction to *E.coli* and 6.5% for the MFI of this response (PHAGOBURST® test kit data sheet, Orpegen Pharma). It is thus likely that it is biological rather than experimental variations leading to this interindividual effect.

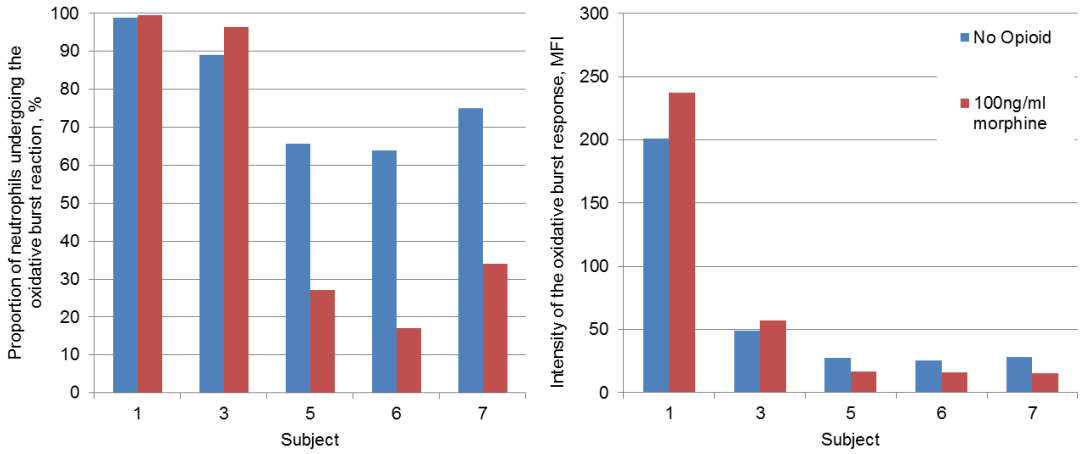

Figure 3-65: Effect of morphine on neutrophil oxidative burst for individuals.
The proportion of neutrophils undergoing the oxidative burst reaction to *E.coli* (**left panel**) and the intensity of this response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 100ng/ml of morphine. Whole blood was incubated with either no opioid or 100ng/ml of morphine for 60 min prior to assessing the oxidative burst reaction to *E.coli*.

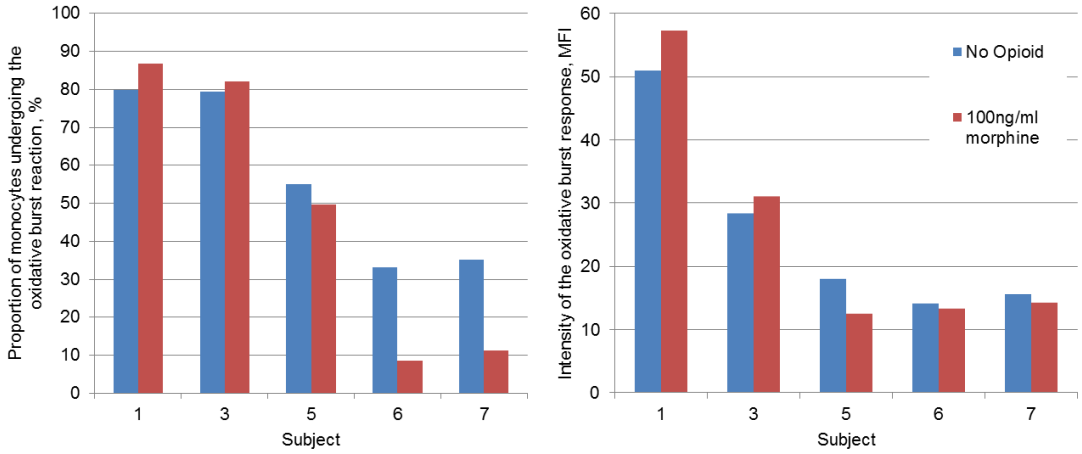

Figure 3-66: Effect of morphine on monocyte oxidative burst for individuals.
The proportion of monocytes undergoing the oxidative burst reaction to *E.coli* (**left panel**) and the intensity of this response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 100ng/ml of morphine. Whole blood was incubated with either no opioid or 100ng/ml of morphine for 60 min prior to assessing the oxidative burst reaction to *E.coli*.

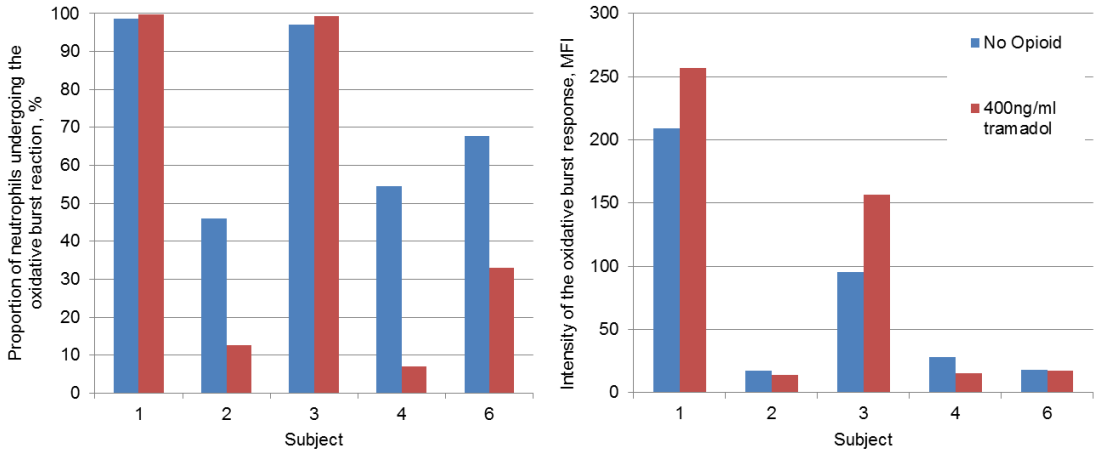

Figure 3-67: Effect of tramadol on neutrophil oxidative burst for individuals.
The proportion of neutrophils undergoing the oxidative burst reaction to *E.coli* (**left panel**) and the intensity of this response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 400ng/ml of tramadol. Whole blood was incubated with either no opioid or 400ng/ml of tramadol for 60 min prior to assessing the oxidative burst reaction to *E.coli*.

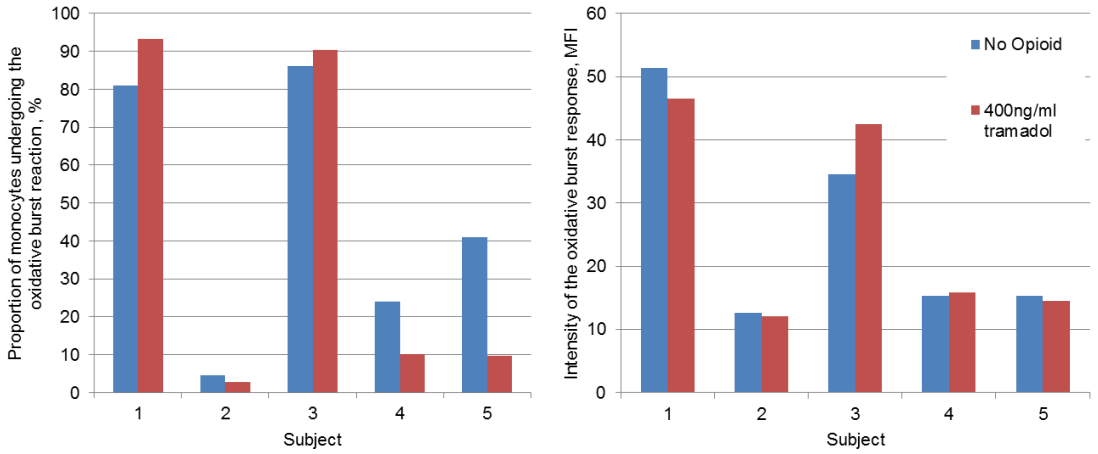

Figure 3-68: Effect of tramadol on monocyte oxidative burst for individuals.
The proportion of monocytes undergoing the oxidative burst reaction to *E.coli* (**left panel**) and the intensity of this response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 400ng/ml of tramadol. Whole blood was incubated with either no opioid or 400ng/ml of tramadol for 60 min prior to assessing the oxidative burst reaction to *E.coli*.

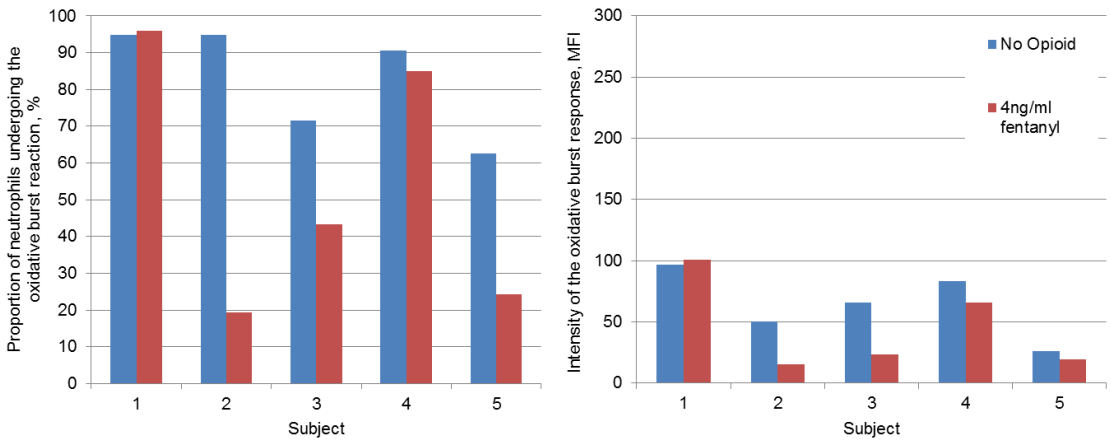

Figure 3-69: Effect of fentanyl on neutrophil oxidative burst for individuals.
The proportion of neutrophils undergoing the oxidative burst reaction to *E.coli* (**left panel**) and the intensity of this response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 4ng/ml of fentanyl. Whole blood was incubated with either no opioid or 4ng/ml of fentanyl for 60 min prior to assessing the oxidative burst reaction to *E.coli*.

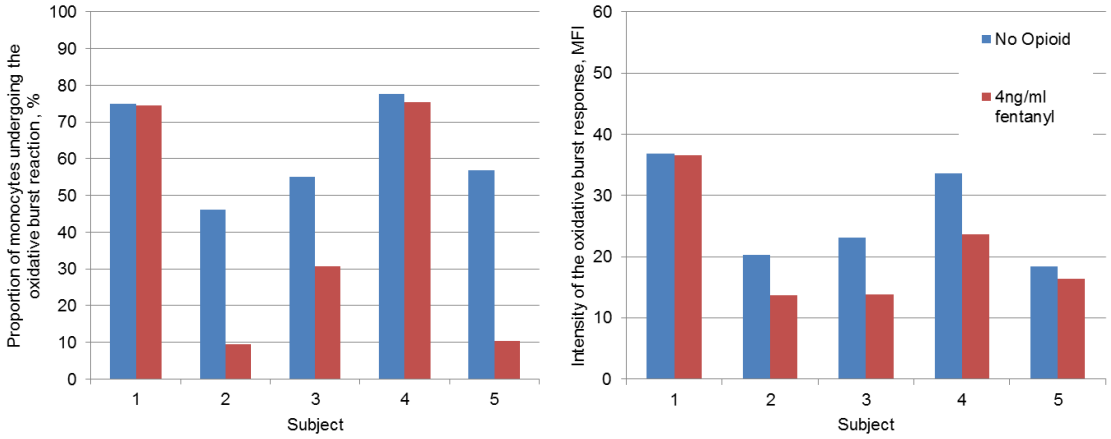

Figure 3-70: Effect of fentanyl on monocyte oxidative burst for individuals.
The proportion of monocytes undergoing the oxidative burst reaction to *E.coli* (**left panel**) and the intensity of this response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 4ng/ml of fentanyl. Whole blood was incubated with either no opioid or 4ng/ml of fentanyl for 60 min prior to assessing the oxidative burst reaction to *E.coli*.

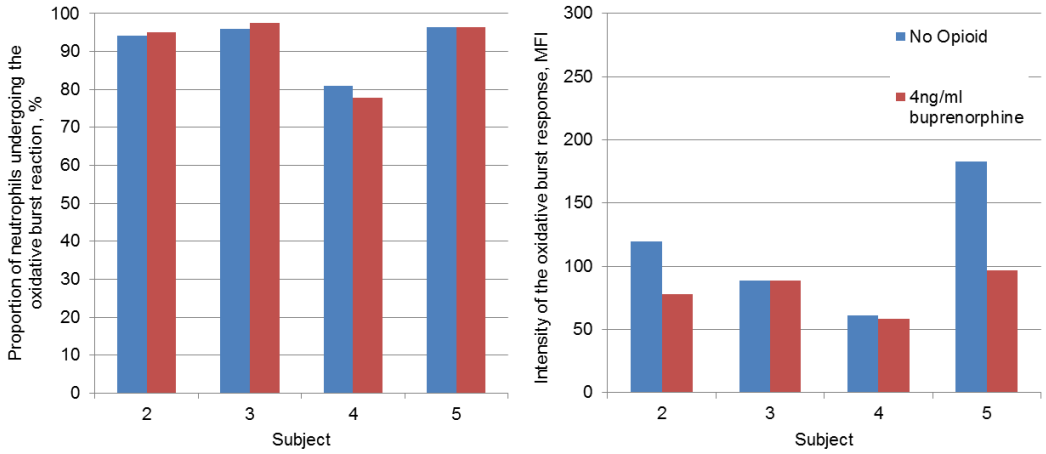

Figure 3-71: Effect of buprenorphine on neutrophil oxidative burst for individuals.
The proportion of neutrophils undergoing the oxidative burst reaction to *E.coli* (**left panel**) and the intensity of this response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 4ng/ml of buprenorphine. Whole blood was incubated with either no opioid or 4ng/ml of buprenorphine for 60 min prior to assessing the oxidative burst reaction to *E.coli*.

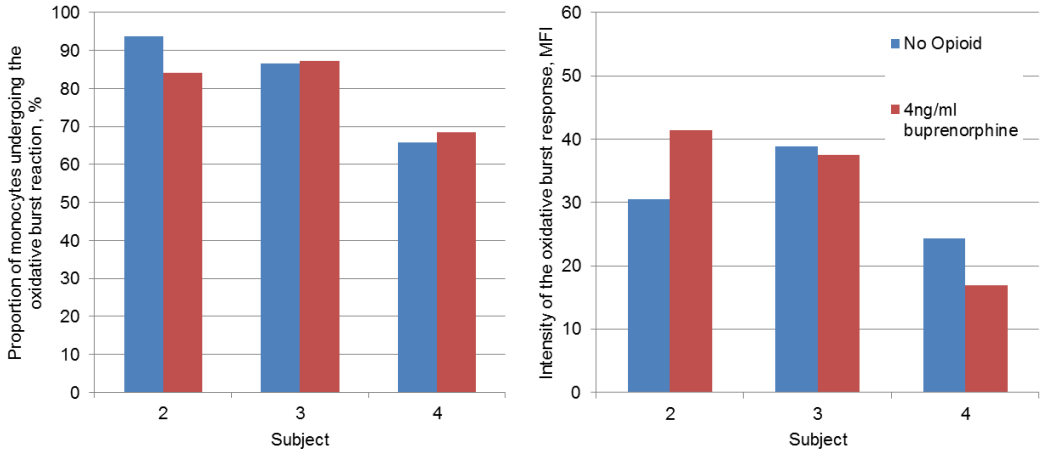

Figure 3-72: Effect of buprenorphine on monocyte oxidative burst for individuals.
The proportion of monocytes undergoing the oxidative burst reaction to *E.coli* (**left panel**) and the intensity of this response (median channel of fluorescent intensity, MFI; **right panel**), presented for each subject individually, with the no opioid control and 4ng/ml of buprenorphine. Whole blood was incubated with either no opioid or 4ng/ml of buprenorphine for 60 min prior to assessing the oxidative burst reaction to *E.coli*.

Oxycodone, diamorphine and codeine had no effect on neutrophil and monocyte oxidative burst responses to *E.coli* in any of the subjects, methadone decreased the intensity of the oxidative burst reaction only in subject 4 (Table 3.3). There are clear differences in the oxidative burst response to opioids between the individuals tested. In general, responses for an individual, to least some of the opioids, are either stimulatory (subject 1 and 3) or inhibitory (all other subjects). For some individuals, the effect is marginal, but for others (i.e. subject 2 and 5 to fentanyl) this is marked and could

potentially translate to a clinically important effect. Even though some opioids appeared to be stimulatory in some individuals, the magnitude of this effect tended to be less than their suppressive effects.

Due to these differences between individuals, any potentially important effects might be diminished when statistical analysis is performed across the whole group. Both the total amount of oxidative burst reaction occurring and the number of neutrophils and monocytes performing this reaction were affected in varying amounts by the different opioids across the individuals. In certain individuals some opioids might be detrimental if they were to have a bacterial or fungal infection, whereas in other individuals some opioids may have no effect or even be beneficial. The effects of the opioids, at all concentrations tested, on the oxidative burst reaction of neutrophils and monocytes to *E.coli* for the individual subjects are summarised below (Table 3.3 - Table 3.4). When assessing the effect of individuals across the range of opioid concentrations for the oxidative burst reaction, only when an opioid increased or inhibited the immune response by at least 20% across the range of the concentrations tested was this response considered to be a trend and represent as so in the tables below.

Table 3.3: Effect of opioids on the oxidative burst reaction of neutrophils for individuals.

Opioid / Subject	Morphine	Tramadol	Fentanyl	Buprenorphine	Methadone	Oxycodone, diamorphine, codeine
1	No effect	Increase	No effect		No effect	No effect
2		Decrease	Decrease	Decrease	No effect	No effect
3	No effect	Increase	Decrease	No effect		No effect
4		Decrease	No effect	No effect	Decrease	No effect
5	Decrease		Decrease	Decrease		
6	Decrease	Decrease				
7	Decrease					

The effect of the opioids on total neutrophil oxidative burst reaction (MFI) to *E.coli* for individual subjects. Red indicates an inhibitory effect, green a stimulatory effect and yellow no effect (boxes are uncoloured if not tested).

Table 3.4: Effect of opioids on the oxidative burst reaction of monocytes for individuals.

Opioid / Subject	Morphine	Tramadol	Fentanyl	Buprenorphine	Methadone	Oxycodone, diamorphine, codeine
1	No effect	No effect	No effect		No effect	No effect
2		Decrease	Decrease	No effect	No effect	No effect
3	No effect	No effect	No effect	No effect		No effect
4		No effect	Decrease	Decrease	Decrease	No effect
5	No effect		No effect			
6	No effect	No effect				
7	No effect					

The effect of the opioids on total monocyte oxidative burst reaction (MFI) to *E.coli* for individual subjects. Red indicates an inhibitory effect, green a stimulatory effect and yellow no effect (boxes are uncoloured if not tested).

For the opioids that did influence the oxidative burst reaction (morphine, tramadol, fentanyl, buprenorphine and methadone) this was more dependent on the subject than on the opioid. Although these opioids suppressed neutrophil oxidative burst reaction in most individuals, the majority of the monocytes had no sizable consistent effect from any of the opioids. The majority of the effects were inhibitory and only tramadol had stimulatory effects in two subjects and these were marginal. Sometimes there was an effect on the proportion of neutrophils and monocytes undergoing the oxidative burst reaction, without much effect on the total oxidative burst reaction (i.e. total amount of oxygen reduction in neutrophils and monocytes, denoted as MFI). There was not always correlation between the two and although both are important, the latter is more likely to be relevant to bacterial killing and this is what was tabulated (Table 3.3 - Table 3.4). It may be that morphine, tramadol, fentanyl, buprenorphine and methadone by decreasing bacterial killing in certain individuals may be detrimental if they had a bacterial or fungal infection. Although oxycodone, diamorphine and codeine did not affect oxidative burst when evaluated on a group or individual basis, diamorphine and codeine are pro-drugs and as they are converted to morphine *in vivo* they will have the immune effects of morphine as well as any intrinsic effect they may have themselves. Although 100 ng/ml of oxycodone statistically significantly inhibited the oxidative burst response of monocytes to *E.coli*, the effect size was very small and would unlikely be clinically significant. Oxycodone might have less of an effect on oxidative burst in some individuals and might be safer, but this hypothesis would need confirmation in clinical studies and with larger numbers of subjects.

3.2.7 Discussion

The current study assessed the effects of eight commonly used opioids at three concentrations which approximated to those found clinically, on the *in vitro* ability of human neutrophils and monocytes to undergo the oxidative burst reaction in response to fMLP, PMA and *E.coli* as measured by flow cytometry. There were no consistent statistically significant effect from any of the opioids, but variability between individuals was detected.

Retrospective power and sample size calculations were performed using DSS research programmes, as some of the opioids seemed to have an effect which did not reach statistical significance and others were statistically significant, but with a very small effect size. The power calculations used a two-tail, two sample test, using average values and a 95% confidence interval. The sample size calculations used a two samples using average values test with a 95% confidence interval. The sample size

calculations for those concentrations of opioids which seemed to have a non-statistically significant effect (i.e. for morphine), showed that although the power was low (less than 80%) the sample size needed to potentially detect an effect was as low as six, which is comparable with the number of samples undertaken for some of these assays. For other samples, even those that were statistically significant (i.e. oxycodone) the power was very low (5%). For many of the tests, with either very small effects from the opioids, or large standard deviations due to interindividual variability, the power was low and sample sizes required consequently high.

The previous *in vitro* studies which assessed the effect of opioids on the oxidative burst reaction are reviewed below and their findings brought into context with the current study.

The effect of morphine on neutrophil killing has been evaluated in 16 male rhesus monkeys (Liu et al., 1992). Neutrophils were isolated from the blood of opioid naïve rhesus monkeys and incubated with 50 fM and 50 pM of morphine (equivalent to 1.4 $x10^{-5}$ and 0.014 ng/ml) for 4 hr, before being incubated with *Candida albicans* blastospores for 45 min. Blastospore viability was assessed using fluorescent microscopy. The killing of the blastospores was inhibited by the pM (≈ 0.014 ng/ml), but not fM (≈ 1.4 $x10^{-5}$ ng/ml) concentrations of morphine and the inhibition was reversed by naloxone (Liu et al., 1992).

Compared with the current study, this experiment by Liu and colleagues assessed the effect of much lower concentrations of morphine on isolated neutrophils from a different species on killing of fungal blastospores (as opposed to oxygen free radial production). There is an advantage in the study by Liu as the function of these cells against a potentially pathogenic fungus was assessed. The concentrations used were far below those that are detected clinically and the use of isolated neutrophils could prime or influence their activation status and function. Furthermore, neutrophil isolation removes the complex immune interactions which occur in whole blood.

Peterson and colleagues evaluated the effect of morphine on the oxidative burst reaction of PBMCs and monocytes from three healthy volunteers (Peterson et al., 1987). Human PBMCs and purified monocytes were incubated with 10^{-10} – 10^{-6} M of morphine (equivalent to 0.03 – 285 ng/ml) for 48 hr and were then stimulated with a 60-min incubation with opsonised zymosan or PMA. Variable incubation times (1, 2, 3 and 24 hr) with 10^{-8} M of morphine (≈ 2.9 ng/ml) were then assessed on zymosan-

stimulated O_2^- release. O_2^- generation was evaluated by a superoxide dismutase inhibitable reduction of ferricytochrome *c* using a photometer. H_2O_2 production was measured using the horseradish peroxidase-mediated oxidation of phenol red using a photometer. 10^{-6} M of morphine increased resting O_2^- and H_2O_2 production. All concentrations of morphine suppressed the response to zymosan and PMA stimulation by over 50%. This was blocked by naloxone. The longer the incubation with 10^{-8} M of morphine the more the suppression of zymosan-stimulated O_2^- production, with less than 20% suppression within 3 hr, increasing to 83% at 48 hr (Peterson et al., 1987).

This study by Peterson and colleagues used similar effector cells to the current study (although the cells were purifying as opposed to using whole blood), but used a longer incubation period (48 hr vs. 1 hr) with overlapping concentration of morphine (although they also used much lower concentrations). When Peterson and colleagues used 1 or 2 hr incubations with morphine, they too showed minimal inhibition of the oxidative burst reaction (Peterson et al., 1987). They also demonstrated an increased response of unstimulated cells to 10^{-6} M of morphine, but only after a 1-hr incubation. Differences between Peterson's study and the current study include the method of stimulation and the measurement technique. Although the current study did not show any significant results, there was a trend for morphine to inhibit the proportion (but not the total response) of monocytes undergoing the oxidative burst reaction to the PMA stimulus.

From the same laboratory, Molitor and colleagues investigated the *in vitro* effects of methadone on swine PBMCs (Molitor et al., 1992). Cells were incubated for 48 hr with 10^{-18} – 10^{-6} M of methadone (equivalent to $3x10^{-10}$ – 285 ng/ml). O_2^- produced by PBMCs was determined using the superoxide dismutase-inhibitable reduction of ferricytochrome *c*. PBMCs were stimulated with a 60-min incubation with PMA. A spectrophotometer was used to determine O_2^- release. Oxidative burst was dose-dependently suppressed by methadone (significantly at concentrations above 10^{-12} M), becoming almost completely blocked at 10^{-6} M, with similar results from all 10 pigs. This effect was blocked by naloxone (Molitor et al., 1992).

The current study only showed a decrease in the *E.coli* stimulated neutrophil and monocyte oxidative burst reaction by 20 ng/ml of methadone (and no effect of methadone on the PMA stimulated cells). In contrast, Molitor reported that methadone inhibited monocytes at concentrations which overlapped with those used in the current study, and also much lower concentrations. Molitor used a longer incubation time (48

hr vs. 1 hr) and only used PMA stimulation. The current study also used the physiological *E.coli* stimulus. The measurement technique also differed. Probably the most important difference between the studies was the species investigated (swine vs. human) which might have caused the discrepancy in the results.

Jaeger and colleagues evaluated the effect of fentanyl on oxidative burst responses in 10 healthy volunteers (Jaeger et al., 1998). Leukocytes were isolated from the venous blood of 10 healthy volunteers by density gradient sedimentation. The cells were incubated with 5 and 50 ng/ml of fentanyl for 15 min, after which their oxidative burst capacity was assessed following stimulation with PMA, *E.coli* or priming by TNF-α followed by stimulation with fMLP for 20 min. Superoxide anion formed during the oxidative burst was measured by intracellular oxidation of dihydrorhodamine to rhodamine and the proportion of activated neutrophils was measured by flow cytometry. Fentanyl had no significant effect on human neutrophil oxidative burst responses at clinically relevant and 10 fold higher concentrations, irrespective of the activation stimulus. The variability for *E. coli* stimulation, with and without fentanyl, was about 40% between individuals (Jaeger et al., 1998).

This study by Jaeger and colleagues is similar to the current study, with the exception of isolating leukocytes as opposed to using whole blood (the latter is more physiological). Nevertheless, the results are similar, with fentanyl at doses comparable to the current study having no effect on the neutrophil oxidative burst reaction to any of the stimuli, although there was a trend for inhibition in the current study to the *E.coli* stimulus. A similar level of variability was detected in both this and the current study. A limitation, as in the current study, is that healthy volunteers were used as the neutrophils in patients with cancer or infection are likely to be modulated by these as well as by host immune processes.

3.2.8 Conclusions

The effect of three concentrations of eight opioids on the ability of neutrophils and monocytes to perform the oxidative burst reaction was assessed in the whole blood of healthy volunteers. None of the opioids had a consistent statistically significant effect on the oxidative burst response; however, by evaluating each subject individually it seems that some subjects might be more susceptible to the *in vitro* effects of morphine, tramadol, fentanyl, buprenorphine and methadone rather than oxycodone, diamorphine and codeine (see section 3.2.6). As diamorphine and codeine are pro-drugs of morphine, and thus potentially have similar effects *in vivo*, oxycodone might be the

opioid with the least impact on the oxidative burst reaction in some individuals. This could be relevant in terms of bacterial and fungal infection but would need confirmation in clinical studies.

3.3 Effect of opioids on T cell activation

T cells are central to the adaptive immune response. $CD4^+$ T cells coordinate the immune response, whereas $CD8^+$ T cells are involved in selected killing of tumour cells, virally infected cells and transplanted tissue to which the adaptive immune response has been primed. One of the means by which the immune response is controlled and target cell killing is enhanced or down regulated is via the production of specific cytokines (results in section 3.4). The effect of opioids on CD25 and CD69 expression on T cells is discussed in this section.

3.3.1 Effect of opioids on the activation of anti-CD3/anti-CD28 mAb stimulated T cells

For these experiments, PBMCs were incubated with Miltenyi anti-CD3/anti-CD28 mAb-coated beads (at a 1:1 bead:PBMC ratio) for 3 days, in order to induce a cellular activation cascade which mimics the responses that are induced when TCRs are triggered and essential co-stimulatory molecules are delivered following T cell interactions with APCs. The influence of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine, added at the same time as the beads, on $CD4^+$ and $CD8^+$ T cell activation, on the basis of CD69 and CD25 expression, was determined by flow cytometry (Figure 3-73 - Figure 3-88).

Using FACSDiva™ data analysis software, T cells were selected on the basis of their size, granularity and expression of CD4 or CD8 (see Figure 2-4). CD25 and CD69 expression was subsequently analysed and the proportion of $CD4^+$ and $CD8^+$ cells expressing CD25 and CD69 was determined (Figure 2-22). From this positive population the total amount (MFI) of CD25 and CD69 mAb bound to these $CD4^+$ and $CD8^+$ cells was measured. The total CD25 and CD69 expression by all $CD4^+$ and $CD8^+$ cells was also measured (data not shown). As there is a population of activated cells and the proportion of $CD25^+$ and $CD69^+$ cells and the intensity of expression by these cells give more useful information than the composite measure of intensity in all cells.

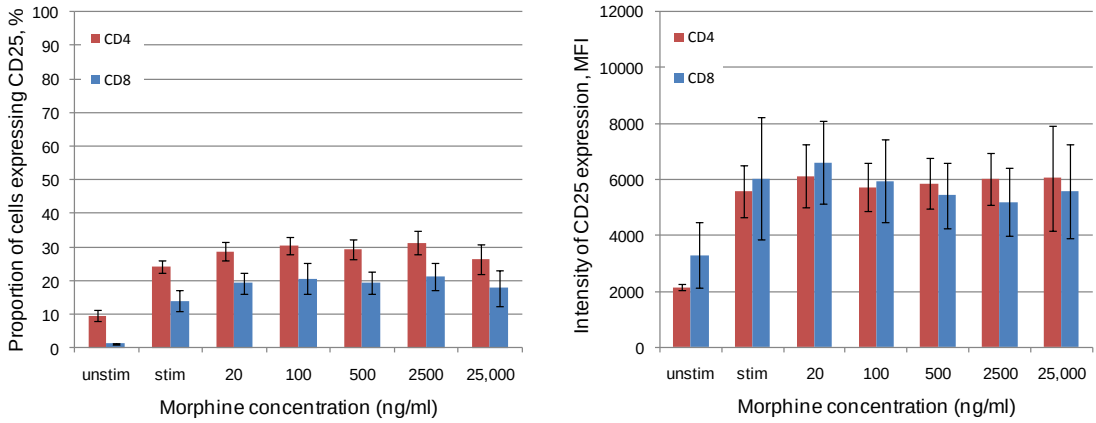

Figure 3-73: Effect of morphine on CD25 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD25 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of morphine for 3 days prior to assessing CD25 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

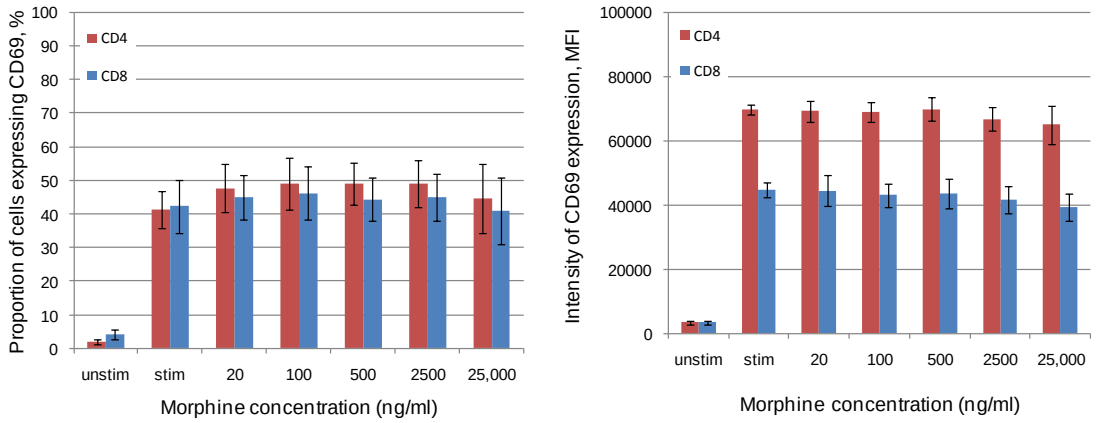

Figure 3-74: Effect of morphine on CD69 expression in $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD69 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of morphine for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

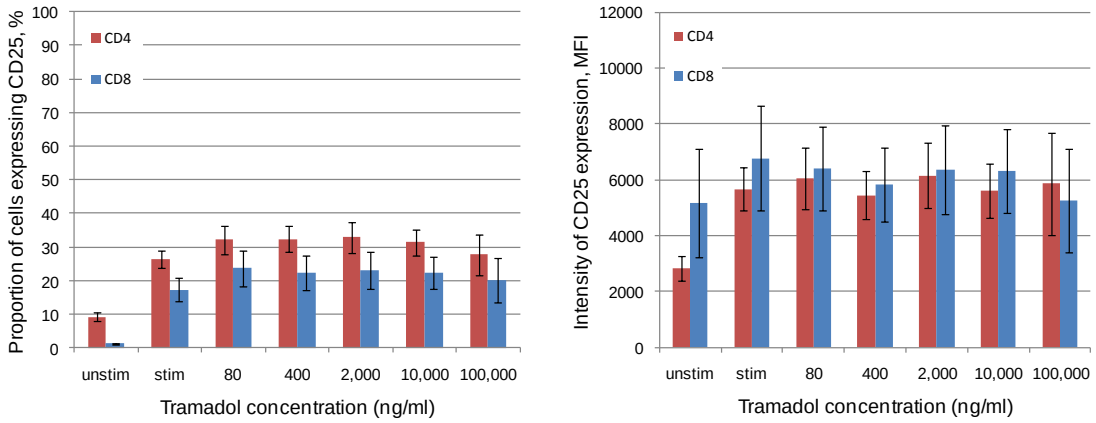

Figure 3-75: Effect of tramadol on CD25 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD25 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of tramadol for 3 days prior to assessing CD25 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

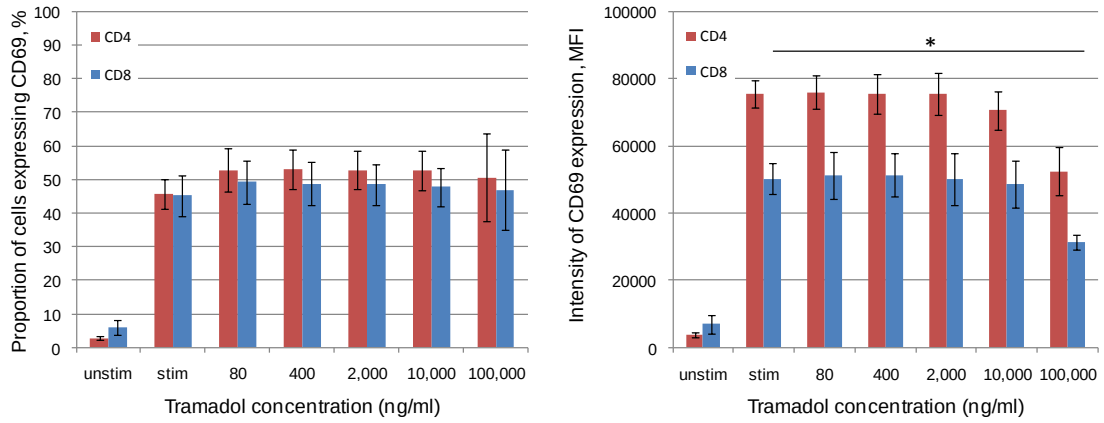

Figure 3-76: Effect of tramadol on CD69 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD69 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of tramadol for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. 100,000 ng/ml of tramadol significantly inhibited the intensity of CD69 expression in anti-CD3/anti-CD28 mAb stimulated $CD8^+$ cells (* = $P<0.05$; paired Student *t*-test).

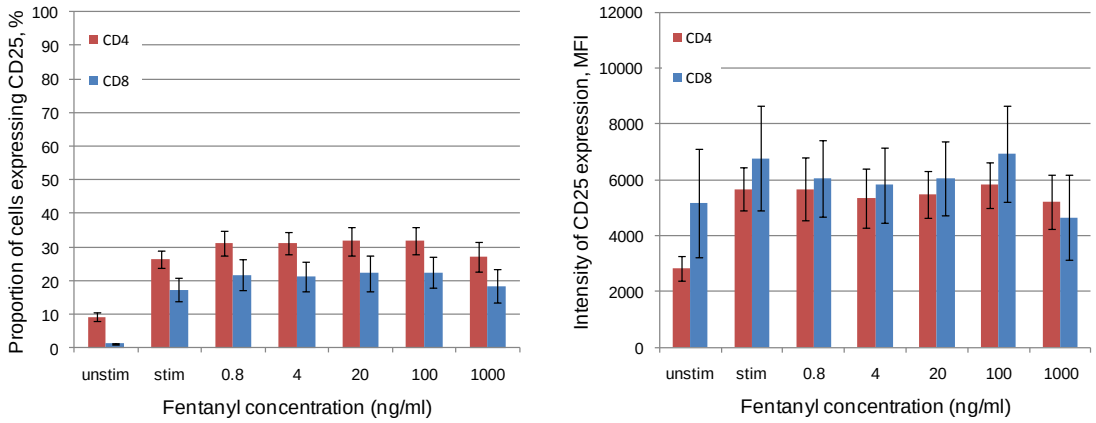

Figure 3-77: Effect of fentanyl on CD25 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD25 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of fentanyl for 3 days prior to assessing CD25 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

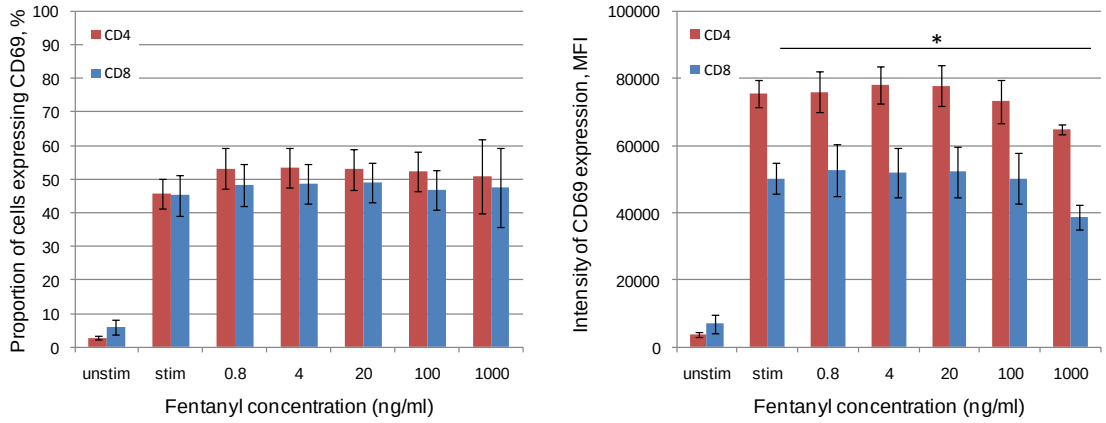

Figure 3-78: Effect of fentanyl on CD69 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD69 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of fentanyl for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. 1000 ng/ml of fentanyl significantly inhibited the intensity of CD69 expression in anti-CD3/anti-CD28 mAb stimulated $CD8^+$ cells (* = $P<0.05$; paired Student *t*-test).

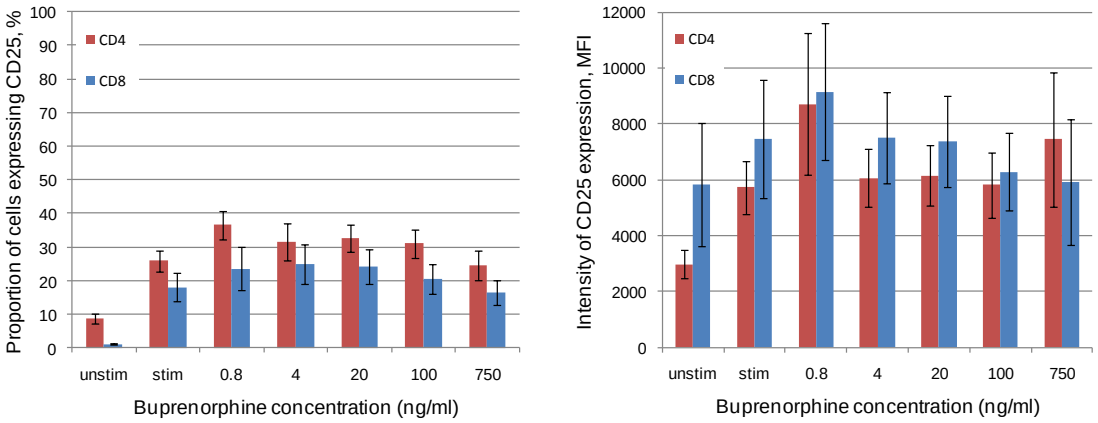

Figure 3-79: Effect of buprenorphine on CD25 expression by CD4$^+$ and CD8$^+$ T cells.
The proportion (%) of CD4$^+$ and CD8$^+$ cells expressing CD25 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of buprenorphine for 3 days prior to assessing CD25 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

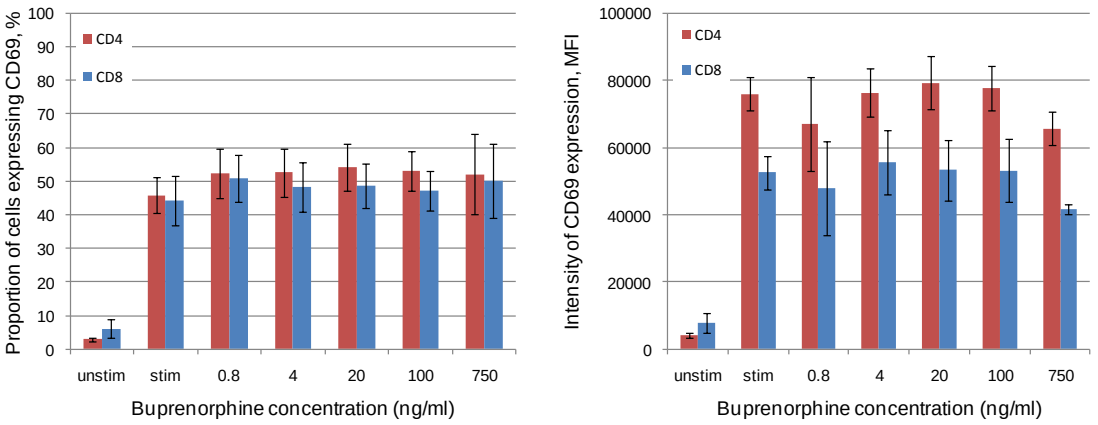

Figure 3-80: Effect of buprenorphine on CD69 expression by CD4$^+$ and CD8$^+$ T cells.
The proportion (%) of CD4$^+$ and CD8$^+$ cells expressing CD69 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of buprenorphine for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

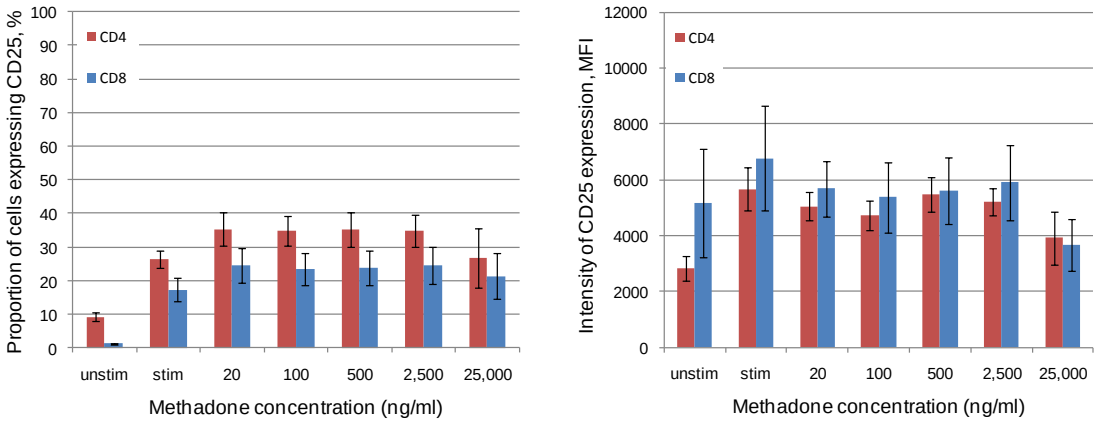

Figure 3-81: Effect of methadone on CD25 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD25 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of methadone for 3 days prior to assessing CD25 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

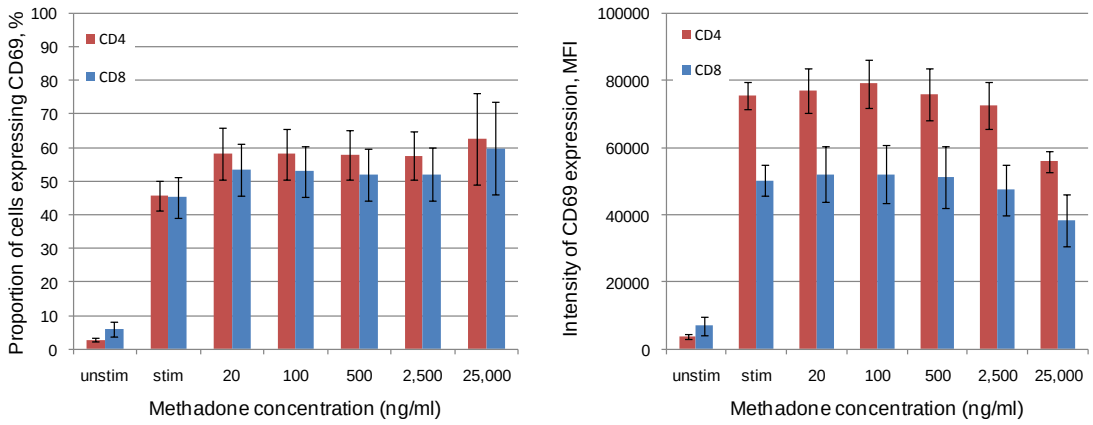

Figure 3-82: Effect of methadone on CD69 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD69 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of methadone for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

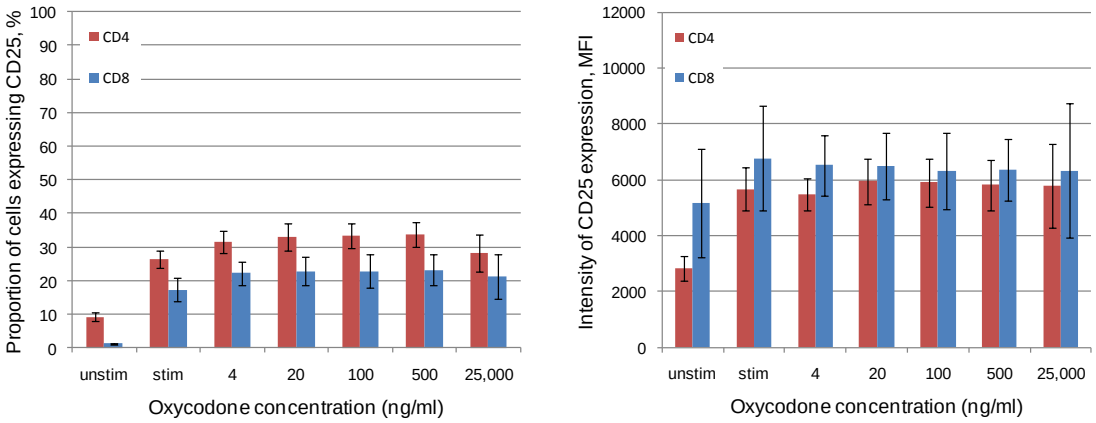

Figure 3-83: Effect of oxycodone on CD25 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD25 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of oxycodone for 3 days prior to assessing CD25 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

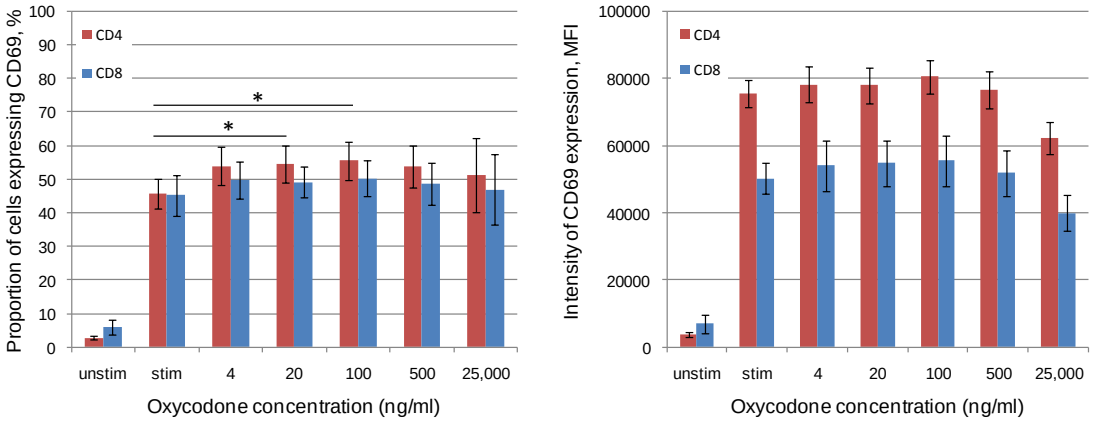

Figure 3-84: Effect of oxycodone on CD69 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD69 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of oxycodone for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. 20 and 100 ng/ml of oxycodone significantly increased the % of anti-CD3/anti-CD28 mAb stimulated $CD4^+$ cells expressing CD69 (* = $P<0.05$; paired Student *t*-test).

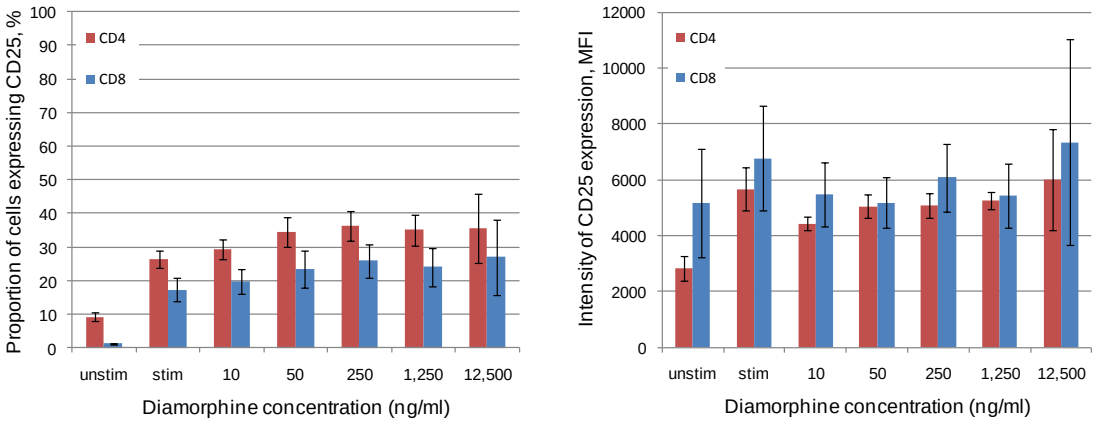

Figure 3-85: Effect of diamorphine on CD25 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD25 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of diamorphine for 3 days prior to assessing CD25 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

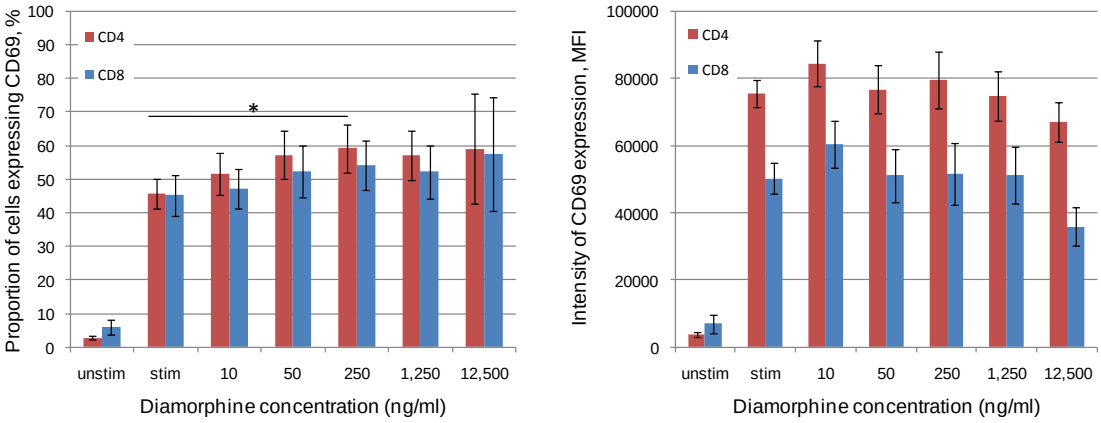

Figure 3-86: Effect of diamorphine on CD69 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD69 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of diamorphine for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. 250 ng/ml of diamorphine significantly increased the proportion of anti-CD3/anti-CD28 mAb stimulated $CD4^+$ cells expressing CD69 (* = $P<0.05$; paired Student *t*-test).

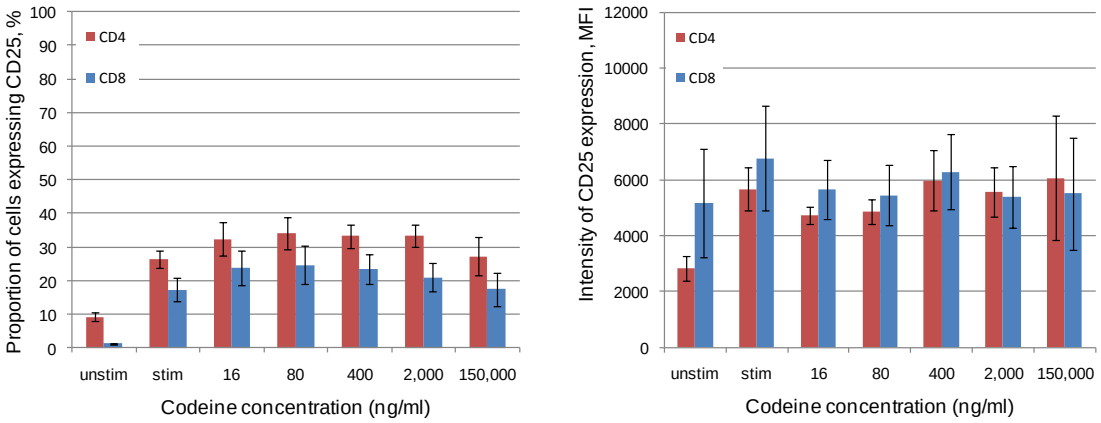

Figure 3-87: Effect of codeine on CD25 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD25 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of codeine for 3 days prior to assessing CD25 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student *t*-test).

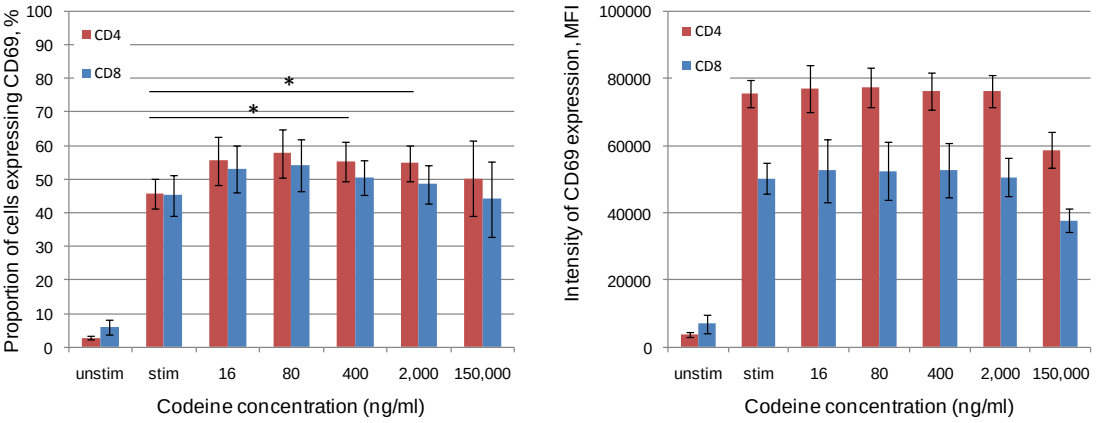

Figure 3-88: Effect of codeine on CD69 expression by $CD4^+$ and $CD8^+$ T cells.
The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD69 (**left panel**) and the intensity of this expression in these positive cells (median channel of fluorescent intensity, MFI; **right panel**). PBMCs were incubated with the indicated concentrations of codeine for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. Data are means ± SEM from 5 independent experiments. 400 and 2000 ng/ml of codeine significantly increased the proportion of anti-CD3/anti-CD28 mAb stimulated $CD4^+$ cells expressing CD69 (* = $P<0.05$; paired Student *t*-test).

The above data illustrate that opioids have no consistent effect on T cell activation (CD25 and CD69 expression) which is induced following a 3-day incubation of PBMCs with anti-CD3/anti-CD28 mAb coated beads. Although occasional results were of statistical significance, the lack of consistency across a range of doses for an opioid or in both the proportion and intensity of response suggests that in view of the number of tests carried out this is likely due to chance. As there were small numbers of subjects, this might have caused a type II error, but as there was no consistent effect of any opioids, increasing the number of subjects would not have led to meaningful, statistically significant results.

3.3.2 Effect of opioids on the activation of anti-CD3/anti-CD28 mAb pre-stimulated T cells

Opioids had no effect on T cell activation when added at the same time as the Miltenyi anti-CD3/anti-CD28 mAb coated beads. It may be that opioids were just having no effect, or that any effect of the opioids could not overcome the effect of the Miltenyi anti-CD3/anti-CD28 mAb coated beads when they were added together. The lack of observed effect might also be due to MOR regulation and that the T cells needed to be activated for receptor expression and for the opioids to have a direct effect (Borner et al., 2008). Thus, additional experiments determined the effect of opioids on the activation status of pre-stimulated T cell populations.

For these, PBMCs were stimulated for 1 day with Miltenyi anti-CD3/anti-CD28 mAb coated beads (at a 1:1 bead:PBMC ratio) before the addition of opioids for a further 2 days. Cells were harvested and stained at this point (after 3 days in total) and the expression of CD25 and CD69 by $CD4^+$ and $CD8^+$ T cells was determined by flow cytometry and analysed using FACSDiva™ data analysis software.

Morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine were assessed at five concentrations, the same as in the previous experiment. None of the opioids had an effect on the proportion or intensity of CD25 and CD69 expression by $CD4^+$ and $CD8^+$ T cells pre-stimulated with Miltenyi anti-CD3/anti-CD28 mAb coated beads (data not shown).

3.3.3 Effect of pre-incubation of T cells with opioids before anti-CD3/anti-CD28 mAb-coated bead stimulation

Opioids had no effect on T cell activation when added at the same time or 1 day after the anti-CD3/anti-CD28 mAb coated beads. As it might be that the stimulation was too potent to be overcome by the opioids, additional experiments evaluated the activation status of T cells, when PBMCs were pre-incubated with opioids prior to polyclonal stimulation with Miltenyi anti-CD3/anti-CD28 mAb coated beads.

For these experiments the opioids were added to the PBMCs and incubated for 1 day before the Miltenyi anti-CD3/anti-CD28 mAb coated beads (at a 1:1 bead:PBMC ratio) were added to this culture. Cells were incubated for a further 3 days, after which CD25 and CD69 expression by $CD4^+$ and $CD8^+$ T cells was determined by flow cytometry. All eight opioids were assessed at five concentrations, the same as in the previous experiments. None of the opioids had an effect on the proportion or intensity of CD25 and CD69 expression by $CD4^+$ and $CD8^+$ T cells (data not shown).

3.3.4 Effect of opioids on the activation of anti-CD3/anti-CD28 mAb stimulated T cells, without platelet removal

When opioids were added at the same time, 1 day after or 1 day before the anti-CD3/anti-CD28 mAb coated beads they had no effect on T cell activation. It is likely that either the opioids were not having a direct effect on T cells. However, as other studies have used PBMC preparations which contained platelets, and Platelet-derived Growth Factor has been shown to modulate the T cell response (Daynes et al., 1991), one further experimental paradigm was used to ensure that the platelet removal step was not affecting the potential effect of opioids on T cell activation.

For these experiments, the opioids and Miltenyi anti-CD3/anti-CD28 mAb coated beads, were added at the same time to the PBMCs (which did not undergo the second density gradient centrifugation, which is to remove the platelets) and incubated for 3 days. Cells were then harvested and stained for the expression of CD25 and CD69 by $CD4^+$ and $CD8^+$ T cells which was determined by flow cytometry. None of the opioids tested had any effect on the proportion or intensity of the expression of CD25 and CD69 by $CD4^+$ and $CD8^+$ T cells (data not shown).

3.3.5 Effects of opioids on the activation T cells in individual subjects

The data in the previous sections are the pooled data for the effect of opioids on T cell activation and show no clear trends for statistical significance from any of the opioids. There is however interindividual variability reducing any potential for a statistically significant effect. For an individual, the effects of certain opioids on the activation of T cells might be clinically significant. When PBMCs were stimulated for 3 days with Miltenyi anti-CD3/anti-CD28 mAb coated beads at the same time as being incubated with opioids, they mostly had no consistent effect on T cells in most of the subjects tested. When the opioids had an effect, this was more dependent on the individual than the opioid used. Subject 1 was generally unaffected by any of the opioids tested. In subjects 2, 4 and 5 most of the opioids tended to increase the proportion of $CD4^+$ and $CD8^+$ T cells expressing CD25 and CD69, with only occasional increases in the MFI. In subject 3 almost all of the opioids increased the proportion of $CD4^+$ and $CD8^+$ T cells expressing CD25 and CD69, with a reduction in the total expression (MFI) of these cell surface markers.

3.3.6 Discussion

None of the opioids, at clinically relevant concentrations, had a consistent effect on the activation of T cells by Miltenyi anti-CD3/anti-CD28 mAb coated beads. Several experimental paradigms were used with the opioids added at the same time as the beads, 1 day before the beads and 1 day after the beads. In this series of experiments, some of the opioids at certain concentrations reached the $p<0.05$ level of significance on some of the parameters tested, but in view of multiple statistical testing and that there was no statistical consistency across the concentrations of any opioid tested on the different measures of activation, it is probable that these occurred by chance and would not be clinically relevant. Despite the small sample size, there was no clear trend from any of the opioids and thus increasing numbers would be unlikely to lead to a statistical, or potential clinical, significant effect. It is likely that if opioids are to influence T cell activation and proliferation *in vivo* that they will do this via indirect mechanisms (e.g. via the SNS and HPA axis), rather than a direct effect on T cells.

Despite many previous studies on the effect of opioids on the activation of T cells *in vivo,* both in animal models and clinically (reviewed in section 1.12.4), and the consequences on cytokine production (reviewed in sections 1.12.5 and 3.4), no previous *in vitro* studies have assessed the effect of opioids on the activation of T cells using CD25 and CD69 expression as an index of activation. Other research groups have assessed the effect of morphine, methadone and diamorphine on the cytotoxicity

of T cells, these are described below. No study has evaluated the direct effects of opioids on the activation and function of $CD4^+$ T cell populations.

Murine splenic lymphocytes from female B6C3F1 mice were stimulated *in vitro* for 5 days with mitomycin C-inactivated P815 cells and 0.0001 – 100 μM of morphine (equivalent to 0.03 – 28,530 ng/ml) (Thomas et al., 1995a). These cells were then incubated with radiolabelled P815 target cells for 4 hr. Cytotoxic T cell activity was assessed by the radioactivity released into the supernatant as measured by a γ counter. Cytotoxicity was not affected by any of the concentrations of morphine (Thomas et al., 1995a).

The same group also evaluated the potential effects of methadone and diamorphine using a similar methodology, with a 5-day incubation of murine splenocytes with 0.0001 – 100 μM of methadone (equivalent to 0.03 – 30,940 ng/ml) or diamorphine (equivalent to 0.03 – 36,940 ng/ml) (Thomas et al., 1995b). Only 100 μM of diamorphine (≈ 36,940 ng/ml), which far exceeds that found clinically, inhibited cytotoxic T cell activity, by about 50%. Methadone had no effect. The responses to both methadone and diamorphine were reported as variable between mice (Thomas et al., 1995b).

A further study from the same group evaluated the *in vitro* effect of 0.0001 – 100 μM of fentanyl (equivalent to 0.03 – 33,650 ng/ml) on cytotoxic T cell killing using splenocytes from female B6C3Fl mice (House et al., 1995). For these experiments, splenocytes were cultured with fentanyl and mitomycin C-inactivated P815 cells for 5 days. The resultant effector cells were harvested and incubated with ^{51}Cr labelled P815 target cells for 4 hr. Only the highest concentration of fentanyl (100 μM; equivalent to 33,650 ng/ml), which far exceeds that found clinically, inhibited cytotoxic T cell killing, which was by 60% (House et al., 1995).

Compared to the current study, these three similar studies by the same group used a different species and cell type (murine splenic vs. human peripheral blood lymphocytes) incubated with a large range of morphine, fentanyl, methadone and diamorphine concentrations, (overlapping with those used in the current study) for a longer duration (5 vs. 3 days). The stimuli used differed (anti-CD3 vs. anti-CD3/anti-CD28 mAb), as did the cytotoxicity detection method, with these using a functional measurement. The results of these studies were consistent with the current study, as none of the opioids had an effect on $CD8^+$ cytotoxic T cells at clinically relevant

concentrations (although supra-pharmacological concentrations of diamorphine and fentanyl were inhibitory in the studies by Thomas and House).

The effect of morphine on T cell proliferation has been evaluated using cells from CB6F1/J, Balb/c female and 3C57BL/6 male mice (Wang et al., 2001). Lymph node T cells were incubated with between 1 nM – 10 μM of morphine (equivalent to 0.3 – 3,365 ng/ml) and stimulated with Con A for 48 hr. Proliferation was measured with a ^{3}H-thymidine uptake assay. 10 nM – 10 μM of morphine inhibited T cell proliferation in a dose-dependent manner, by up to 60% (Wang et al., 2001). Compared with the current study, this study by Wang and colleagues has a very different methodology with different species, cells, stimulation and a functional outcome measure. Although, the concentrations of morphine overlapped, this study had very different results compared with the current study, showing that non-physiologically stimulated murine cytotoxic T cells were inhibited by morphine, whereas it had no effect on activation markers in the current study. This is likely to be due to the differing species, cells and stimulation method used, as activation marker expression has been demonstrated to correlate to T cell proliferation (Maino et al., 1995, Mardiney et al., 1996, Simms and Ellis, 1996).

Other groups have assessed the effect of opioids on T cells using non physiological activation pathways (i.e. Con A) and have shown no effect (personal communication: Dr Nick Gough, University College London, UK).

3.3.7 Conclusion

In this series of experiments the effect of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on T cell activation (CD25 and CD69 expression) was assessed. T cells were stimulated with Miltenyi anti-CD3/anti-CD28 mAb coated beads, added before, at the same time or after the opioids. There was good activation of both $CD4^{+}$ and $CD8^{+}$ T cells by the beads but none of the opioids had a consistent effect on the activation of T cells. Despite using different methodologies, the results from previous *in vitro* studies mostly correlate to those of the current study. The effect of morphine in previous *in vivo* experiments was therefore unlikely to be a result of a direct effect of the opioids on the T cells, but an indirect effect, possibly via the SNS or HPA axis.

3.4 Effect of opioids on cytokine production

Cytokines are intercellular signalling molecules that play a pivotal role in the regulation of immune function. The effect of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on the production of a range of cytokines in unstimulated, IL-2 stimulated and Miltenyi anti-CD3/anti-CD28 mAb coated bead stimulated PBMCs were assessed. For these experiments, PBMCs were incubated with four concentrations of each opioids and 100U/ml IL-2 or Miltenyi anti-CD3/anti-CD28 mAb beads (at a 1:1 bead:PBMC ratio) for 3 days before the supernatants were removed and stored at -80°C until cytokine analysis using BD Bioscience CBAs and a BD FACSArray™ flow cytometer.

The cytokines analysed in the supernatants of activated PBMC cultures were IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN-α, IFN-γ, TNF-α and IP-10. Cytokine levels in the unstimulated samples were at, or below the detection limit of the respective assays and opioids had no effect on these (data not shown). IL-4, IL-12p70, IP10 and IFN-α were below the detection limit of the IL-2 and Miltenyi anti-CD3/anti-CD28 mAb stimulated assays and no data were obtained for IL-2. The results presented below are for IL-1β, IL-6, IL-8, IL-10, IL-17A, IFN-γ and TNF-α (Figure 3-89 - Figure 3-96). The major comparison to be considered in the following data sets is the change from the “no opioid” stimulated baseline, rather than the absolute concentrations.

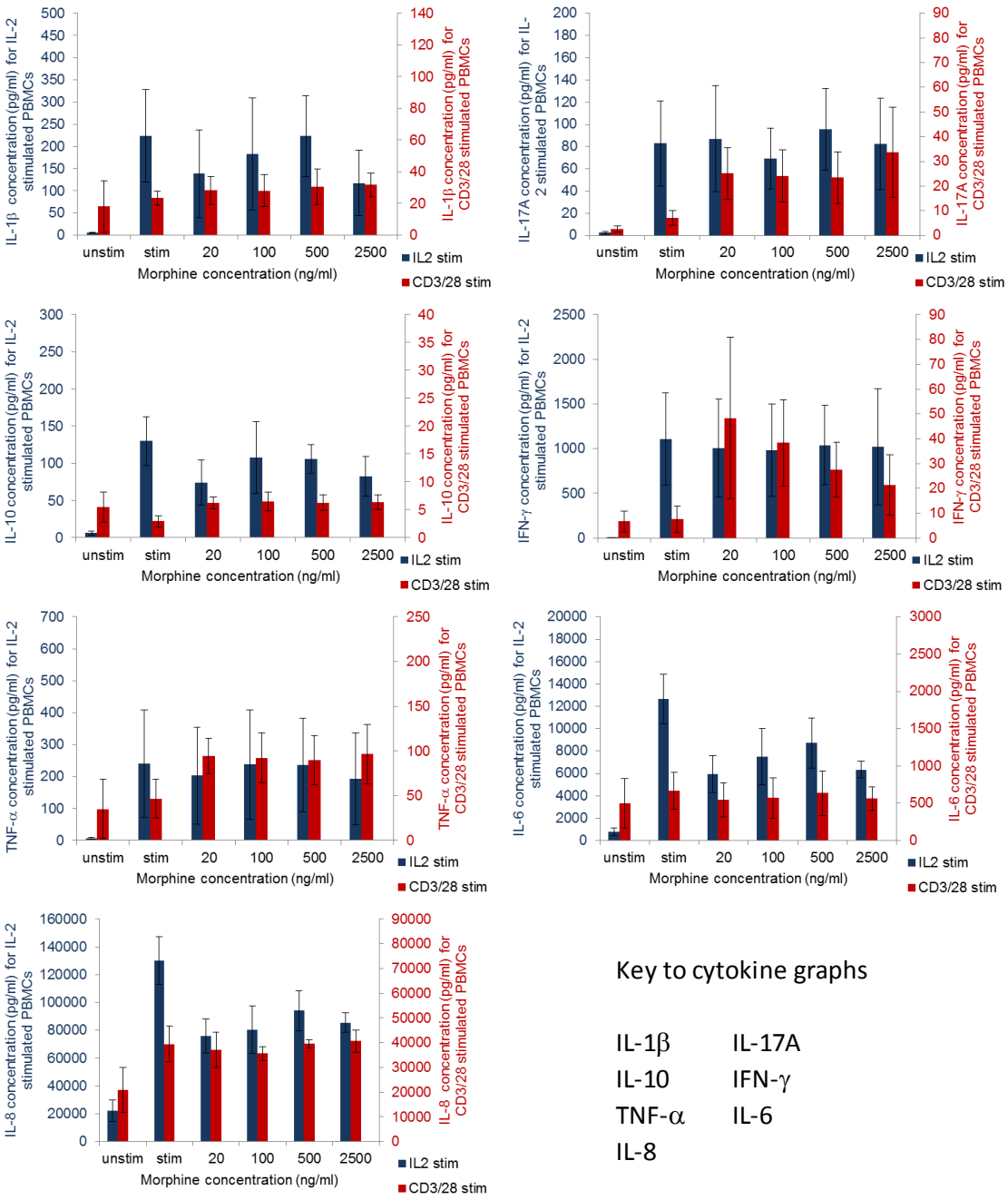

Figure 3-89: Effect of morphine on cytokine production.

Effect of morphine on cytokine production by IL-2 (**left Y axis, blue**) and Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y axis, red**) stimulated PBMCs. IL-1β (**top left panel**), IL-17A (**top right panel**), IL-10 (**upper middle left panel**), IFN-γ (**upper middle right panel**), TNF-α (**lower middle right panel**), IL-6 (**lower middle left panel**) and IL-8 (**bottom left panel**) concentrations are presented on the y axes, in pg/ml. PBMCs were incubated with the indicated concentration of morphine for 3 days, with either IL-2 or anti-CD3/anti-CD28 mAb coated beads. Unstimulated (unstim) and stimulated (stim) controls without morphine are included. Data are means ± SEM from 3 independent experiments for IL-2 stimulated and 4 independent experiments (except IL-6 and 8 at 20 ng/ml where n=3) for anti-CD3/28 stimulated. No statistical differences between the cytokine concentrations of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells were observed (paired Student t-test). **20 ng/ml of morphine significantly decreased IL-1β and IL-8 levels and 500 ng/ml of morphine increased IL-17A levels in IL-2 stimulated PBMCs** (P<0.05; paired Student *t*-test).

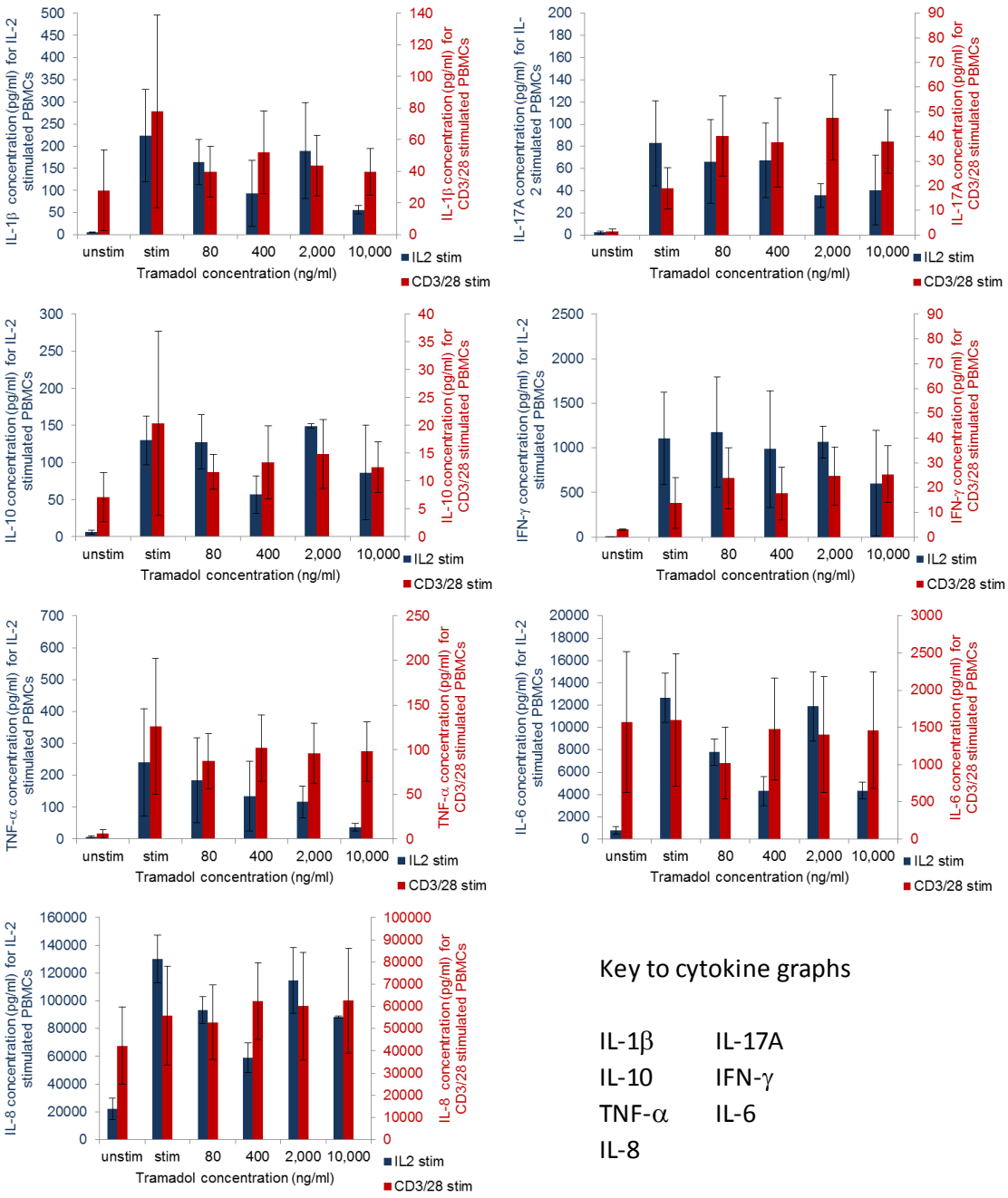

Figure 3-90: Effect of tramadol on cytokine production.

Effect of tramadol on cytokine production by IL-2 (**left Y axis, blue**) and Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y axis, red**) stimulated PBMCs. IL-1β (**top left panel**), IL-17A (**top right panel**), IL-10 (**upper middle left panel**), IFN-γ (**upper middle right panel**), TNF-α (**lower middle right panel**), IL-6 (**lower middle left panel**) and IL-8 (**bottom left panel**) concentrations are presented on the y axes, in pg/ml. PBMCs were incubated with the indicated concentration of tramadol for 3 days, with either IL-2 or anti-CD3/anti-CD28 mAb coated beads. Unstimulated (unstim) and stimulated (stim) controls without tramadol are included. Data are means ± SEM from 3 independent experiments for IL-2 stimulated (except at 2,000 and 10,000 ng/ml where n=2) and 3 independent experiments (except for IL-6 and 8 where n=4) for anti-CD3/28 stimulated. No statistical differences between the cytokine concentrations of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells were observed (paired Student t-test). **80 ng/ml of tramadol significantly decreased IL-8 levels in IL-2 stimulated PBMCs** (P<0.05; paired Student *t*-test).

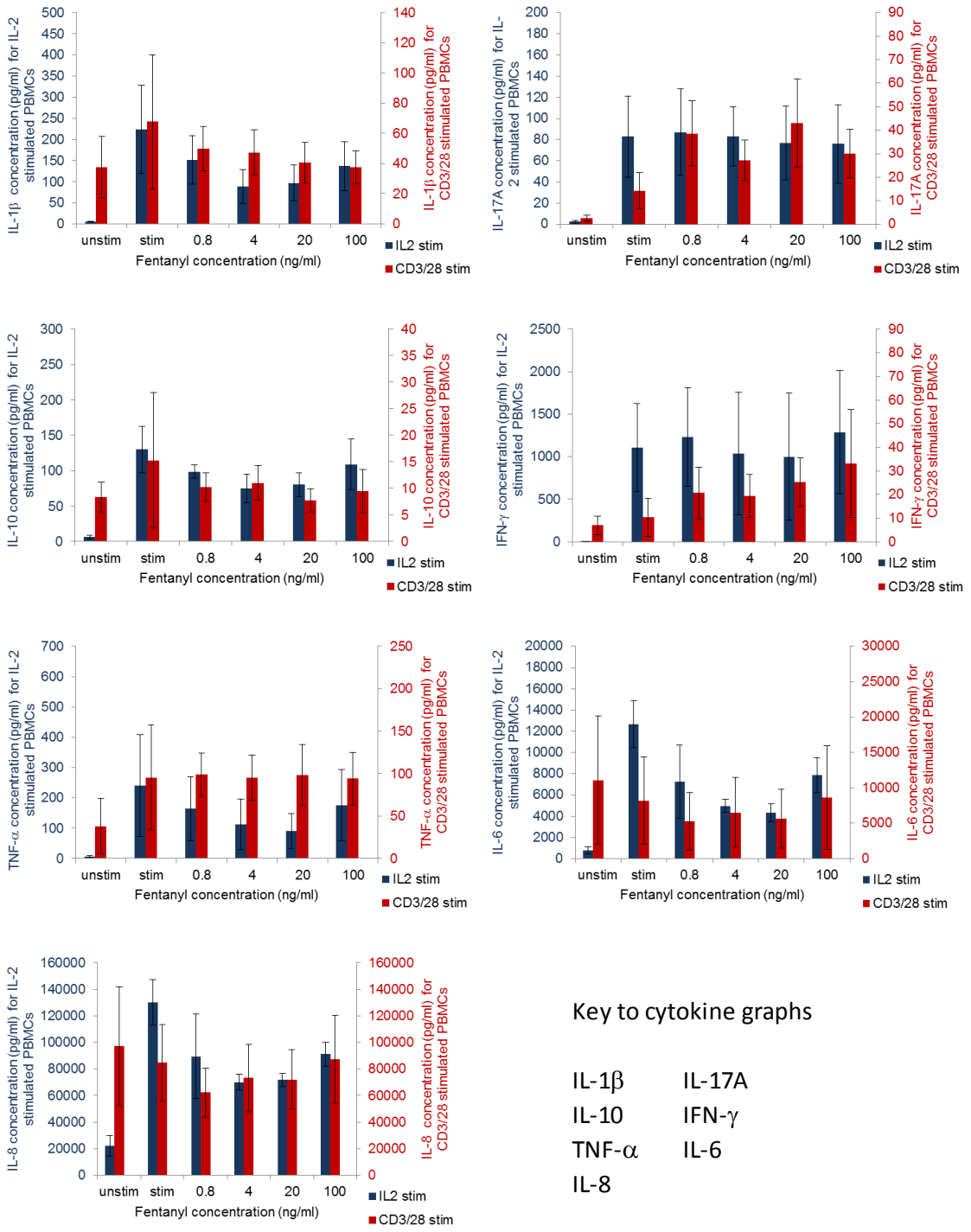

Figure 3-91: Effect of fentanyl on cytokine production.

Effect of fentanyl on cytokine production by IL-2 (**left Y axis, blue**) and Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y axis, red**) stimulated PBMCs. IL-1β (**top left panel**), IL-17A (**top right panel**), IL-10 (**upper middle left panel**), IFN-γ (**upper middle right panel**), TNF-α (**lower middle right panel**), IL-6 (**lower middle left panel**) and IL-8 (**bottom left panel**) concentrations are presented on the y axes, in pg/ml. PBMCs were incubated with the indicated concentration of fentanyl for 3 days, with either IL-2 or anti-CD3/anti-CD28 mAb coated beads. Unstimulated (unstim) and stimulated (stim) controls without fentanyl are included. Data are means ± SEM from 3 independent experiments for IL-2 stimulated and 4 independent experiments (except at 20 ng/ml where n=3) for anti-CD3/28 stimulated. No statistical differences between the cytokine concentrations of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells were observed (paired Student t-test). **20 ng/ml of fentanyl significantly decreased IL-6 levels in IL-2 stimulated PBMCs** (P<0.05; paired Student *t*-test).

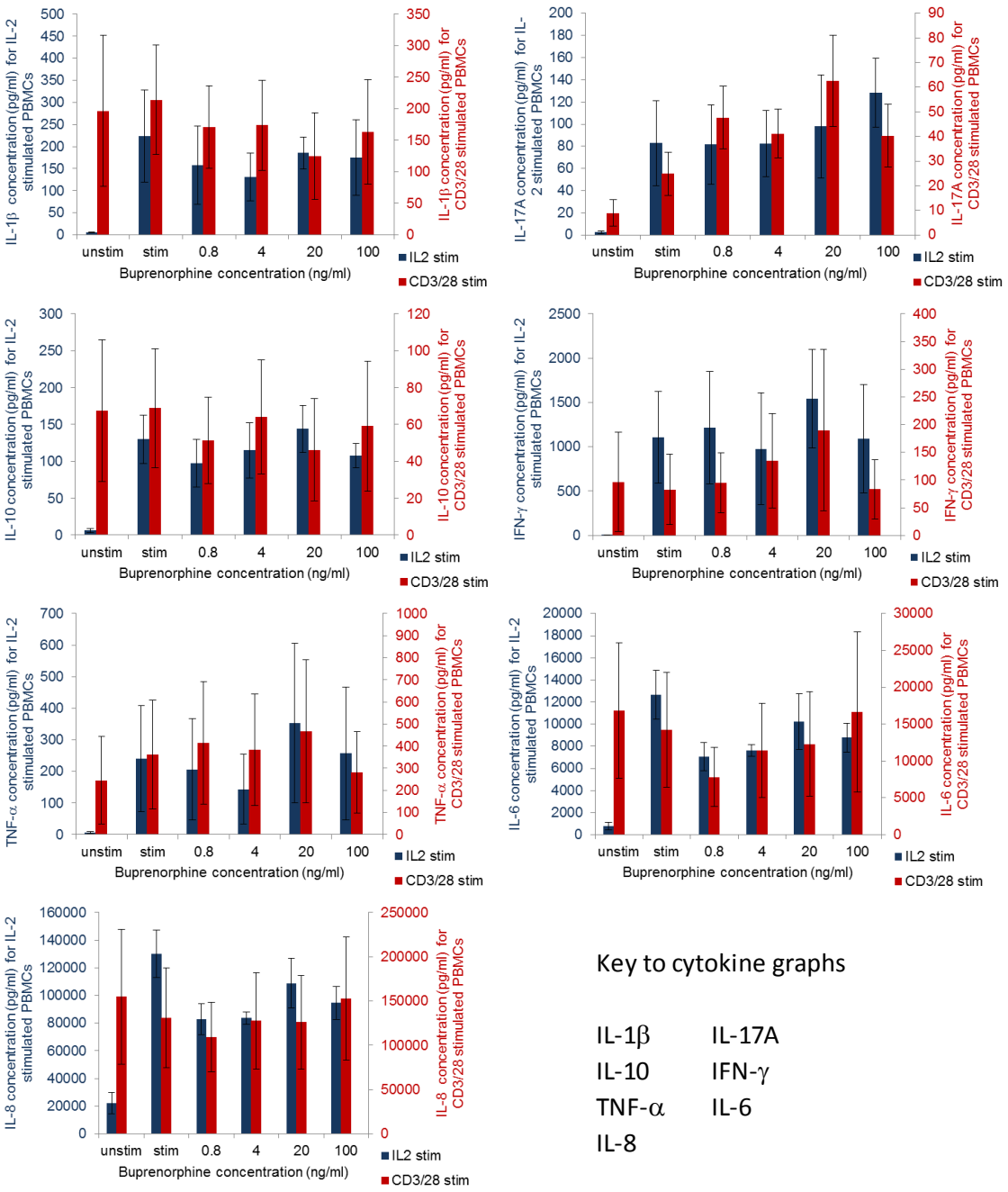

Figure 3-92: Effect of buprenorphine on cytokine production.

Effect of buprenorphine on cytokine production by IL-2 (**left Y axis, blue**) and Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y axis, red**) stimulated PBMCs. IL-1β (**top left panel**), IL-17A (**top right panel**), IL-10 (**upper middle left panel**), IFN-γ (**upper middle right panel**), TNF-α (**lower middle right panel**), IL-6 (**lower middle left panel**) and IL-8 (**bottom left panel**) concentrations are presented on the y axes, in pg/ml. PBMCs were incubated with the indicated concentration of buprenorphine for 3 days, with either IL-2 or anti-CD3/anti-CD28 mAb coated beads. Unstimulated (unstim) and stimulated (stim) controls without buprenorphine are included. Data are means ± SEM from 3 independent experiments for IL-2 stimulated and between 3 and 5 independent experiments for anti-CD3/28 stimulated. No statistical differences between the cytokine concentrations of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells were observed (paired Student t-test). **20 ng/ml of buprenorphine significantly increased IFN-γ levels in IL-2 stimulated PBMCs** (P<0.05; paired Student *t*-test).

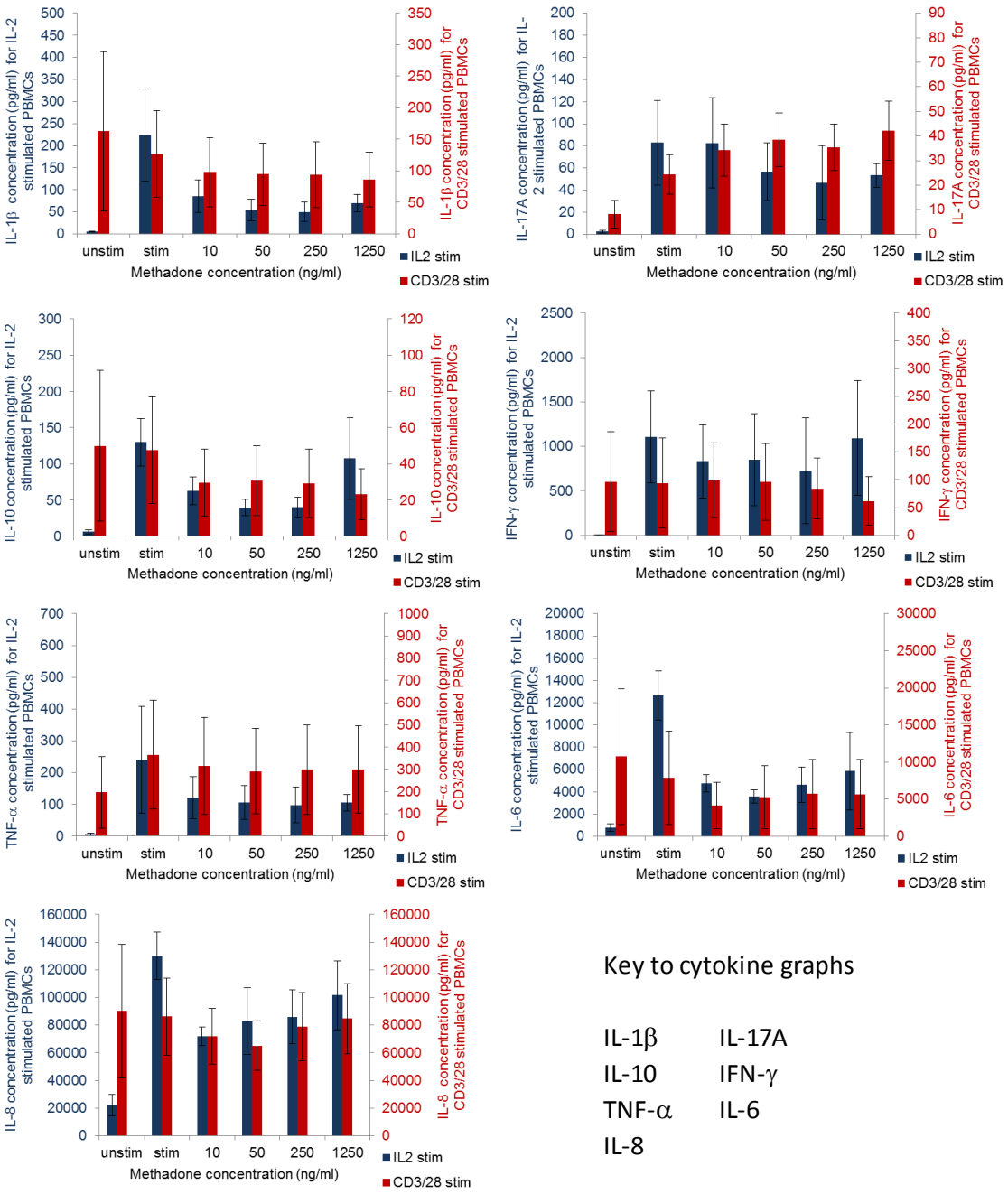

Figure 3-93: Effect of methadone on cytokine production.

Effect of methadone on cytokine production in IL-2 (**left Y axis, blue**) and Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y axis, red**) stimulated PBMCs. IL-1β (**top left panel**), IL-17A (**top right panel**), IL-10 (**upper middle left panel**), IFN-γ (**upper middle right panel**), TNF-α (**lower middle right panel**), IL-6 (**lower middle left panel**) and IL-8 (**bottom left panel**) concentrations are presented on the y axes, in pg/ml. PBMCs were incubated with the indicated concentration of methadone for 3 days, with either IL-2 or anti-CD3/anti-CD28 mAb coated beads. Unstimulated (unstim) and stimulated (stim) controls without methadone are included. Data are means ± SEM from 3 independent experiments for IL-2 stimulated and 5 independent experiments (except for IL-6 and 8 where n=4) for anti-CD3/28 stimulated. No statistical differences between the cytokine concentrations of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells were observed (paired Student t-test). **Methadone at all concentrations decreased IL-6 levels; 10 and 250 ng/ml decreased IL-8 levels and 50 ng/ml decreased IFN-γ levels in IL-2 stimulated PBMCs** ($P<0.05$; paired Student *t*-test).

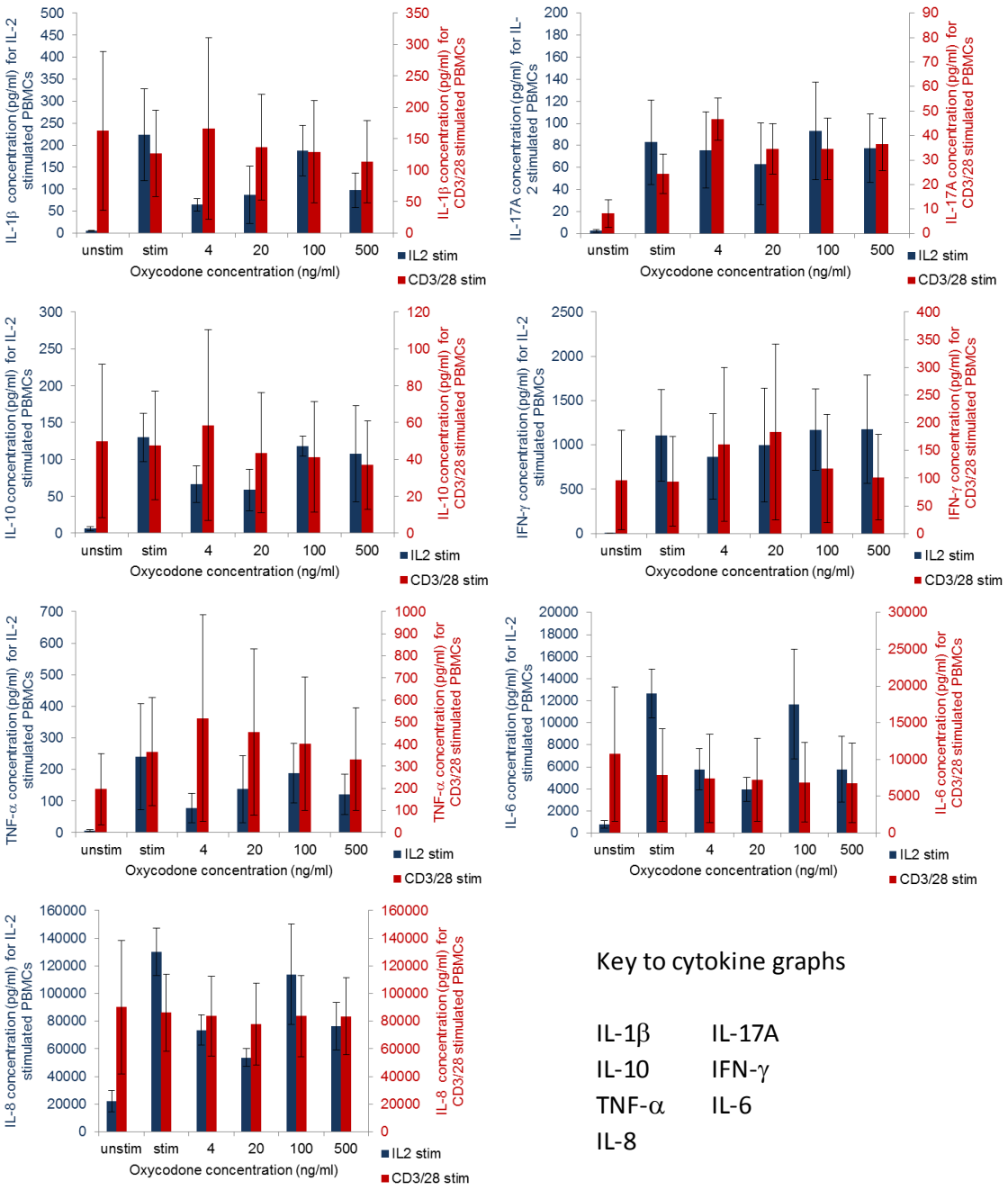

Figure 3-94: Effect of oxycodone on cytokine production.

Effect of oxycodone on cytokine production in IL-2 (**left Y axis, blue**) Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y axis, red**) stimulated PBMCs. IL-1β (**top left panel**), IL-17A (**top right panel**), IL-10 (**upper middle left panel**), IFN-γ (**upper middle right panel**), TNF-α (**lower middle right panel**), IL-6 (**lower middle left panel**) and IL-8 (**bottom left panel**) concentrations are presented on the y axes, in pg/ml. PBMCs were incubated with the indicated concentration of oxycodone for 3 days, with either IL-2 or anti-CD3/anti-CD28 mAb coated beads. Unstimulated (unstim) and stimulated (stim) controls without oxycodone are included. Data are means ± SEM from 3 independent experiments for IL-2 stimulated and between 3 and 5 independent experiments (n=4 for IL-6 and 8) for anti-CD3/28 stimulated. No statistical differences between the cytokine concentrations of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells were observed (paired Student t-test). **4, 20 and 500 ng/ml of oxycodone decreased IL-6 levels; 4 and 500 ng/ml of oxycodone decreased IL-8 levels; 4 ng/ml of oxycodone decreased IL-10 and IFN-γ; and 20 ng/ml of oxycodone decreased IL-17A levels in IL-2 stimulated PBMCs** ($P<0.05$; paired Student *t*-test).

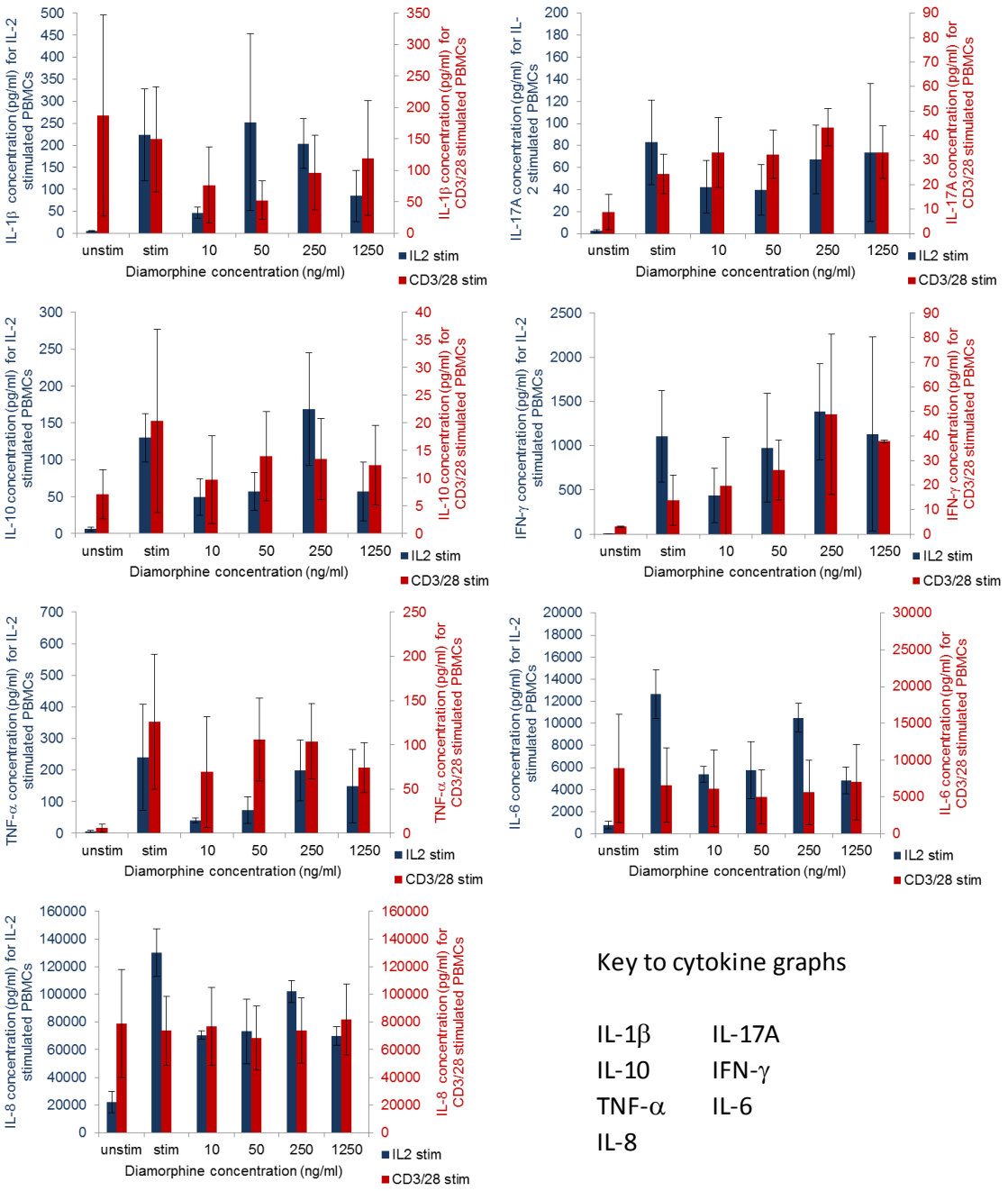

Figure 3-95: Effect of diamorphine on cytokine production.

Effect of diamorphine on cytokine production in IL-2 (**left Y axis, blue**) and Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y axis, red**) stimulated PBMCs. IL-1β (**top left panel**), IL-17A (**top right panel**), IL-10 (**upper middle left panel**), IFN-γ (**upper middle right panel**), TNF-α (**lower middle right panel**), IL-6 (**lower middle left panel**) and IL-8 (**bottom left panel**) concentrations are presented on the y axes, in pg/ml. PBMCs were incubated with the indicated concentration of diamorphine for 3 days, with either IL-2 or anti-CD3/anti-CD28 mAb coated beads. Unstimulated (unstim) and stimulated (stim) controls without diamorphine are included. Data are means ± SEM from 3 independent experiments (except n=2 for 1250 ng/ml; n=3 for IL-6 and 8) for IL-2 stimulated and 3 independent experiments (except n=2 for 10ng/ml and n=5 for IL-6 and 8 [n=4 for 10ng/ml]) for anti-CD3/28 stimulated. No statistical differences between the cytokine concentrations of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells were observed (paired Student t-test). **10, 50 and 1250 ng/ml of diamorphine decreased IL-6 levels and 50 and 1250 ng/ml of diamorphine decreased IL-8 levels in IL-2 stimulated PBMCs** (P<0.05; paired Student *t*-test).

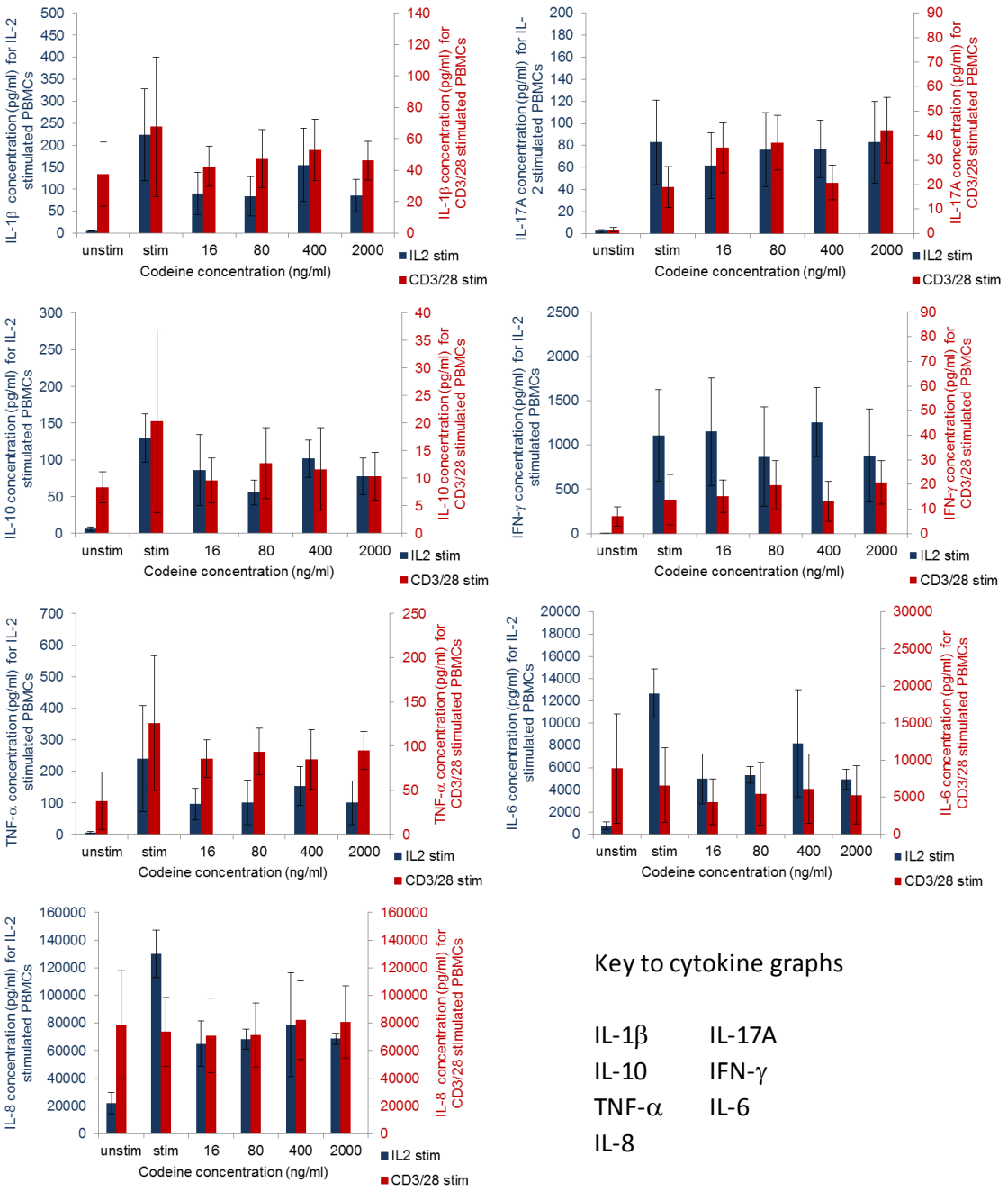

Figure 3-96: Effect of codeine on cytokine production.

Effect of codeine on cytokine production in IL-2 (**left Y axis, blue**) Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y axis, red**) stimulated PBMCs. IL-1β (**top left panel**), IL-17A (**top right panel**), IL-10 (**upper middle left panel**), IFN-γ (**upper middle right panel**), TNF-α (**lower middle right panel**), IL-6 (**lower middle left panel**) and IL-8 (**bottom left panel**) concentrations are presented on the y axes, in pg/ml. PBMCs were incubated with the indicated concentration of codeine for 3 days, with either IL-2 or anti-CD3/anti-CD28 mAb coated beads. Unstimulated (unstim) and stimulated (stim) controls without codeine are included. Data are means ± SEM from 3 independent experiments for IL-2 stimulated and 3 or 4 independent experiments (except n=2 for 400ng/ml for IL-17A, [n=5 for IL-6 and 8, except for 400 and 2,000ng/ml where n=4]) for anti-CD3/28 stimulated. No statistical differences between the cytokine concentrations of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells were observed (paired Student t-test). **10 and 1250 ng/ml of codeine decreased IL-6 and IL-8 levels, 1250 ng/ml of codeine also decreased IL-10 and IFN-γ levels in IL-2 stimulated PBMCs** (P<0.05; paired Student *t*-test).

Stimulation of PBMCs with IL-2 increased the production of IL-1β, IL-6, IL-8, IL-17A, IL-10, IFN-γ and TNF-α. This was more variable with the Miltenyi anti-CD3/anti-CD28 mAb coated bead stimulation. This increased IL-17A, did not affect IL-1β, IL-6 and IL-8, and either did not affect or increased IL-10, IFN-γ and TNF-α production, depending on the subject. This is considered in more detail in the following section and in Table 3.13.

None of the opioids had a statistically significant effect on cytokine secretion in PBMCs stimulated with Miltenyi anti-CD3/anti-CD28 mAb coated beads. Some of the cytokine concentrations in the supernatants of IL-2 stimulated PBMCs were significantly influenced by certain concentrations of some opioids but the only clear trends in the statistical significance were for methadone, oxycodone and diamorphine which all decreased IL-6 production from IL-2 stimulated PBMCs at several concentrations. Although conventionally considered a pro-inflammatory cytokine as IL-6 initiates the acute phase response during infection and after trauma, it can also have anti-inflammatory effects as it can inhibit IL-1 and TNF-α (Xing et al., 1998). Thus the potential clinical impact of IL-6 inhibition by these opioids would depend on various other factors including the hosts level of immune activation, the concentrations of other cytokines and cytokine regulators such as the suppressor of cytokine signalling-3 (Croker et al., 2011). As IL-6 induces CRP production in the liver by activating JAKs (Heikkila et al., 2007), it is possible the inhibition of IL-6 production by these opioids might inhibit the acute phase response which is important during infection and after trauma. However inflammation, along with CRP and IL-6, is associated with poor prognosis and decreased survival in some solid and haematological malignancies (Heikkila et al., 2007, Nikiteas et al., 2005, Pine et al., 2011). IL-6 is also a myokine, being produced from muscles during exercise, to increase lipolysis and fat oxidation (Pedersen, 2011), and is also secreted by osteoblasts to stimulate osteoclasts and promote new bone formation (Eriksen et al., 2010). IL-6 is ubiquitous with many actions and inhibiting its production could potentially have widespread effects.

Even though there were these statistically significant effects, this does not mean that they will translate into clinical significance as they might be due to chance in view of the large number of tests performed (eight opioids, four concentrations, two stimuli and seven cytokines measured) or even a significant effect statistically might only be a small actual effect which may not impact on clinical outcome. Furthermore, the lack of statistical significance was in part due to interindividual variability in cytokine production in response to the different opioids.

3.4.1 Effects of opioids on cytokine production in individual subjects

The graphs in the previous section show the pooled data for the effect of each of the opioids on each cytokine; there is however interindividual variability causing large standard error of the mean and reducing any potential statistical significant effect. However, for an individual the effects of certain opioids on the production of certain cytokines might be clinically significant and the trends for each individual are explored in the sections below.

3.4.1.1 Effects of opioids on IL-2 stimulated cytokine production in individual subjects

The results for IL-2 stimulated cytokine production presented in the graphs of the previous section only show occasional effects of the opioids tested, which is in part due to differences between the individuals tested. In this section the effects of each of the opioids on cytokine production by each individual is described in the tables below (Table 3.5 - Table 3.12). The tables have been colour coded for whether each opioid (at two or more concentrations) has an inhibitory or stimulatory effect in each of the individuals on the different cytokine concentrations.

IL-2 stimulation increased the production of all cytokines in all of the individuals tested, the combined data are presented in the graphs of the previous section (individual data not shown).

Table 3.5: Effect of morphine on IL-2 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
1	Decrease	Decrease	Decrease	No effect	No effect	No effect	No effect
2	No effect	Decrease	Decrease	No effect	No effect	Decrease	Decrease
3	No effect	Decrease	Decrease	No effect	No effect	No effect	No effect

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.6: Effect of tramadol on IL-2 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
1	Decrease	Decrease	Decrease	No effect	No effect	No effect	No effect
2	No effect	No effect	Decrease	No effect	No effect	No effect	No effect
3	Decrease	Decrease	Decrease	No effect	Decrease	No effect	No effect

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.7: Effect of fentanyl on IL-2 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
1	Decrease	Decrease	Decrease	Decrease	No effect	No effect	Decrease
2	Decrease	Decrease	Decrease	No effect	No effect	No effect	Decrease
3	Decrease	Decrease	Decrease	Decrease	No effect	No effect	Decrease

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.8: Effect of buprenorphine on IL-2 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
1	Decrease	Decrease	Decrease	No effect	Increase	No effect	Decrease
2	Decrease	Decrease	Decrease	No effect	Increase	No effect	Decrease
3	Decrease	Decrease	Decrease	No effect	No effect	No effect	No effect

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.9: Effect of methadone on IL-2 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
1	Decrease	Decrease	Decrease	Decrease	No effect	Decrease	No effect
2	Decrease	Decrease	No effect	Decrease	No effect	Decrease	Decrease
3	Decrease	Decrease	Decrease	Decrease	No effect	No effect	Decrease

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.10: Effect of oxycodone on IL-2 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
1	Decrease	Decrease	Decrease	Decrease	No effect	No effect	No effect
2	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease
3	Decrease	Decrease	Decrease	Decrease	No effect	No effect	Decrease

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.11: Effect of diamorphine on IL-2 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A,	IFN-γ	TNF-α
1	Decrease	Decrease	Decrease	No effect	No effect	No effect	No effect
2	Decrease	Decrease	Decrease	Decrease	No effect	No effect	Decrease
3	No effect	Decrease	Decrease	Decrease	Decrease	No effect	Decrease

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.12: Effect of codeine on IL-2 stimulated cytokine production for the individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
1	Decrease	Decrease	Decrease	Decrease	No effect	Decrease	Decrease
2	Decrease	No effect	No effect	Decrease	No effect	Decrease	Decrease
3	Decrease	Decrease	Decrease	Decrease	No effect	No effect	Decrease

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

In general the production of IL-1β, IL-6, IL-8, IL-10 and TNF-α were inhibited by most of the opioids in most individuals. IL-17A and IFN-γ were less affected, only being inhibited by some opioids in some individuals.

For the IL-2 stimulated samples, the same three subjects were used for all the opioids and although the effects of the different opioids varied between the subjects', in general all the opioids decreased IL-1β, IL-6 and IL-8.

3.4.1.2 Effects of opioids on anti-CD3/anti-CD28 mAb stimulated cytokine production in individual subjects

The results for the Miltenyi anti-CD3/anti-CD28 mAb coated bead stimulated cytokine production presented in the graphs previously show variability between subjects, as indicated by the large error bars, and thereby reduced the potential for statistical significance. In this section, the effect of the opioids on cytokine levels for each individual is explored.

In some individuals, the Miltenyi anti-CD3/anti-CD28 mAb coated bead stimulation did not increase cytokine levels, and on occasions (especially for Subject 5) it decreased the concentration of some cytokines (Table 3.13). The tables have been colour coded for whether each opioid (at two or more concentrations) has an inhibitory or stimulatory effect in each of the individuals on the different cytokine concentrations.

Table 3.13: Effect of anti-CD3/28 stimulation on cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
1	Decreased	No effect	Decreased				Decreased
2	Increased	No effect	No effect	Increased	Increased	No effect	Increased
3	Increased	Increased	Increased		Increased		Increased
4	Increased	No effect	Increased	Increased	Increased	Increased	Increased
5	Decreased	Decreased	Decreased	Decreased	Increased	No effect	Increased

Assessing the effect of anti-CD3/28 stimulated, compared with unstimulated, production of cytokines for individuals. Red indicates an inhibitory effect, green a stimulatory effect and yellow no effect or no consistent effect.

The effect of opioids on cytokine production for each individual is described in the tables below (Table 3.14 - Table 3.21).

Table 3.14: Effect of morphine on anti-CD3/28 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
2	No effect	Decreased	Decreased	No effect	Increased	Increased	Increased
3	Increased	Increased	Increased	Increased	Increased	Increased	Increased
4	Decreased	Decreased	Decreased	No effect	Decreased	Increased	Decreased

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.15: Effect of tramadol on anti-CD3/28 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
2	No effect	Decreased	Decreased	No effect	Increased	Increased	Increased
3	Increased	Increased	Increased	No effect	Increased	Increased	Increased
4	Decreased	Decreased	No effect	Decreased	Decreased	Decreased	Decreased

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.16: Effect of fentanyl on anti-CD3/28 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
2	No effect	Decreased	Decreased	No effect	Increased	Increased	Increased
3	Increased	No effect	No effect	Increased	Increased	Increased	Increased
4	Decreased	Decreased	No effect	Decreased	Decreased	Decreased	Decreased

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.17: Effect of buprenorphine on anti-CD3/28 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
2	Decreased	No effect	No effect	No effect	No effect	Decreased	No effect
3	Increased	Increased	Increased	Increased	Increased	Increased	Increased
4	Decreased	Decreased	Decreased	Decreased	Decreased	No effect	Decreased

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.18: Effect of methadone on anti-CD3/28 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
2	No effect	Decreased	Decreased	No effect	Increased	Increased	Increased
3	Increased	Increased	Increased	Increased	Increased	Increased	Increased
4	Decreased	Decreased	No effect	Decreased	Decreased	Decreased	Decreased

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.19: Effect of oxycodone on anti-CD3/28 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
2	No effect	Decreased	Decreased	No effect	Increased	Increased	Increased
3	Increased	Increased	Increased	Increased	Increased	Increased	Increased
4	Decreased	No effect	Increased	Decreased	Decreased	No effect	Decreased

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.20: Effect of diamorphine on anti-CD3/28 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
2	Decreased	Decreased	Decreased	No effect	Increased	No effect	No effect
3	Increased	Increased	Increased	Increased	Increased	Increased	Increased
4	Decreased	Decreased	Decreased	Decreased	Decreased	No effect	Decreased

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

Table 3.21: Effect of codeine on anti-CD3/28 stimulated cytokine production for individuals.

Cytokine / Subject	IL-1β	IL-6	IL-8	IL-10	IL-17A	IFN-γ	TNF-α
2	No effect	Decreased	Decreased	Decreased	Increased	Increased	Increased
3	Increased	Increased	Increased	Increased	Increased	Increased	Increased
4	Decreased	Decreased	Increased	Decreased	Decreased	Decreased	Decreased

Red indicates an inhibitory effect, green a stimulatory effect and yellow no consistent effect.

The effect of Miltenyi anti-CD3/anti-CD28 mAb coated bead stimulation was more variable between individuals compared with IL-2 stimulation. This was mainly studied in subjects 2, 3 and 4. In subject 2, most of the opioids inhibited IL-6 and IL-8 production, increased IL-17A, IFN-γ and TNF-α levels, and had little effect on IL-1β and IL-10 concentrations. In subject 3, production of all of these cytokines tended to be increased by all opioids. Whereas in subject 4, they were mostly inhibited.

Although opioids had variable effects on the secretion of the cytokines analysed, the variation was more related to the individual rather than the opioid (especially in subjects 3 and 4). Thus for an individual subject the effect of all the opioids tested was generally inhibitory, stimulatory or neutral, with minor variations between the opioids. Subjects 3 and 4 had a similar effect from the opioid across all the cytokines tested, so in general, opioids either stimulated (for subject 3) or inhibited (for subject 4) an individual's capacity to produce the array of cytokines which were analysed.

The effect of opioids on cytokine production in pre-stimulated PBMCs (1-day incubation with Miltenyi anti-CD3/anti-CD28 mAb coated beads before a further 2-day incubation with opioids and the beads), as well as a pre-opioid incubation (1-day incubation with opioids before a further 3-day incubation with Miltenyi anti-CD3/anti-CD28 mAb coated beads and the opioids) was also assessed. There was no consistent effect of the opioids on cytokines in these experiments (data not shown). In the pre-stimulated experiments, there were some trends for each individual. In Subject 2, diamorphine decreased IL-17A and IFN-γ; codeine increased IL-6 and IL-8. In Subject 3, all opioids decreased IL-17A and IFN-γ, although buprenorphine had less effect on IFN-γ.

3.4.2 Discussion

In the current study, PBMCs from healthy volunteers were stimulated with IL-2 or Miltenyi anti-CD3/anti-CD28 mAb coated beads for 3 days and the effect of clinically relevant concentrations, of eight opioids on the production of cytokines (IL-1β, IL-6, IL-8, IL-10, IL-17A, IFN-γ and TNF-α) was evaluated. IL-4, IL-12p70, IP10 and IFN-α were also analysed but were below the detection threshold.

IL-2 more potently and consistently stimulated baseline cytokine production compared with Miltenyi anti-CD3/anti-CD28 mAb coated beads, which showed interindividual variability (Table 3.13). Whereas IL-2 directly activated the T cells to induce a cascade of cytokine production, anti-CD3/anti-CD28 mAb need to activate the TCR and its co-receptor in order to trigger intracellular activation cascades and IL-2 secretion before other cytokines could be produced as a secondary result of this. Thus, although 3 days might have been long enough to activate T cells, it might not have been long enough to induce cytokine secretion and the subsequent activation cascades from these. However, this approach might have led to a more sensitive detection of potential stimulatory effects of the opioids, as if they promoted the transduction signalling pathways then this may have resulted in higher levels of cytokines.

There were occasional statistically significant effects from all opioids on some of the cytokines (previously described in section 3.4) although due to multiple statistical tests being performed on the vast numbers of data analysed, it is difficult to determine if this is more by chance or truly significant. Some of the significance is potently lost due to the variability of the subjects tested, as when looked at individually some of the opioids affected cytokine levels several fold, which may be clinically significant for that individual (an example of which is shown in Figure 3-97 and Figure 3-98).

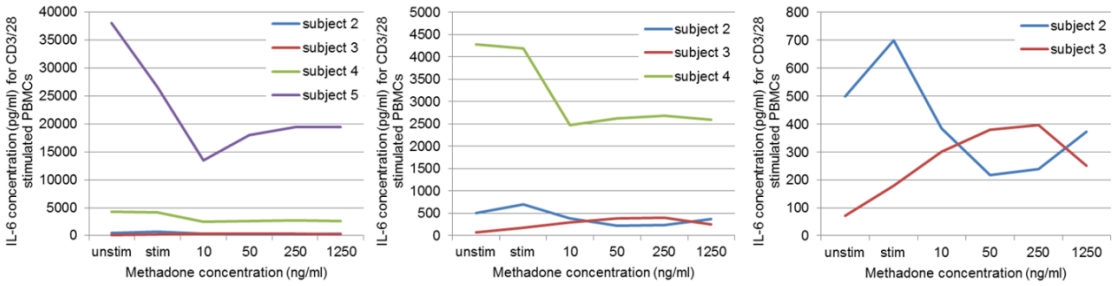

Figure 3-97: Effect of methadone on individual IL-6 production.
Effect of methadone on IL-6 production in different subjects' PBMCs stimulated with Miltenyi anti-CD3/anti-CD28 mAb coated beads. The (**left panel**) shows all four subjects tested, but due to the very high levels of IL-6 in subject 5, this is removed to show trends for the other subjects (**middle panel**), two subjects have much lower levels of IL-6 and subject 4 is removed to enable the trends to be shown in subjects 2 and 3 (**right panel**).

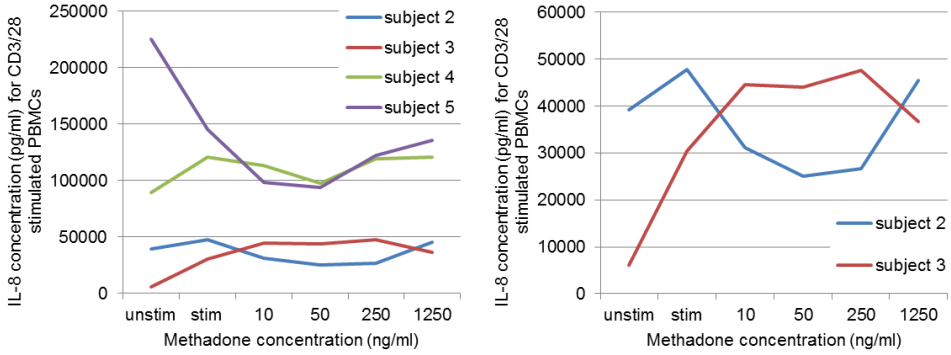

Figure 3-98: Effect of methadone on individual IL-8 production.
Effect of methadone on IL-8 production in different subjects' PBMCs stimulated with Miltenyi anti-CD3/anti-CD28 mAb coated beads. The (**left panel**) shows all four subjects tested, but due to the high levels of IL-8 in subjects 4 and 5, these are removed to show trends for subjects 2 and 3 (**right panel**).

The variation between individuals at baseline, their differential response to Miltenyi anti-CD3/anti-CD28 mAb coated bead stimulation (increasing cytokine levels in some subjects and inhibiting production in others) and the effects of opioids on this are impossible to unravel for the population studied. It is therefore likely to be the case that in a population of patients that some will be very sensitive to certain opioids and others will have minimal opioid derived immune effects. For example, methadone at most concentrations caused about a 50% reduction in IL-6 levels in three out of four subjects tested (Subjects 2, 4 and 5), whereas methadone at intermediate concentrations doubled IL-6 levels in subject 3. Faced with such variability in absolute concentrations of these cytokines and the wide variety of effects induced by opioids in the subjects tested, it cannot be known if these are by chance or could be clinically significant for that subject (providing these *in vitro* changes were to be replicated in a clinical study).

There is also a paucity of data in the literature as to what change (or absolute concentration) of the cytokines would lead to a difference in clinical outcome. Long term clinical studies are needed, in which cytokine levels are measured and the course of the patients' illness is monitored.

Several previous studies have assessed the effect of certain opioids on the production of cytokines. The *in vivo* studies have been discussed in the introduction (section 1.12.5) and the *in vitro* studies are described below and compared to the current study.

The effect of morphine on IL-4 production has been assessed in splenocytes from C57/S129 mice that have been depleted of B cells and $CD8^+$ T cells (Roy et al., 2005). For these experiments, splenocytes were incubated for 2-hr with 200 ng/ml of morphine and then stimulated with BD Pharmingen anti-CD3/anti-CD28 mAbs for 3 days. IL-4 was measured using an ELISA. Morphine increased IL-4 synthesis three fold in splenic $CD4^+$ T cells (Roy et al., 2005). IL-4 was below the detection threshold in all samples analysed in the current study, this might be due to differences in potency of the beads.

In a study from the same group, the *in vitro* effect of morphine on IL-2, IL-4, IL-5 and IFN-γ production by healthy human PBMCs and CB6F1/J murine splenocytes was evaluated (Roy et al., 2001). PBMCs and splenocytes were incubated for 4 days with 10, 30 and 100 ng/ml of morphine and then stimulated with anti-CD3/anti-CD28 mAbs (bead type and duration not stated). Cytokines were measured using ELISAs. Morphine dose-dependently decreased PBMC production of IL-2 (up to 50%) and IFN-γ (up to 70%), and increased IL-4 (up to 10x) and IL-5 (up to 3x) production. Similar results were detected in murine splenocytes, with morphine inhibiting IFN-γ (up to 70%) and stimulating IL-4 (up to 4x) production. These effects were abolished in $MOR^{-/-}$ mice (Roy et al., 2001).

These *in vitro* studies demonstrated that morphine treatment of murine and human cells results in polarisation of cytokine production towards the T_H2 phenotype (Roy et al., 2001, Roy et al., 2005), potentially inhibiting cell mediated immunity. The current study evaluated the effect of comparable doses of morphine on IL-2, IL-4 and IFN-γ production from healthy human PBMCs. However, IL-4 was below the detection limit of the assay used and although IFN-γ levels were increased several fold, this effect was not statistically significant. These differences might be accounted for by the duration and exact stimulation method.

The effect of morphine on IL-2 and IFN-γ production has been evaluated using CB6F1/J, Balb/c female and 3C57BL/6 male mice (Wang et al., 2001). Lymph node cells were incubated with 1 nM – 10 μM of morphine (equivalent to 0.3 – 3,365 ng/ml) for 2 hr and then stimulated with Con A for 24 hr. IL-2 and IFN-γ were measured using ELISAs. IL-2 was inhibited (up to 50%) by 1 nM – 10 μM of morphine and IFN-γ was inhibited (up to 70%) by 100 nM – 10 μM of morphine (Wang et al., 2001). Despite using overlapping concentrations of morphine, this is in contrast to the current study, which may be due to the species and cell type used (lymph node vs. peripheral lymphocytes) and the method and duration of stimulation.

Qian and colleagues assessed the effect of morphine and tramadol on the *in vitro* differentiation of T_H cells taken from 20 human adult outpatients without immune disorders (Qian et al., 2005). Whole blood or PBMCs were stimulated with PMA and Ionomycin, and then incubated with morphine or tramadol for 48 hr (concentrations not stated). The ratio of $CD4^+$ T cells with the intracellular cytokines IFN-γ and IL-2 (T_H1 cells) to $CD4^+$ cells with IL-4 and IL-10 (T_H2 cells) was analysed by flow cytometry. Morphine, and to a lesser extent tramadol, dose-dependently increased IL-4 and IL-10 levels, and decreased IL-2 and IFN-γ, compared with no opioid control groups (effect size is not stated). Opioids might thus direct naïve T_H cells toward the T_H2 phenotype, suppressing cell-mediated immunity (Qian et al., 2005).

This study differed from the current study as it used patients (their illness was not reported) rather than healthy volunteers. Whole blood as well as PBMCs were used and stimulated differently (PMA and Ionomycin vs. IL-2 or anti-CD3/anti-CD28 mAbs), and intracellular rather than secreted cytokine levels were measured. These studies cannot be directly compared as, surprisingly, the concentrations of opioids that were used were not stated. In the current study, neither morphine nor tramadol consistently affected the concentrations of any cytokine, which contrasts to the study by Qian and colleagues. The difference may be due to the subjects used or the intracellular measurement.

Murine splenic lymphocytes and peritoneal macrophages from female B6C3F1 mice have been stimulated *in vitro* by anti-CD3 mAb and incubated for 48 hr with 0.0001 – 100 μM of morphine (equivalent to 0.03 – 28,530 ng/ml) (Thomas et al., 1995a). IL-2 production was measured using a CTLL-2 proliferation assay, and IL-4 and IL-6 by spectroscopy. Only low concentrations of morphine (0.0001 – 0.1 μM; equivalent to

0.03 – 29 ng/ml) enhanced macrophage production of IL-6, with a maximal effect of 8% (which occurred at 0.0001 μM). Splenic lymphocyte production of IL-2 was decreased (by up to 8%) by occasional concentrations of morphine (0.0001, 1 and 10 μM). IL-4 was unaffected at all concentrations tested (Thomas et al., 1995a).

The effect of 0.0001 – 100 μM of methadone (equivalent to 0.03 – 30,940 ng/ml) and diamorphine (equivalent to 0.03 – 36,940 ng/ml) on IL-2, IL-4 and IL-6 production has been evaluated in female B6C3F1 mice (Thomas et al., 1995b). The opioids were cultured with murine splenocytes and thioglycolate-elicted peritoneal macrophages which were stimulated for 48 hr with anti-CD3 mAb and LPS, respectively. IL-2 was measured using the CTLL-2 proliferation assay and IL-4 and IL-6 by spectroscopy. Although the production of IL-2 was slightly, but significantly, suppressed by most concentrations of diamorphine (generally by around 10%, maximum 23%) and methadone (generally by around 15%, maximum 43%), this did not follow any dose relationship. IL-4 production was reduced only by the maximum concentration of diamorphine (by 20%) and methadone (by 88%). IL-6 production by LPS-stimulated peritoneal macrophages was reduced (by 15%) by 0.001 and 100μM of methadone (≈ 0.3 and 30,940 ng/ml) (Thomas et al., 1995b).

These two studies by the same group incubated cells from different species (murine splenic vs. human peripheral blood lymphocytes) with a large range of morphine, methadone and diamorphine concentrations which overlapped with those used in the current study for a similar duration (2 vs. 3 days). The stimulation used differed (anti-CD3 vs. anti-CD3/anti-CD28), as did the cytokine detection method. The current study did not assess macrophage cytokine production and measurable levels of IL-4 were not detected. The only effect seen in both studies was that methadone reduced IL-6 production, albeit inconsistently in the study by Thomas.

Peng and colleagues assessed the *in vitro* and *in vivo* effects of morphine on IL-12 production (Peng et al., 2000). For the *in vitro* studies, murine peritoneal macrophages from female C3HeB/FeJ mice were incubated with 10^{-10} – 10^{-6} M of morphine (equivalent to 0.03 – 285 ng/ml) for 2 hr before stimulation with LPS plus IFN-γ for 48 hr. IL-12 was measured in the supernatants by an ELISA. *In vitro* morphine had no effect on IL-12 production. For the *in vivo* study, female C3HeB/FeJ mice were implanted with a 75 mg morphine pellet, with or without a naloxone pellet for 48 hr, after which peritoneal macrophages were harvested and stimulated *in vitro* with LPS

plus IFN-γ for 48 hr. Cytokine levels were measured by an ELISA. Morphine increased the production of IL-12 (by 4x) and TNF-α (by 2x), but inhibited IL-10 production (by 50%) from peritoneal macrophages. The authors suggested that the modulation of IL-12 by morphine was not a direct effect on macrophages, and that the enhancement of the pro-inflammatory cytokines production by macrophages during morphine treatment was due to morphine-induced occult bacteraemia (Peng et al., 2000). This was demonstrated in their previous study, in which morphine increased bacteria translocating from the gastrointestinal tract, which might act as a stimulus *in vivo* to prime macrophages to increase production of inflammatory cytokines (Hilburger et al., 1997).

These studies potentially elucidate further differences between the *in vitro* and *in vivo* effects of morphine as being more than just the neuro-immune mechanisms. This makes it very difficult to dissect the mechanisms that underlie the effects that have been observed in *in vivo* models. This study used different species, stimulation and measurement compared with the current study, but did not have an effect on IL-12. In the current study IL-12p70 was below the detection threshold in all samples analysed.

Rao and colleagues incubated whole blood from seven healthy male and female volunteers for 6 hr with morphine (2,000 and 200,000 ng/ml) or fentanyl (20 and 2,000 ng/ml) in the presence or absence of LPS. IL-6 and TNF-α concentrations were measured using ELISAs. LPS increased IL-6 and TNF-α concentrations. These opioids did not affect unstimulated cytokine production. Both concentrations of morphine and fentanyl inhibited LPS induced expression of IL-6 (by around 30%) and TNF-α (by around 60%) (Rao et al., 2004). This could have a detrimental effect on the pro-inflammatory immune response.

The same group has investigated the effect of fentanyl on LPS-induced IL-6, IL-10 and TNF-α secretion in whole blood from 10 healthy volunteers (Wu et al., 2009). Whole blood was incubated for 6 hr with fentanyl (2, 20 and 200 ng/ml) in the absence or presence of LPS. IL-6, IL-10 and TNF-α concentrations were measured using ELISAs. The production of all three cytokines was increased by LPS. Fentanyl dose-dependently reduced the production of IL-6 (by up to 85%), IL-10 (by up to 85%) and TNF-α (by up to 40%) in LPS stimulated, but not unstimulated samples. There was variability of both control and opioid tests by up to ± 50% (Wu et al., 2009). By inhibiting both pro- and anti-inflammatory cytokines, the net effect of fentanyl might be neutral,

however the balance of cytokines in the inflammatory microenvironment is under careful control and such significant changes might induce immune dysregulation.

These two studies by the same group used whole blood (as opposed to PBMCs) from similar donors, a shorter incubation time (6 vs. 72 hr) and very high opioid concentrations which overlapped the highest concentrations used in the current study. Different stimuli (LPS vs. IL-2 or anti-CD3/anti-CD28 mAbs) and measurement assays (ELISA vs. CBA) were used. Both ELISAs and CBAs are comparable, provided the same clones of antibodies are used (Elshal and McCoy, 2006). As in the current study, unstimulated cytokine production was unaffected. However, in the current study only 20 ng/ml of fentanyl decreased IL-6 in IL-2 stimulated PBMCs, and IL-10 and TNF-α secretion was unaffected by either opioid. This might be due to the different stimulation method in whole blood, with LPS binding to TLR4 on macrophages and the release of pro-inflammatory cytokines being inhibited by morphine and fentanyl (Hutchinson et al., 2010).

Bonnet and colleagues incubated PBMCs from 11 healthy volunteers with 10^{-5} M of morphine (equivalent to 2854 ng/ml) for 3 hr, before culture with peptidoglycan for 2 hr. TNF and IL-6 were measured using ELISAs (Bonnet et al., 2008). Morphine inhibited the production of TNF (by 55%), but not IL-6. The MOR was not involved in this morphine-induced TNF inhibition in PBMCs as it was not blocked by CTOP, a MOR antagonist (Bonnet et al., 2008).

Bonnet and colleagues used the same cells that were used in the current study, but with a shorter incubation time and a higher concentration of morphine. They also used different stimulation (peptidoglycan vs. IL-2 or anti-CD3/anti-CD28 mAbs) for a shorter duration (2 vs. 72 hr). As in the current study, they showed that morphine did not inhibit the production of IL-6. However, TNF production was not inhibited in the current study, which may be due to the different stimulation, the duration of incubation or the concentration of morphine used.

Peterson and colleagues stimulated PBMCs isolated from six healthy male volunteers with Con A for 72 hr with or without 10^{-8} M of morphine (≈ 3 ng/ml) and measured IFN-γ secretion using a radioimmunoassay. Morphine slightly (by 14%), but significantly decreased IFN-γ production (Peterson et al., 1989). In this similar experiment to the current study, Peterson and colleagues incubated PBMCs for the same duration as the current study but with a lower concentration of morphine (3 vs 20 ng/ml) and stimulated

the PBMCs with Con A (as opposed to IL-2 or anti-CD3/anti-CD28 mAbs). IFN-γ was significantly decreased in this study, although the effect size was small. It was statistically unaffected in the current study, although numerically it was increased several fold by morphine in the anti-CD3/anti-CD28 mAb stimulated cultures. This might be due to the different morphine concentration or stimulation.

Nair and colleagues incubated PBMCs isolated from healthy donors with 10^{-15} – 10^{-5} M of morphine (equivalent to $3x10^{-7}$ – 2,853 ng/ml) for 4 hr (Nair et al., 1997). Cells were then infected with Sendai virus and 24 hr later IFN-α and IFN-β levels were measured spectrophotometrically. Morphine inhibited production of IFN-α (by up to 93%) and IFN-β (by up to 95%) by PBMCs. IFN-α inhibition was maximal at intermediate doses of morphine (10^{-7} – 10^{-11} M; equivalent to 0.003 – 29 ng/ml) (Nair et al., 1997).

Although this study used the same cells and a range of morphine concentrations which overlapped with the current study, the incubation period and mode of stimulation (Sendai virus infection vs. IL-2 or anti-CD3/anti-CD28 mAbs) differed. Interestingly, in this study intermediate concentrations of morphine maximally inhibited IFN-α. In the current study IFN-α was below the detection threshold and IFN-β was not assessed.

Chao and colleagues incubated PBMCs isolated from eight male and 12 female healthy volunteers with 0.1 fM – 10 nM of morphine (equivalent to $3x10^{-5}$ – 3 ng/ml) for 24 hr prior to the addition of LPS or PHA for a further 24 hr (Chao et al., 1993). TNF was measured by a cytotoxicity assay using L929 mouse cells. Morphine had no effect on TNF production from unstimulated PBMCs. Although there was much interindividual variability, morphine reduced TNF production with LPS (by up to 30%) and PHA (by up to 50%), but only the inhibitory effect of morphine to the PHA stimulation was reversed by naloxone (Chao et al., 1993), indicating a non-opioid receptor mediated effect from morphine in LPS stimulated PBMCs. Although not explored in this study it could possibly involve TLRs (Hutchinson et al., 2010).

In this experiment the same cells were used as in current study, but were pre-incubated for a shorter duration with lower concentration of morphine (their maximum was about one tenth that used in the current study) before being stimulated with LPS or PHA (as opposed to IL-2 or anti-CD3/anti-CD28 mAbs). TNF was measured using a cytotoxicity assay rather than an ELISA. Morphine decreased TNF production by stimulated PBMCs in this study, whereas it was unaffected in the current study. This

could be due to the stimulation used, the concentration of morphine or the duration of stimulation. In the current study, pre-incubation with opioids was also assessed, however this did not influence the effect of morphine on TNF-α.

3.4.3 Conclusion

The current study analysed how a range of cytokines produced by unstimulated and stimulated (both IL-2 and anti-CD3/28 for 3 days) PBMCs from healthy volunteers was influenced by eight opioids at four concentrations. Although there were individual differences, with some individuals being sensitive to the effects of some opioids, the differences noted were more dependent on the individual than the opioid or cytokine analysed. There were several statistically significant differences in some of the cytokines to some opioids (see section 3.4); however, the only consistent trends were that methadone, oxycodone and diamorphine all significantly decreased IL-6 levels at several concentrations, which might impact on the acute phase response to infection and trauma.

CHAPTER 4

DISCUSSION

4.1 Introduction

A range of opioid analgesics are used for the management of patients with moderate to severe pain in clinical practice. These opioids exert actions on most organ systems and can elicit a range of effects and side-effects. The effects of opioids on the immune system might be especially important in patients with cancer, infection and those undergoing surgery. Morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine are some of the most commonly used opioids. Although all of these interact with opioid receptors, they have very different physicochemical and pharmacodynamic properties which might explain their different clinical effects and potentially different immunoregulatory properties. They can also have effects via different non-opioid receptors. Furthermore, some opioids might have direct effects on immune responsiveness, as morphine, fentanyl, buprenorphine, methadone and oxycodone are also TLR4 agonists (Hutchinson et al., 2010).

The key properties of these opioids are:

- Morphine is a naturally-occurring alkaloid, moderately potent hydrophilic MOR agonist
- Tramadol is a synthetic, relatively weak MOR agonist (with 1/6000th the affinity of morphine), with noradrenergic and serotinergic re-uptake inhibitory actions
- Fentanyl is a very potent (over 100x the potency of morphine) synthetic, lipophilic MOR agonist
- Buprenorphine is a synthetic, potent MOR partial agonist, a partial KOR antagonist, a DOR antagonist and an ORL1 agonist, without effect on the SNS and HPA axis
- Methadone is a synthetic, moderately potent, lipophilic MOR agonist, with NMDA antagonistic properties
- Oxycodone is a synthetic, moderately potent, hydrophilic KOR and MOR agonist
- Diamorphine is a synthetically derived lipophilic pro-drug of morphine, which may also have a specific MOR subtype to which it can bind
- Codeine is a naturally-occurring alkaloid, which is converted to morphine to have its principal effects

Although studies have previously shown that these opioids have differential effects on the immune system, these effects depend on the model being used. Furthermore, none of these effects have been directly compared and investigated in a systematic manner.

Studies investigating the mechanisms which underlie the immunomodulatory actions of opioids suggest that these are manifested via direct effects on immune cell opioid receptors (Wang et al., 2002, Wu et al., 2009) and non-opioid receptors (Roy et al., 1998a, Roy et al., 1998b) as well as via indirect actions that are mediated by the CNS (Weber and Pert, 1989). The centrally mediated effects depend on the duration of opioid use. In the short term, these depend on the SNS and the systemic release of immunosuppressive amines, whereas in the longer term they involve the production of glucocorticoids which are induced by the action of opioids on the HPA axis (Mellon and Bayer, 1998).

The overall purpose of this PhD programme was to systematically analyse the *in vitro* (direct) effects of commonly used opioids on the aspects of innate and adaptive immunity that are involved in anti-tumour immune surveillance and protective immunity against viral and bacterial infection. This is the first study to systematically compare a range of commonly used opioids at clinically relevant concentrations across a range of innate and adaptive immune tests.

Although this is an *in vitro* study, the aim was to undertake experiments that were as relevant as possible to the clinical setting. The concentrations of the opioids used throughout the study are representative of those detected in patient groups. Fresh human blood was used, albeit from healthy volunteers and not patients. The analysis of phagocytosis and oxidative burst responses was undertaken using a whole blood approach and phagocytosis was triggered using *E.coli* bacteria. The analysis of the oxidative burst used three stimuli, including *E.coli*. Although platelet-free PBMC preparations were used in order to remove the inhibitory effect of platelet activating factor on the NK cell assay, further isolation of NK or T cell populations were not performed in order to allow interactions between the different mononuclear cell populations. T cells were activated physiologically via the TCR and co-receptor signalling using anti-CD3/anti-CD28 mAbs.

Lymphocytes were analysed using multiparameter flow cytometric analysis in order to enable specific activation markers on defined NK cells and T cell subsets to be evaluated without the need to isolate individual sub-populations. Older studies often used tritiated thymidine radioisotope incorporation to detect DNA synthesis, which does not allow the responsiveness of individual cell subsets in a more physiologically-relevant mixed cell population to be monitored. It has been demonstrated that there is a good correlation between measurements of proliferation that are made using

radionuclide incorporation assays and the measurement of activation on the basis of CD69 expression, with the latter being more sensitive (Simms and Ellis, 1996).

A major difference from the clinical situation in which opioids are prescribed was that this study used blood from healthy volunteers and it might be that the immune status of patients with cancer, infection or inflammatory conditions might influence the responsiveness to opioid mediated effects. Furthermore, opioids were not metabolised as they would be *in vivo* and the effect of metabolites could not be evaluated with this experimental regimen. Likewise, any potential centrally mediated actions via the SNS or HPA axis could not be assessed by these *in vitro* assays. Notwithstanding these limitations, the experimental approach adopted tests the hypothesis that opioids have direct, immunoregulatory effects on innate and adaptive immune responses.

4.2 Natural killer cell cytotoxicity

NK cells, which are part of the innate immune system, are important in immunosurveillance and control of tumour development and viral infections. They constitute about 10 – 15% of the PBMC fraction of whole blood. This study assessed the effects of opioids on the activation status (CD69 and CD25 expression) and cytotoxic capacity of unstimulated and IL-2 stimulated NK cells. The effects of opioids on the activation status of NK cells in anti-CD3/anti-CD28 mAbs stimulated cultures were also determined.

None of the opioids at the concentrations tested had any direct effect on the activation status or cytotoxic capacity of unstimulated and stimulated human NK cells in the PBMC preparations. There was variability between individuals, with occasional effects from some opioids on some subjects (section 3.1.6) which most likely resulted from phenotypic differences rather experimental variability, as the assay which was used is standardised and the activity of NK cells is known to vary between individuals, even when directed against the NK cell sensitive K562 cells (Hopkinson et al., 2007).

Although results from *in vivo* studies in animal, healthy human and patient groups are conflicting, in general the published literature reports that morphine (Weber et al., 2006, Yeager et al., 1995), fentanyl (Martucci et al., 2004, Shavit et al., 2004), methadone (Van Der Laan et al., 1996) and diamorphine (Novick et al., 1989) all decrease NK cell cytotoxicity. Buprenorphine does not affect NK cell cytotoxicity (Martucci et al., 2004) and tramadol enhances it (Gaspani et al., 2002, Sacerdote et al., 2000). Exceptions are

that acute fentanyl in humans increases NK cell numbers and cytotoxicity (Jacobs et al., 1999, Yeager et al., 2002), low-dose morphine stimulates NK cell cytotoxicity in pigs (Borman et al., 2009) and morphine selectively stimulates peritoneal and pulmonary NK cells in rats (Van Der Laan et al., 1996). Interestingly, in a clinical study, morphine has been reported to have no effect on NK cell function (Sacerdote et al., 2000). This change in NK cell cytotoxicity has been demonstrated to correlate with tumour growth and metastasis in animal models (Gaspani et al., 2002, Shavit et al., 2004). Although this might also hold true for patients, the magnitude of change in NK cell cytotoxicity which is needed to produce a clinically relevant effect is unknown.

There is no clear clinical correlation between NK cell cytotoxicity and clinical outcome to cancer or infection, it is also unclear what change in NK cell cytotoxicity could be considered clinically significant. It is therefore difficult to definitively attribute a decrease in NK cell cytotoxicity with poorer outcomes in patients with cancer. However, animal studies have shown there to be a relationship between NK cell cytotoxicity and cancer, in that Gaspani and colleagues showed that tramadol prevents the effect of surgery on NK cell cytotoxicity and reduces metastasis in rats (Gaspani et al., 2002). Furthermore, Shavit and colleagues have shown that fentanyl decreases NK cell cytotoxicity and increases cancer burden in rats (Shavit et al., 2004). NK cell activity is also considered to be a critical endpoint in the immunotoxicological evaluation of pharmaceuticals (European Medicines Evaluation Agency. Note for Guidance on Repeated Dose Toxicity. CPMP/SWP/1042/99. EMEA: London, 2000) and a Consensus Statement proposes that the effects of opioids on immune function should be considered, recommending buprenorphine over morphine and fentanyl (Pergolizzi et al., 2008).

The results of the current study generally correlate with the previous *in vitro* studies, but conflict with reported effects of opioids *in vivo*. One of the major limitations of the current study is that in whole animal and human studies, the effects of opioids may be mediated indirectly by their action on the CNS and release of immunomodulatory mediators. From this study, the effects of clinically-relevant concentrations of opioids on NK cells are not being mediated by direct effects on these cells. Thus the effects opioids have *in vivo* are more likely to be due to a systemic action (via the SNS or HPA axis) and an indirect effect on NK cell cytotoxicity. This would have occurred in the volunteer studies in which fentanyl increased NK cell numbers and cytotoxicity (Jacobs et al., 1999, Yeager et al., 2002), in the animal studies in which morphine inhibited NK cell cytotoxicity (Weber et al., 2006) and in animal and clinical studies in which tramadol enhanced NK cell cytotoxicity, (Gaspani et al., 2002, Sacerdote et al., 2000).

It is likely that the immune enhancing effects of tramadol are predominant *in vivo*, as tramadol increases serotonin levels by inhibiting its reuptake into cells, having most of its effect in the CNS. To have a direct effect on the immune cells *in vitro* serotonin would need to be released in the cell culture and its re-uptake inhibited. Although serotonin might be produced by immune cells, it is likely to be more relevant as a neuro-immune modulator *in vivo* (Mossner and Lesch, 1998).

It is important for future work to systematically analyse a range of opioids in *in vivo* models. It would be preferable if these studies were performed in defined patient groups, as this would enable evaluation of the indirect, as well as the direct, effects of opioids on immune cells, which have been physiologically activated *in vivo* by disease. The findings of such studies would be more readily applicable to patients than further *in vitro* and animal studies.

4.3 Neutrophil and monocyte phagocytosis

Neutrophils and monocytes have a central role in the control of bacterial and fungal infections and the removal of foreign particles. After being brought into proximity with the pathogen/particle by chemotaxis, they then engulf this material and move it into a phagosome for subsequent degradation, a process called phagocytosis. As phagocytosis is critical for a number of immunological processes, the effects of opioids on neutrophil and monocyte phagocytosis were assessed using concentrations of opioids that are consistent with those that are found in patients' plasma and a physiological approach was used for measuring phagocytosis.

Although initial experiments (n=3) showed that morphine, tramadol and fentanyl inhibited the ability of neutrophils and monocytes to phagocytose *E.coli*, this statistical significance was negated when subsequent volunteers were added (n=5). This prompted the analysis of individual responses to opioids which showed that there is marked variation in the sensitivity of phagocytic responses to morphine, tramadol, fentanyl and buprenorphine between individual subjects. For these potentially suppressive opioids, the interindividual variability was more dependent on the individual tested rather than the opioid to which their blood had been exposed.

Overall, although no statistically significant effects were observed, results from the current study suggest that morphine, tramadol, fentanyl and buprenorphine had the

capacity to inhibit neutrophil and monocyte phagocytosis in some individuals. The apparent ability of tramadol to suppress phagocytosis in some individuals has not been reported in other studies. It is possible that these opioids could negatively impact on the outcome of some patients to bacterial and fungal infections, although this hypothesis would need confirmation in clinical studies. Methadone, oxycodone, diamorphine and codeine had no consistent effect on phagocytosis in any of the subjects tested.

In vivo, morphine has been demonstrated to suppress murine and rabbit phagocytosis (Rojavin et al., 1993, Shirzad et al., 2009, Tubaro et al., 1987), although acutely it may initially be stimulatory (Pacifici et al., 1994). In rhesus monkeys neutrophil phagocytosis was not affected by morphine (Liu et al., 1992). In intravenous drug users morphine reduced phagocytic activity (Tubaro et al., 1985). The majority of previous *in vitro* studies have shown morphine to be inhibitory (Beilin et al., 2005, Sowa et al., 1997, Szabo et al., 1993), although other studies have shown either no effect (Liu et al., 1992) or stimulation of phagocytosis by morphine (Lee et al., 1995b, Peterson et al., 1995). *In vitro* tramadol had no effect on healthy volunteer phagocytosis (Beilin et al., 2005), but *in vivo* enhanced it in mice (Shirzad et al., 2009). Fentanyl has not been assessed in this way before, however it has been reported not to affect phagocytosis in a healthy volunteer study (Yeager et al., 2002). Methadone has been reported to have no effect on phagocytosis in *in vitro* and *in vivo* animal models (Molitor et al., 1992, Pacifici et al., 1994), but did reduce it at intermediate concentrations in hMDMs (Delgado-Velez et al., 2008). Chronic methadone suppressed murine and rabbit phagocytosis (Tubaro et al., 1987). In rehabilitated intravenous heroin users who were maintained on methadone, there was no effect on phagocytosis (Tubaro et al., 1985). Oxycodone, diamorphine and codeine have not previously been studied in this context.

From the current study, future research should initially focus on exploring the prevalence of sensitive subjects to morphine, tramadol, fentanyl and buprenorphine. Clinical studies should focus on the impact of these four opioids on clinical outcome. If in larger studies, these opioids were proven to be inhibitory, further laboratory research could try and elucidate the mechanism of action, including studies into phagocyte activation, such as effects on CD64 (Fc gamma receptor, which mediates phagocytosis of opsonised particles) and CD66 (neutrophil activation marker) cell surface expression and the intracellular signalling events that follow cell surface ligation events.

4.4 Neutrophil and monocyte oxidative burst reaction

Following phagocytosis, neutrophils and monocytes generate toxic oxygen free radicals as part of the oxidative burst reaction which is aimed at killing phagocytosed organisms. This too is an important element of anti-bacterial and anti-fungal defence and the influence of opioids on this reaction was evaluated. The oxidative burst reaction was stimulated with fMLP (a synthetic, chemotactic peptide which mimics the activity of bacterially-derived peptides) which is a mild stimulus, PMA (an activator of PKC) which is a potent stimulus and *E.coli* which is a physiological stimulus of moderate potency. fMLP did not induce many of the neutrophils or monocytes to undergo the oxidative burst reaction, and of those cells that did, it was at a very low level. This weak stimulus was included in the study as it might have enabled opioid-mediated stimulatory effects to be identified. However, no stimulatory effects were detected.

For the neutrophils, the intensity of the oxidative burst response was dramatically increased by the PMA stimulus, however no measurable opioid-mediated effects were observed. PMA, by activating PKC, might have been too potent a stimulus for the opioids to overcome. Opioids also exert some of their intracellular effects by inhibiting PKC, although they are likely to be far less potent than PMA. *E.coli* induced over 80% of the neutrophils to undergo the oxidative burst reaction, and this was at an intermediate intensity, as defined by the MFI. This was significantly inhibited by 500 ng/ml of morphine and 20 ng/ml of methadone, and stimulated by 16 ng/ml of codeine. However, there were no consistent effects across the range of opioid concentrations that were used. For the monocytes, about 50% of cells underwent the oxidative burst reaction in response to PMA and *E.coli*, although the intensity of this reaction was low for all stimuli. Although some of the inhibitory effects of morphine, tramadol, fentanyl, buprenorphine, oxycodone and methadone on the oxidative burst response induced by *E.coli* were statistically significant, there were no consistent effects across the range of concentrations tested and the statistical power of some of the effects was quantitatively low.

There were marked differences between individuals, both in their baseline oxidative burst response and the effect of certain opioids on the oxidative burst reaction. Although not statistically significant, it is possible that, morphine, tramadol, fentanyl, buprenorphine and methadone might have an effect on the oxidative burst response for certain individuals. Although this was mostly inhibitory, morphine and tramadol marginally increased the proportion and intensity of neutrophils and monocytes

undergoing the oxidative burst reaction in two of the subjects. The response of an individual to these potentially immunologically active opioids, depended more on the individual than the opioid, as each individual tended to respond in a similar way to these five opioids. None of the subjects had any effect from oxycodone, diamorphine and codeine.

Previous *in vitro* studies have shown that low concentrations of morphine inhibited the oxidative burst reaction and killing by neutrophils and monocytes (Liu et al., 1992, Peterson et al., 1987). *In vivo* morphine inhibited murine and rabbit oxidative burst activity (Tubaro et al., 1987), and the killing capacity of monkey neutrophils (Liu et al., 1992). Acute administration of morphine has been shown to stimulate the killing capacity of murine neutrophils, although it was inhibited at 24 hr (Pacifici et al., 1994). In opioid addicts, morphine reduced the ability of neutrophils and monocytes to kill, whereas superoxide anion production is only decreased in neutrophils (Tubaro et al., 1985). Fentanyl did not affect neutrophil oxidative burst responses in healthy volunteers (Jacobs et al., 1999, Jaeger et al., 1998). Both *in vitro* swine, and *in vivo* murine and rabbit studies have reported methadone to suppress the oxidative burst reaction (Molitor et al., 1992, Tubaro et al., 1987), although this was unaffected in another murine study (Pacifici et al., 1994). In intravenous drug users, methadone reduced the ability of neutrophils and increased the capacity of monocytes to kill, whereas superoxide anion production was decreased only in neutrophils, but to a lesser degree than with morphine (Tubaro et al., 1985).

In summary, none of the opioids tested in the current study had a consistent statistically significant effect on the oxidative burst reaction of neutrophils and monocytes when they were stimulated with fMLP, PMA or *E.coli*. However, when *E.coli* was used as the stimulus, morphine, tramadol, fentanyl, buprenorphine and methadone were inhibitory for some individuals. If these results were to hold true in *in vivo* studies, then these data suggest that opioid choice might influence oxidative burst capacity and this could impact on an individual's susceptibility to bacterial and fungal infections. However, this hypothesis would need confirmation in clinical studies, which should focus on these five opioids and evaluate the prevalence of their immune effects and how they impact on clinical endpoints as well as their mechanism of inhibition, possibly via TLRs.

4.5 T cell activation

T cells are part of the regulatory arm ($CD4^+$ cells) and cytotoxic arm ($CD8^+$ cells) of the adaptive immune response. $CD4^+$ cells are critical in ensuring that the rest of the immune system produces an appropriate response to pathogens without causing inappropriate damage to host tissue. $CD8^+$ T cells form part of this effector response. The activation of both of these T cell types is critical in the maintenance of an intact immune system and interference with this could be detrimental to the host.

The effect of opioids on the activation status of T cells was evaluated in several experimental regimens. Isolated PBMCs were incubated with Miltenyi anti-CD3/anti-CD28 mAb-coated beads, added before, at the same time as or after the opioids, and T cell activation status was evaluated by measuring CD69 and CD25 expression, determined by flow cytometry. There was good activation of both $CD4^+$ and $CD8^+$ T cells by the beads but none of the opioids had a consistent effect on their activation.

Previous *in vitro* studies are sparse, but despite using very different methodologies, they generally agree with the current study, in that T cell-mediated cytotoxicity of murine spleen cells is unaffected by clinically relevant concentrations of morphine, methadone and diamorphine (House et al., 1995, Thomas et al., 1995a, Thomas et al., 1995b). However, in one study morphine inhibited the proliferation of murine lymph node T cells (Wang et al., 2001). There have not been any studies using human cells.

The *in vivo* effects of morphine, tramadol, fentanyl, methadone and diamorphine on T cell function have also been assessed. In mice, morphine caused atrophy of the spleen and thymus, decreased mitogen-stimulated T cell proliferation and reduced the thymic $CD4^+$:$CD8^+$ cell ratio (Bryant et al., 1987, Bryant et al., 1988, Fecho et al., 1996, Gaveriaux-Ruff et al., 1998, Singhal et al., 1998, Weber et al., 2006). Morphine has also decreased splenic and peritoneal cytotoxic T cell activity (Carpenter et al., 1995, Carpenter et al., 1994). In rats, morphine has been shown to suppress mitogen-stimulated splenic T cell proliferation, (West et al., 1997). In healthy volunteers, fentanyl increased in the percentage of $CD8^+$ cells in the circulation in one study (Yeager et al., 2002), but not in another (Jacobs et al., 1999). One drug addiction study reported that diamorphine increased lymphocyte numbers and that methadone had no effect (Novick et al., 1989). In a surgical study, morphine suppressed T cell proliferation more than tramadol (Sacerdote et al., 2000).

There have not been enough previous *in vitro* or *in vivo* studies into the effects of opioids on T cells to draw any definitive conclusions. However, the current study did not detect any direct effects of opioids on T cells and it is likely that the effects detected *in vivo* are by indirect mechanisms, via central pathways, such as the SNS and HPA axis. This is in contrast to the detection of opioid receptors on activated T cells (Kraus, 2009), although there is some debate as to the presence of these receptors on T cells (Williams et al., 2007) and the presence and function of opioid receptors can be difficult to determine in the immune system as they are in much lower numbers than in the CNS (Borner et al., 2007), reviewed in section 1.6. Comparative clinical studies are essential in order to establish if opioids influence T cell function and if so, the relative impact of each opioid. These would ideally include surgical and substance abuse groups, as well as patients with cancer and infection.

4.6 Cytokine production

Cytokines are cell signalling molecules which are used, amongst other functions, to regulate immune function. They are secreted by a wide range of cells types and their concentration can increase by several orders of magnitude during immune system activation. In the current study, the effect of clinically relevant concentrations of the opioids was assessed on the production of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN-α, IFN-γ, TNF-α and IP-10 in unstimulated, IL-2 stimulated and Miltenyi anti-CD3/anti-CD28 mAb coated bead stimulated PBMCs, using BD Bioscience CBAs and a BD FACSArray™ flow cytometer.

The current study revealed several statistically significant effects from opioids on cytokine production, but the only consistent effect was that several concentrations of methadone, oxycodone and diamorphine significantly decreased IL-6 concentration. Although IL-6 is often considered a pro-inflammatory cytokine and its levels are correlated with the acute phase reactant, CRP (Heikkila et al., 2007), it can also have anti-inflammatory effects (Xing et al., 1998). This makes it difficult to predict what the net effect of these opioids would be. They are however likely to reduce the acute phase inflammatory response and might therefore impair the immune response to infection. As IL-6 also influences lipid (Pedersen, 2011) and bone metabolism (Eriksen et al., 2010), any inhibition of its production could have downstream effects on several organ systems.

In the current study, there were differences between individuals in cytokine production, both at baseline and to the opioids. However, the differences in response reflected more on the individual than on the opioid tested, with some individuals being sensitive (either stimulated or inhibited) to a range of opioids and other being resistant. This is explored in the following section.

From the previous studies described earlier, morphine generally decreased the production of IL-1β, IL-2, IL-4, IL-6, IL-12 and IFN-γ in mice (Bhargava et al., 1994, Martucci et al., 2007, Roy et al., 1998a, Wang et al., 2002). Whereas they show mixed effects of morphine on IL-10 and TNF levels (Martucci et al., 2007, Wang et al., 2002). Fentanyl decreased IL-2 and IFN-γ production in mice, whereas buprenorphine had no effect (Martucci et al., 2004). Codeine increased the production of IL-8 and TNF-α in human derived mast cells (Sheen et al., 2007). Diamorphine addicts exhibit lower IL-4, IFN-γ and TNF-α levels, normal IL-2 levels and increased IL-10 levels (Azarang et al., 2007), these were normalised by methadone and buprenorphine treatment (Sacerdote et al., 2008). In post-operative studies, morphine has been shown to inhibit IL-2, whereas this was unaffected by fentanyl and either unaffected or increased by tramadol (Liu et al., 2006, Wang et al., 2005a).

From these heterogeneous studies in a variety of models, it appears that morphine has mixed effects on pro- and anti-inflammatory cytokines in mice, making it difficult to draw definitive conclusions about its overall effect. However, it did decrease IL-2 production following surgery (Liu et al., 2006) and this could potentially be clinically deleterious as IL-2 stimulates the proliferation, differentiation and survival of lymphocytes. Although the data on the other opioids are sparse, buprenorphine is likely to be immune neutral in terms of cytokine production and tramadol enhances IL-2 levels and could even have beneficial clinical effects. These sporadic clinical findings would need confirmation in long term patient studies.

It is important that the effect of these opioids are assessed on a range of cytokines in various clinical scenarios in which immune regulation is critical, as in patients with cancer, infection and autoimmune disease.

4.7 Individual variability

In the current study none of the opioids at the concentrations tested had a consistent statistically significant effect on immune function (apart from methadone, oxycodone and diamorphine inhibiting IL-6). There were however variable effects between individuals both in their baseline immune function and in their responses to the different opioids, this was more pronounced with the phagocytosis and oxidative burst assays. These findings concur with the clinical observations that the analgesic and toxic effects of opioids vary markedly between individuals (Campa et al., 2008, Oertel et al., 2006, Rakvag et al., 2005, Somogyi et al., 2007). This could arise from multiple single-nucleotide polymorphisms (SNPs), and the potential genetic and molecular basis underlying this is explored below.

The multifactorial nature of pain and its expression makes it difficult to quantify the genetic influences on the analgesic response which are reported by the patient. The total opioid doses that are needed for analgesia are often used in studies assessing the effect of genetic variations on pain. In the studies outlined below, total opioid doses are sometimes used as a surrogate for analgesic response of individuals. As these studies are assessing the effect of a limited number of SNPs, a major limitation is that the other variable genetic (and environmental) factors will induce further variability, also confounding this measurement.

In addition to patients exhibiting large variations in their response to opioids, it should be noted that these drugs also have a narrow therapeutic index (Somogyi et al., 2007). The pharmacokinetics and pharmacodynamics of opioids are regulated by genetic factors which contribute to this variability. There are many variants within the MOR gene, OPRM1, the most studied being the c.118A>G SNP. Healthy volunteers with one or two copies of the G allele experience ~33% of the analgesic effect of alfentanil to experimental pain and ~10% of the respiratory depressant effect of this opioid, compared to people with no copies of the G allele (Oertel et al., 2006).

The c.118A>G SNP was studied in patients with cancer on morphine. Four patients who had two copies of the G variant allele required a higher dose (mean ± SD; 225 ± 143 mg/day) compared to 17 patients with one copy of the variant (66 ± 50 mg/day) or 78 patients with no copy (97 ± 89 mg/day) (Klepstad et al., 2004). However, the number of homozygous variants was small and there was a large variability in dosages between patients.

In a study of the c.118A>G SNP in 175 patients with cancer pain being initiated on morphine, the G variant allele number had no effect of on the opioid dose. However, after one week patients with two copies of this allele experienced a smaller change in pain (mean 0.2 unit change on an 11 point scale), compared to those with one copy (1.8 unit change) and those with no copies (3.8 unit change) (Campa et al., 2008).

The c.118A>G SNP G variant allele occurs in 10 – 15% of Caucasians and almost 50% of Asians (Tan et al., 2009). In a study of post-operative pain in almost 1,000 women undergoing elective caesarean section under spinal anaesthesia from Singapore, the morphine dose (adjusted for weight) in the homozygous variant (GG) group was 10.9 μg/kg, almost twice that of the wild-type (AA) group, 5.8 μg/kg. Usage was intermediate (8.8 μg/kg) in the heterozygotes (AG) (Tan et al., 2009).

In a meta-analysis the c.118A>G SNP showed no consistent association with phenotype (Walter and Lotsch, 2009). However, the studies that were included originated from diverse patient groups, including chronic cancer and non-cancer pain, as well as post-operative and labour pain, often with small numbers of patients.

The COMT (catechol-O-methytransferase) enzyme system metabolises catecholamines (noradrenaline, adrenaline and dopamine). In animal models, COMT inhibitors are pronociceptive in acute and inflammatory pain (Kambur et al., 2010). The Val158Met polymorphism of the human COMT gene influences morphine requirements in cancer pain patients, with the Met form being less active (Rakvag et al., 2005, Rakvag et al., 2008). COMT knockout mice are more sensitive to nociceptive stimuli, whereas mice with a high COMT activity have a decreased sensitivity to pain (Kambur and Mannisto, 2010). Low COMT activity, associated with the met allele, increases the availability of opioid receptors and might enhance opioid analgesia in cancer pain (Kambur and Mannisto, 2010). In 207 patients with cancer, those with the Met-Met genotype required less morphine (mean ± SD; 95 ± 99 mg/day) than those with the Met-Val genotype (117 ± 100 mg/day). Those with the Val-Val genotype required the highest doses of morphine (155 ± 160 mg/day) (Rakvag et al., 2005).

P-glycoprotein is a member of the ATP-binding cassette (ABC) efflux transporters, which is in the blood-brain barrier and limits the entry of certain opioids (morphine, fentanyl and methadone) into the CNS (Mercer and Coop, 2010). In acute pain, a variant of the gene that encodes for p-glycoprotein (ABCB1) has been shown to cause more respiratory depression following a single intravenous dose of fentanyl (Park et al.,

2007). Haplotype (multiple SNP) analysis for ABCB1 has shown that dosage requirements of methadone in opioid dependence maintenance treatment were related to the number of copies of the variant haplotypes c.3435C>T and c.2677G>T. Patients with two wild-type alleles required twice the methadone dose compared to those with both variant alleles (Coller et al., 2006). In 175 patients with cancer pain starting on morphine, patients with two copies of the ABCB1 variant allele c.3435C>T experienced an increased analgesic effect with a decrease in pain score of 4.4 (on an 11 point scale), compared to those with one variant whose decrease in pain score was 3.2 and those with wild-type with a decrease in pain score of 2.3 (Campa et al., 2008).

The combination of variants in OPRM1 and ABCB1 can have opposite effects on the effect of opioids, including analgesia. Thus, the overall analgesic response in an individual will depend on which combination of these two genetic variants are present. Campa and colleagues showed that the best response to morphine in patients with cancer was in those with a combination of wild-type OPRM1 plus ABCB1 variant with a 4.8 unit change in pain score and the worst response was in those with a combination of OPRM1 variant and wild-type ABCB1 with a 1.3 unit change in pain score (Campa et al., 2008).

Opioids that are O-demethylated to more potent opioid metabolites by the polymorphic CYP2D6 enzyme, such as codeine to morphine, tramadol to O-desmethyltramadol and oxycodone to oxymorphone, have variable effects in subjects with mutations in the CYP2D6 gene (de Leon et al., 2003, Maddocks et al., 1996, Somogyi et al., 2007). These mutations may result in the poor metaboliser (PM) phenotype which comprises about 7% of the Caucasian population who do not convert the opioid to the more active form, or ultrarapid metabolisers (UM), comprising about 2% of Caucasians, having multiple copies of the CYP2D6 gene, who rapidly produce the more potent opioid. In UMs, more adverse effects have been reported due to increased conversion of codeine to morphine (Dalen et al., 1997). Adverse CNS reactions to oxycodone have been reported in patients with the UM phenotype (de Leon et al., 2003). CYP2D6 PMs administered codeine had a reduced analgesic response to experimental pain and less respiratory depression (Somogyi et al., 2007). In post-operative patients, there has been shown to be a higher number of non-responders to tramadol among PMs (81%) compared to extensive metabolisers (17%) (Stamer et al., 2007). In a small study with oxycodone, the patient who required the highest dose of oxycodone and the most breakthrough analgesia was the only PM (Maddocks et al., 1996).

GTP cyclohydrolase 1 (GCH1) is the rate-limiting enzyme in the synthesis of tetrahydrobiopterin, a cofactor in the biosynthesis of serotonin, noradrenaline and NO. In over 250 patients with cancer, a reduced-function GCH1 variant delayed the onset of pain and need for opioid therapy (Lotsch et al., 2010). In an observational study of 100 patients, associations between COMT, MOR and GCH1 genes and the development of pain 3 months after third molar extraction showed that seven patients experienced persistent pain. There were associations between SNPs in these genes, post-operative pain and effect of analgesia. In patients after surgery, the number of days until effective analgesia was associated with GCH1 SNPs (Lee et al., 2011).

In summary, people with the OPRM1 gene variant c.118A>G SNP have a reduced response, those with variants in the p-glycoprotein efflux transporter gene ABCB1 have an enhanced response and those with CYP2D6 gene mutations can have a variable response to certain opioids (Coller et al., 2009). Reduced function of GCH1 tends to reduce pain and opioid use and patients with the COMT SNP c.474G>A require less morphine. However, the clinical usefulness of pharmacogenetic testing for predicting analgesic response to opioids remains uncertain (Peiro et al., 2010).

Although just a few of the most common mutations have been covered here, there are numerous other genetic factors which can affect the analgesic and adverse effect profile of the different opioids used clinically (Droney et al., 2011, Kadiev et al., 2008, Ross et al., 2006). There is so much genetic variation that when studied together the effect of an individual SNP may be impossible to determine, especially as some of the variants increase response to a particular opioid and others reduce it. A recent study in over 2,000 cancer pain patients found no relationship between doses of opioids and 112 SNPs in 25 genes involved in the effect of opioids and pain. (Klepstad et al., 2011).

In addition to differences at the genetic level potentially having a vital role in individual responses to opioid effects, there is also evidence that interindividual variation in responses to opioids can occur at a molecular level due to differing receptor dimerisations (Chakrabarti et al., 2010). Dimerisation can be between opioid receptors of the same class (i.e. two MORs – homodimerisation) or between opioid receptors of different classes (i.e. MOR and KOR – heterodimerisation) and this changes the activation and response of these receptor compounds to opioids (Chakrabarti et al., 2010, Faras-Melainis et al., 2009, Rozenfeld and Devi, 2007).

Variability between subjects has been alluded to in previous immunological studies although it is rarely directly reported. Jacobs and colleagues assessed the *in vivo* effects of fentanyl on NK cells in volunteers. Fentanyl increased the number of circulating NK cells by more than 250% in four out of seven subjects. Naloxone was administered to these four volunteers, which blocked the increase in NK cells in only two of these subjects (Jacobs et al., 1999). A study assessing the effect of morphine on the release of TNF from PBMCs isolated from healthy volunteers has also alluded to variability between subjects. They showed that individuals differed in both their baseline TNF production (to LPS and PHA) and in the sensitivity of this to the suppressive effects of morphine (Chao et al., 1993). In a study assessing PHA and LPS stimulated IFN-γ and IL-10 production in whole blood from healthy controls and heroin (diamorphine) addicts, there was marked variability in the production of these cytokines in both groups (Azarang et al., 2007). In healthy volunteers, as well as in morphine and methadone treated drug addicts, there was large variation between individuals in their neutrophil and monocyte phagocytic and oxidative burst capacity (Tubaro et al., 1985). Yeager and colleagues investigated the *in vitro* effect of β-endorphin on NK cell cytotoxicity and showed that 26 of the 34 subjects had an enhancement of NK cell cytotoxicity, only these subjects were used for the subsequent analysis of the interaction of β-endorphin with morphine, in a type of enrichment methodology (Yeager et al., 1992). In rhesus monkeys, the *in vivo* inter- and intra-individual variability in neutrophil phagocytosis was up to 50% over the 30 week study. There was also a variable effect of morphine with some monkeys showing inhibition at some time points and others showing an increased phagocytic capacity, although this was not consistent over time (Liu et al., 1992).

In clinical studies of analgesics, an enrichment methodology is sometimes used to select those patients who respond to a drug and only putting these responders into the main drug trial (Gilron et al., 2011, Hale et al., 2010, Schwartz et al., 2011), illustrating the interindividual response to analgesics in various clinical settings.

In both animal and human studies, gender also affects the sensitivity to pain and the responsiveness to treatment, with differences in the effects of opioids between individuals (Andreassen et al., 2011, Mogil and Bailey, 2010, Paller et al., 2009). This may in part be due to sex hormones which have a complex role in inflammatory processes and the pain response (Manson, 2010), the types of opioid receptor present (Chakrabarti et al., 2010) and the effects of KOR agonists (Lawson et al., 2010).

Many animal studies use a single strain of a species, which is likely either to respond to opioids or not. This is certainly true in pain research, with different animal strains exhibiting a different response to opioids pain (Lariviere and Mogil, 2010). This is compounded, as the sensitivity to pain varies depending on the social context with social threat modulating pain behaviour in a sex-specific manner, even in mice (Langford et al., 2011). It is likely only those that have an effect from opioids will be published and further researched.

The effect of morphine on different types of pain is variable between mouse strains (Lacroix-Fralish and Mogil, 2009). This variability has also been reported for social investigation behaviour (social responsiveness and motivated behaviour), especially during adolescence (Kennedy et al., 2011). It may be that the different effects between species and even individual strains are due to alterations in the endogenous opioid systems. Studying a single inbred strain removes the variability which was seen in the current study and clinically, allowing for specific genetic variability to be assessed. However, it does not reflect the vast genetic differences that lead to different responses between patients.

In the current study there was variability between the different subjects, but as their immune function was only evaluated once, this will need verification. All the assays will have some intrinsic variability, as will the subjects, so confirmation of this potential effect is essential. Much of the variability did not follow a consistent pattern across different opioid concentrations and although this could represent a u or n shaped effect, at this stage it has to be considered that opioids have no overall net *in vitro* effect.

There was a trend for many of the immune function tests performed (i.e. phagocytosis and oxidative burst) to be influenced more by the individual than the opioid, leading to no clear effect between subjects and no statistical significance when the data were combined for analysis. Although diamorphine and codeine did have some small direct effect on immune cells, it is likely *in vivo* these will have the same effect as morphine. Methadone and oxycodone were generally immune neutral for most subjects and any effects they did have were quantitatively very small. If these results were replicated clinically, it might be that these would be immunologically safer options.

4.8 Effect of pain on immune function

From a clinical perspective, it is believed that pain itself is immunosuppressive (Page, 2003, Page et al., 2001) and that the reduction of pain might also attenuate an immunosuppressed state (Gaspani et al., 2002). In the case of potentially immunosuppressive opioids, there will undoubtedly be a complex balance between the immunosuppressive effect of the opioid and the reduction of putative immunosuppression of pain (Figure 4-1) (Page, 2005, Rittner et al., 2008). As cytokines not only influence immune activity, but also modulate pain signalling (Gordin, 2010, Hutchinson et al., 2008), and with immune cells producing endogenous morphine when activated (Glattard et al., 2010), there are likely to be complex tri-directional interactions between pain, analgesia (especially opioids) and the immune response.

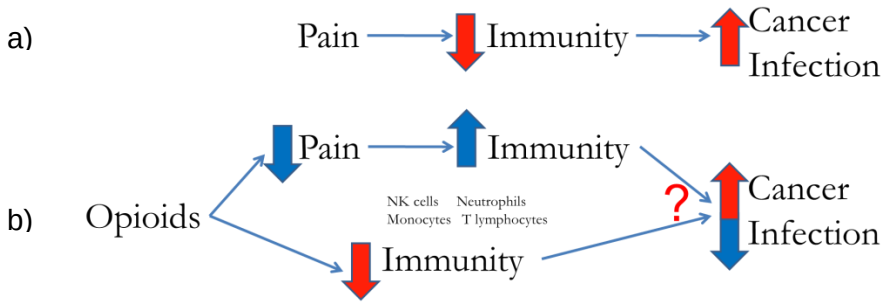

Figure 4-1: Complexity of the action of opioids on immunity and cancer.
a) Pain is immunosuppressive and may worsen outcome in cancer and infection (Page, 2003, Page et al., 2001). b) Opioids, by reducing pain, might have a beneficial effect on immune function, cancer and infection (Gaspani et al., 2002, Page, 2005). Some opioids might have a suppressive effect on immune function; this may in turn decrease anti-tumour and anti-infective immunity and promote the development of cancer or infection (Shavit et al., 2004, Wang et al., 2008). The balance between the immune enhancing and suppressing effects of prescribed opioids is therefore complex (red arrows represent a detrimental effect and blue arrows a beneficial effect).

In a retrospective analysis of two clinical trials of patients with cancer cachexia, the relationship between cancer pain and systemic inflammation (measured by CRP) was evaluated. Pain (using the pain subscale of the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire C-30) was statistically correlated with CRP (Laird et al., 2011).

In male Wistar rats administered chronic morphine, spinal IL-1β, IL-6 and TNF-α gene expression was increased (Shen et al., 2011). These pro-inflammatory cytokines were normalised by TNF-α blockade with etanercept acting on microglia, as determined by spinal cord immunocytochemistry. Etanercept also reduced tolerance and restored the antinociceptive effect of morphine (Shen et al., 2011). Opioids, by acting on glial TLR4, can antagonise their own neuronal MOR-mediated analgesic effect and increase opioid

tolerance (Watkins et al., 2009). Glial TLR4 are also involved in neuropathic pain. Thus the beneficial effects of opioids (mediated via the opioid receptor) may have a different mechanism from some of the unwanted toxicities (TLR4 and glial mediated) and this leads to further potential interactions between opioids, pain and the immune system (Watkins et al., 2009).

Several retrospective clinical studies assessed the opioid-sparing effect of regional analgesia with general anaesthesia on the recurrence rate in the surgical removal of tumours. Exadaktylos and colleagues looked retrospectively at 129 medical records of patients that had undergone mastectomy for primary breast cancer and found lower cancer recurrence and metastasis at 3 years in patients receiving paravertebral analgesia with general anaesthesia compared to those that that had received general anaesthesia alone (Exadaktylos et al., 2006). Biki and colleagues showed that in 225 patients undergoing radical prostatectomy, general anaesthesia with epidural analgesia (as opposed to systemic opioids) was associated with a lower risk of biochemical cancer recurrence (prostate-specific antigen increase), over 3 – 13 years (Biki et al., 2008). In 177 patients undergoing colonic cancer resection, epidural analgesia with general anaesthesia increased survival rate in patients with non-metastatic, but not metastatic, disease (Christopherson et al., 2008). However, several other retrospective studies have not shown any recurrence or mortality benefits for regional analgesia in prostate, colorectal, and cervical cancers even though there was a reduced need for postoperative opioids (Gottschalk et al., 2010, Ismail et al., 2010, Tsui et al., 2010). A prospective multicentre randomised, controlled clinical trial of 503 patients undergoing abdominal surgery for cancer resection, compared the effect of general anaesthesia with either epidural analgesia or postoperative systemic opioids. This showed no difference between the two groups in cancer recurrence and mortality at 2 – 3 years (Myles et al., 2011).

Although there are many potential toxicities associated with opioids (outlined in Table 1.1) and each of the opioids can produce any of these toxicities, it is likely that certain opioids cause some toxicities more than others. We have some understanding of the relative risk of some opioids in certain patient groups for some of their toxicities, i.e. fentanyl is less constipating and less sedating that morphine, and there is a difference in response between patients with cancer pain and non-cancer pain (Clark et al., 2004). However, the relative adverse effect profile for each opioid for the whole range of toxicities still needs to be elucidated, this is particularly true for the immunological effects of the opioids.

There is likely to be a different risk:benefit ratio for the clinically used opioids for different patients. In the context of this study there will be patients who need an opioid analgesic and in whom immune suppression would be very detrimental (i.e. some patients with cancer or infection and pain). For these patients, potential immune effects from opioids could form a necessary part of the decision making process in analgesic prescribing.

4.9 Limitations of study

Although this is a comprehensive study of a range of opioids on several aspects of immunity, several key factors currently limit its impact on clinical practice. Despite using physiologically-relevant concentrations of opioids, this is an *in vitro* study using healthy volunteers which does not take into account the existing immune status of individual patients, the influence of drug metabolism or effects that involve the SNS and HPA axis. It is also unknown what *in vitro* effect size might correlate to a potential clinical effect.

With regards to the NK cell cytotoxicity assay, a potential limitation was that this was based on the killing of NK cell sensitive K562 cells, which were used as these cells are MHC class I negative and can be used as targets for NK cells in PBMC preparations. Although the use of appropriate solid tumour cell lines that have variable sensitivities to NK cells could have been used, some of these are MHC class I positive and therefore sensitive to $CD8^+$ T cells in the PBMC preparation. The use of such targets would require the isolation of NK cells from PBMCs. The removal of opioids from the PBMCs before incubation with the K562 cells (to ensure the opioid did not affect K562 cells) might also influence the NK cells by causing opioid withdrawal.

The T cell studies assessed the effect of opioids over several days and opioids might only transiently affect immune function by inducing receptor desensitisation and down regulation. Thus, the acute effect of opioids on immune cells could be very different from those applied over days (Bidlack et al., 2006). However, opioids are often prescribed to patients over days or often much longer and if immune tolerance occurs to opioids, then this is less likely to be a clinically significant problem in patients on regular opioids. It may, however, be problematic to patients given short term opioids, for trauma or surgery.

Another potential limitation is that activation markers were used to assess T cell activation rather than using functional tests. However, a profile of cytokines was also analysed to give a balanced picture of T cell activation; additionally previous studies have correlated activation marker expression with T cell proliferation (Maino et al., 1995, Mardiney et al., 1996, Simms and Ellis, 1996). Although using activation marker expression with multiparameter flow cytometric analysis did not give a direct measure of cell proliferation, it allowed the activation of lymphocyte subsets to be analysed simultaneously.

Although the majority of previous *in vivo* studies show that opioids have an effect on some aspects of immune function, many *in vitro* studies show no effect from opioids at clinically relevant concentrations, and often those that show an effect used inbred animals. It is not possible to draw clear, statistically supported, conclusions between the opioids in the current study due to variability between the subjects being used in the study. The effect of all of the opioids on the range of immune assays could be assessed in larger numbers of subjects, but they are all likely to be variable and thus the clinical relevance of this would not inform decision making for any individual patient.

A key finding of this study is the differential sensitivities of the subjects to the modulatory effects of the opioids. However a major limitation with this is that each subject has only been tested once, with the results from several subjects being pooled for the analysis. As individual differences were potentially detected, confirmation of this effect over time in the same individuals would be needed.

Being a small study, this could have led to the analysis being underpowered and caution is needed as to not over-interpret the results. However, retrospective power and sample size calculations have been performed for those assays for which opioids potentially showed an effect and some of these calculations have a power greater than 80% and a sample size compatible with that performed. Other experiments, which did not seem to show an effect of opioids, are very poorly powered and would need many samples to confidently show significance or non-significance. However, the need for very large sample sizes draw into doubt the clinical relevance of any observed effect at the patient level.

4.10 Potential impact of this study

Currently, opioids are primarily prescribed on the basis of their overtly clinically recognisable toxicities (i.e. sedation, constipation, nausea and vomiting) and the preferences of the clinical team, which may be based on familiarity and cost. The findings that opioids differentially influence neutrophil and monocyte phagocytosis and oxidative burst and IL-6 production for some subjects, means that if these *in vitro* results are replicated *in vivo*, then opioid choice would have to take into account the immune status and risk for certain patients, including those with bacterial infection.

If opioids do indeed affect immune function and this in turn influences infection or cancer progression, then the prescription of opioids should be based on a better understanding of their immunobiological properties. Although the clinical impact of opioids on immune function cannot yet be quantified (Manchikanti et al., 2011), clinical guidelines now include a consideration for opioid mediated immune suppression (Pergolizzi et al., 2008).

In the previous literature, there have been many differences in the methodologies used between the studies, including: incubation times and concentrations of opioids; opioid tolerance/withdrawal; peripheral blood vs. splenic/peritoneal cells; isolation of specific cells vs. PBMCs vs. whole blood; the cell isolation method; baseline activation status; stimulation methods at variable concentrations and durations; animals/species; male vs. female. Furthermore, there are variances in methodology for each assay type, i.e. for NK cell cytotoxicity differences between previous studies include: E:T ratios; total cell numbers; effector cells; target cells; incubation times and methods of measuring NK cell cytotoxicity. Unfortunately, not all studies mention all the relevant variables in their methodology which makes it even more difficult to compare and extrapolate their results.

This is the first study to systematically assess the *in vitro* effect of a range of opioids on both innate and adaptive immunity using standardised assays, and is the first to have evaluated the immune effects of certain opioids, such as oxycodone. It has highlighted that morphine, tramadol, fentanyl and buprenorphine could be more likely to inhibit phagocytosis in some individuals; and that morphine, tramadol, fentanyl, buprenorphine and methadone could inhibit the oxidative burst reaction of neutrophils and monocytes in some individuals (statistically oxycodone was also inhibitory but this was quantitatively very small). It also demonstrated that methadone, oxycodone and diamorphine inhibited IL-6 production in IL-2 stimulated PBMC cultures, which could

compromise the acute phase response to infection. This study has also reinforced the need to systematically analyse a range of opioids in *in vivo* models as it is likely that the indirect effects of opioids are essential to evaluate, along with their direct effects.

One advantage of studying opioids *in vitro* is that it reduces many of the influences relating to the enzymatic metabolism of drugs (e.g. codeine by CYP2D6) and the transport of drugs (e.g. fentanyl by p-glycoprotein) which cause interindividual variability. Other factors such as renal function (relevant in the elimination of many opioids), liver function (important for the elimination of oxycodone) and interactions with other drugs (such as those that induce or inhibit the cytochrome P450 enzymes) are eliminated *in vitro*. Although this means that *in vitro* data may not be directly applicable to patients, it does enable a more focused assessment of specific elements of a biological effect and provides insight into the comparative influence of the opioids.

This systematic *in vitro* study, using cells from healthy humans, provides a "clean" baseline from which future clinical studies can be compared. In patient studies, there are many potential modulations of the immune system caused by different diseases, presence of infection and effects of treatment (i.e. chemotherapy).

In previous studies variability has been alluded to, but is rarely reported, even though there are large error bars, even when SEM is being reported (Azarang et al., 2007, Beilin et al., 2005, Chao et al., 1993, Jacobs et al., 1999, Tubaro et al., 1985). Many of the animal studies used inbred animals, which are genetically similar, leading to less variability than would be found in a heterogeneous human population. Many previous studies also only examined one or sometimes two opioids and thus it would be difficult to know if the effect of the opioid studied was a group effect (which would occur with all opioids) or an effect from that specific opioid.

The main finding of this study is the disparate effects that certain opioids can have at a cellular level on immune function, especially phagocytosis, oxidative burst and IL-6 production at an individual level. This means that some patients might be put at risk if they are administered immune suppressing opioids during a time when they are susceptible to infection. As this effect might only be relevant for an as yet undetermined proportion of patients, it would be difficult to detect them clinically. If future clinical studies were to confirm this effect, it may be possible to detect these patients prior to the administration of opioids, and if patients were at risk of immune suppression and needed an opioid, it might be that oxycodone would be a safer option.

From this study it is possible to hypothesis that administering opioids to human subjects could lead to three possible effects on their immune function: a) adversely affect aspects of their immune function; b) improve immune function; c) no effect of opioids on their immune function. Larger studies will be needed in order to evaluate the relative proportion of people in each group and to potentially evaluate the risk to the whole population. However, if the results from the whole population are combined they may show no difference due to the differences between the groups. It is also currently not known what *in vitro* effect size would be of clinical significance.

Furthermore, it seems likely that as there are individual variations in the direction and magnitude of the effect of opioids on aspects of the immune response, that randomised controlled trials might hide the variations in group results. Thus, other methodologies (i.e. phenotypic screening) may be needed to study these effects.

4.11 Future Work

If opioids do differentially affect immune function for some individuals, then it is of vital importance to ascertain the clinical significance of this. There have been a few *in vivo* human studies, some in volunteers and others in different patient groups. The clinical studies assessing the effects of opioids on immune function have included cancer patients and surgical patients, but hard clinical endpoints (such as survival or disease recurrence) are often lacking. There is an urgent need for well-designed long term clinical studies in various patient groups that are aimed at better understanding the impact of the commonly used opioids on disease progression and patient outcome. Although the latter will not be answered as part of this project, the results generated will provide preliminary data on which to base such clinical studies.

From the previous literature and data from the current study, a range of opioids need to be systematically analysed in defined patient groups, as this would enable evaluation of the indirect, as well as the direct, effects of opioids on immune cells, which have been physiologically activated *in vivo* by disease. In the evaluation of neutrophil and monocyte phagocytosis, morphine, tramadol, fentanyl and buprenorphine had the capacity to inhibit in some subjects and in the oxidative burst reaction of neutrophils and monocytes to *E.coli*, morphine, tramadol, fentanyl, buprenorphine and methadone were inhibitory in some individuals, future research in patients should focus on these opioids on clinical outcome. A range of clinical studies are essential in order to confirm

any effect of opioids in patient groups and these would ideally include surgical and substance abuse groups, as well as patients with cancer and infection.

There are countless permutations for all of the *in vitro* assays with different durations of stimulation, shorter and longer incubations with opioids and different incubation times with target cells. The effect of further agents including the endogenous opioids, ORL1 agonists and opioid antagonists (as well as the combinations of these) could be assessed. The underlying mechanisms for any detectable effects, including CD64 (Fc gamma receptor) and CD66 (neutrophil activation marker) expression on neutrophils and NO production as well as intracellular pathways (i.e. NFκB) could be elucidated. However, the *in vitro* work should ideally have functional endpoints (i.e. target cell killing), as alterations in markers and messengers are only indicative of a change in cell function.

The current study indicates that there is individual effects of some opioids on neutrophil and monocyte phagocytosis and oxidative burst reaction; however the frequency of these effects in the general population is unknown. As each subject was only tested once, individuals would need to be studied over time to determine the stability of the effects. Although the intra-assay precision has been shown to be high by the manufacturers (PHAGOBURST® test kit data sheets, Orpegen Pharma) and a consistent measurement for baseline phagocytosis and oxidative burst responses over time has been demonstrated in the host laboratory, it would need to be confirmed for the effect of opioids on phagocytosis and oxidative burst in five separate samples from the same individuals. Once confirmed, to take this study forward, the prevalence of the potential immune effects of opioids in individuals could be evaluated in 50 healthy subjects and 50 patients with cancer, where they would be phenotyped into having stimulatory, inhibitory or no effect from *in vitro* morphine, tramadol, fentanyl, buprenorphine and methadone on their neutrophil and monocyte phagocytosis and oxidative burst response. This would enable initial identification of the prevalence of this effect and the distribution of the response, as if a large number of people are potentially at risk then the effect would be more relevant to the population.

A subsequent study could phenotype the effect of these opioids on the *in vitro* phagocytosis and oxidative burst reaction in patients who had an infective complication after surgery and compare these to matched controls without infections. The *in vitro* and *in vivo* effects of the opioids having the most effect in these studies could then be evaluated in the same patient group. If the effects of the *in vitro* and *in vivo* studies

correlated, then a simple *in vitro* assay could detect the patients who would have a detrimental response to certain opioids before they are administered (i.e. pre-operatively) and these could be avoided and alternative analgesics used. If these studies replicate the results of the current study and the inhibition of phagocytosis and oxidative burst is shown to correlate with infective complications in patients, then by screening patients this could reduce potentially serious infections, antibiotic use and duration of hospital admission.

Despite opioid receptors being present on activated immune cells, it might be that the SNS and HPA axis are the principal mediators of the effects of opioids on the immune system (especially NK and T cells) and that future studies should be focused on the *in vivo* effects of opioids in humans. To make this most clinically relevant, this would need to be in appropriate patient groups, where their immune system is activated and the interaction of pain, opioids and immunity can be explored.

A clinical study which would help elucidate this, would evaluate the immune effects of morphine vs. tramadol vs. oxycodone in patients undergoing curative resection of breast carcinoma. In view of potential variability between patients, if a randomised controlled trial is used (as this is considered the "gold standard"), either immune phenotyping may be necessary to identify at risk patients to be entered into the study, or the results might have to be analysed as subsets (responders vs. non responders). These patients would all need intra- and post-operative analgesia and the effects of morphine, tramadol and oxycodone could be evaluated on NK cell cytotoxicity and activation, neutrophil and monocyte phagocytosis and oxidative burst and T cell activation and cytokine production.

Preliminary studies would assess the kinetics of this response to opioids, before a larger trial measuring the effect of these opioids at a specific time point. Using a whole blood approach, the activation status of $CD16^+$ and $CD56^+$ NK cells (using CD69 and CD107A), and $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells (using CD25 and CD69) could be evaluated using multiparameter flow cytometry. PBMCs could be isolated from the sample and cytokine levels in the supernatants measured by CBAs. The cytotoxic capacity of the NK cells in the PBMCs could also be measured. Neutrophil and monocyte activation could be evaluated by measuring functional capacity of these cells to phagocytose labelled *E.coli* and the cell surface expression of CD64 and CD66 using multiparameter flow cytometry. Their killing capacity could be measured by their production of oxygen free radicals and NO to *E.coli*. Alongside this, long-term clinical

follow-up would elucidate if the opioids affected clinical outcome, including, infective complications, tumour recurrence and mortality rate.

It is important to use a defined patient group as the intrinsic immune activation in patients with different cancers or inflammatory and infective conditions might vary as might the outcome to different opioids. The results of such studies in a defined patient group might not however be applicable to other patients with different diseases.

The optimisation of patient care requires the clarification of where opioids lie in the spectrum of toxicities and the sensitivity of a particular patient to the influences of a given opioid, such that the opioid which maximises benefit and minimises risk for the individual patient can be prescribed. This would need systematic clinical studies in patients with cancer and other chronic illness both before starting opioids and while on opioids, comparing the effects of opioids on infection and tumour progression, with clinical outcome, as well as immunological, endpoints (Afsharimani et al., 2011, Brack et al., 2011, Roy et al., 2011, Sacerdote, 2008).

4.12 Conclusion

This systematic analysis assessed the effect of eight commonly used opioids, at concentrations found in the clinical setting, on both innate and adaptive immunity. The only consistent significant effect detected, was that methadone, oxycodone and diamorphine decreased IL-6 levels in IL-2 stimulated PBMCs. One of the major findings of this study is that there is cellular level interindividual variability in the response to morphine, tramadol, fentanyl and buprenorphine for phagocytosis, and morphine, tramadol, fentanyl, buprenorphine and methadone (although oxycodone had a significant effect, it was quantitatively very small) for the oxidative burst reaction, and that these changes were not in a common direction, although they were generally inhibitory. Furthermore, the change in immune function with opioids was more dependent on the individual rather than the opioid, such that if an opioid was to influence immune function and whether this was enhancing or suppressive, would depend more on the individual subject than the opioid used.

IL-6 production by IL-2 stimulated PBMCs was decreased by methadone, oxycodone and diamorphine, which could potentially inhibit the acute phase response to infection. There was less effect of the opioids tested on NK cells and T cells, and it may be that these cells are principally affected indirectly *in vivo*, by neuro-immune effects (via the

SNS and HPA axis). However, this study used a small sample size and although it has been detected that variability could exist, it has not been possible to quantify the variability or the distribution of response.

Despite being a systematic *in vitro* analysis of a range of opioids, the current study does not enable clear stratification of the risk of the different opioids on all the aspects of immune function studied. Drawing together the previous studies, from the diverse and often discrepant literature, it may be that there is a spectrum of opioid immunomodulation (Figure 4-2). Although this is speculative at this stage, further comparative studies between the opioids in various clinical settings and clinical studies with defined immunological endpoints could allow such stratification. Eventually, this could then potentially be combined with relative risks of the other toxicities from the various opioids (for the individual patient) to enable a clearer risk:benefit ratio to be devised for an individual, to help inform optimal (safety and efficacy) prescribing of opioids. Without large, controlled, clinical studies in appropriate patient populations, it is necessary to try to synthesis the results from diverse literature in an attempt to stratify the opioids into their likely effect on immune function.

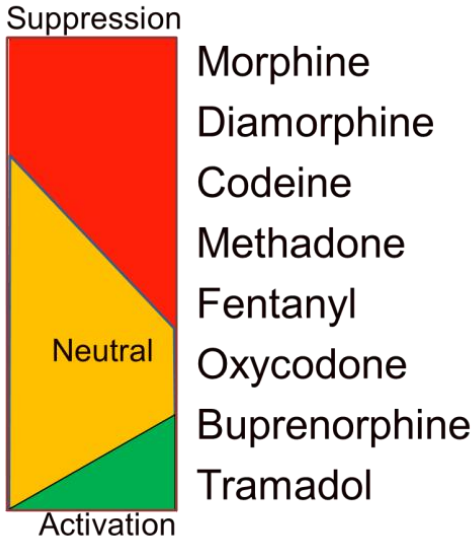

Figure 4-2: Speculative distribution of the effect of opioids on immune function.
Although difficult to quantify with the currently available data, it is likely that there is a spectrum of opioid immunomodulation, with morphine, methadone and codeine being more immunosuppressive than oxycodone and buprenorphine and tramadol being immune enhancing, although this is likely to vary between different aspects of immune function and between individuals.

Taking this work forward, a larger sample of volunteers would enable elucidation of the distribution of response to the effects of morphine, tramadol, fentanyl and buprenorphine on phagocytosis and morphine, tramadol, fentanyl, buprenorphine and methadone on the oxidative burst reaction. If a large proportion of people are potentially at risk of suppression from these opioids and this could be correlated to infective complications, this would be very relevant on a population level.

In view of interindividual variability in aspects of the immune response to opioids and the relatively small effect sizes, future clinical studies would need large numbers of patients. It might also be necessary to analyse the results as subsets (responders vs. non responders) or use immune phenotyping to identify at risk patients. Infection or antibiotic use would be the most relevant clinical outcome, however long-term follow up would capture other clinical end-points, such as malignancy and mortality.

Unlike with most of the effects of opioids, their potential immune effects may take a long time to become apparent, yet the harm might be done during their acute administration. This leads to one of the ultimate clinical challenges in pain management, which is how to distinguish which patients will have a deleterious or beneficial response to which opioids.

Appendix

Related presentations, abstract publications and manuscripts in progress.

Presentations:

The data generated in this thesis has been presented at both local, national and international meetings including:

- Association of palliative medicine conference, Dublin 2011. The *in vitro* effect of opioids on adaptive and innate immunity.
- Association of palliative medicine research meeting, Bristol 2011. Research in Palliative medicine.
- Transpennine research meeting, Sheffield 2010. Effect of morphine and fentanyl on innate and adaptive immune function.
- 6th Annual Conference of the Canadian Society of Palliative Care Physicians 2009. The Immunomodulatory Effects of Opioids.
- Presented locally at palliative medicine, immunobiology, section of oncology and medical school meetings, Sheffield 2009 - 2011.

Key Abstract publications:

- **Boland J**., Foulds G., Ahmedzai S.H., Pockley A.G. Influence of opioids on the phagocytic activity of peripheral blood neutrophils and monocytes: a systematic in vitro analysis. Immunology, 2010; 131 (supplement 1): 79.
- **Boland J**, Foulds G, Ahmedzai SH, Pockley AG. A systematic analysis of the *in vitro* effects of opioids on natural killer cell function. Support Care Cancer 2010; 18 (Suppl 3):S67–S220 (15-135)
- **Boland J**, Foulds G, Ahmedzai SH, Pockley AG. Effect of morphine and fentanyl on immune function. Palliat Med, 2010; 24. 4 suppl S5-S239
- **Boland J**, Foulds G, Hopkinson K, Newton S, Ahmedzai SH, Pockley AG. Establishing an in vitro assay for evaluating the influence of opioids on T cell activation. Eur J immunol 2009; S1/09; 757
- **Boland J,** Ahmedzai SH. Are opioids good for cancer? A review of the immunomodulatory effects of opioids. Support Care Cancer. 2007; 15 (6) 713

Manuscripts in preparation:

- **Boland J**, Ahmedzai SH, Pockley AG. A review of the effects of opioids on innate and adaptive immune function.
- **Boland J**, Foulds G, Ahmedzai SH, Pockley AG. A systematic analysis of the *in vitro* effects of opioids on innate and adaptive immune function.

References

Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. Nature reviews. Immunology 2003; **3:** 939-51.

Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000; **406:** 782-7.

Adorini L. Cytokine-based immunointervention in the treatment of autoimmune diseases. Clin Exp Immunol 2003; **132:** 185-92.

Afsharimani B, Cabot P, Parat MO. Morphine and tumor growth and metastasis. Cancer Metastasis Rev 2011.

Ahn S, Kim J, Lucaveche CL, Reedy MC, Luttrell LM, Lefkowitz RJ, Daaka Y. Src-dependent tyrosine phosphorylation regulates dynamin self-assembly and ligand-induced endocytosis of the epidermal growth factor receptor. J Biol Chem 2002; **277:** 26642-51.

Al Omar SY, Marshall E, Middleton D, Christmas SE. Increased killer immunoglobulin-like receptor expression and functional defects in natural killer cells in lung cancer. Immunology 2011; **133:** 94-104.

Alexander M, Daniel T, Chaudry IH, Schwacha MG. Opiate analgesics contribute to the development of post-injury immunosuppression. J.Surg.Res. 2005; **129:** 161-68.

Alhashemi JA, Kaki AM. Effect of intrathecal tramadol administration on postoperative pain after transurethral resection of prostate. Br J Anaesth 2003; **91:** 536-40.

Alsayed Y, Uddin S, Ahmad S, Majchrzak B, Druker BJ, Fish EN, Platanias LC. IFN-gamma activates the C3G/Rap1 signaling pathway. Journal of immunology 2000; **164:** 1800-6.

Ambach A, Bonnekoh B, Gollnick H. Perforin granule release from cytotoxic lymphocytes ex vivo is inhibited by ciclosporin but not by methotrexate. Skin Pharmacol Appl Skin Physiol 2001; **14:** 249-60.

Ames IH, Gates CE, Garcia AM, John PA, Hennig AK, Tomar RH. Lysis of fresh murine mammary tumor cells by syngeneic natural killer cells and lymphokine-activated killer cells. Cancer Immunol Immunother 1987; **25:** 161-8.

Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. J Allergy Clin Immunol 2010; **125:** 1114-24 e8.

Andreassen TN, Klepstad P, Davies A, Bjordal K, Lundstrom S, Kaasa S, Dale O. Influences on the pharmacokinetics of oxycodone: a multicentre cross-sectional study in 439 adult cancer patients. European journal of clinical pharmacology 2011; **67:** 493-506.

Angiolillo AL, Sgadari C, Taub DD, Liao F, Farber JM, Maheshwari S, Kleinman HK, Reaman GH, Tosato G. Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. The Journal of experimental medicine 1995; **182:** 155-62.

Appel NM, Kiritsy-Roy JA, van Loon GR. Mu receptors at discrete hypothalamic and brainstem sites mediate opioid peptide-induced increases in central sympathetic outflow. Brain Res 1986; **378:** 8-20.

Azarang A, Mahmoodi M, Rajabalian S, Shekari MA, Nosratabadi J, Rezaei N. T-helper 1 and 2 serum cytokine assay in chronic opioid addicts. Eur.Cytokine Netw. 2007; **18:** 210-14.

Azaryan AV, Coughlin LJ, Buzas B, Clock BJ, Cox BM. Effect of chronic cocaine treatment on mu- and delta-opioid receptor mRNA levels in dopaminergically innervated brain regions. J Neurochem. 1996; **66:** 443-48.

Bach EA, Aguet M, Schreiber RD. The IFN gamma receptor: a paradigm for cytokine receptor signaling. Annu Rev Immunol 1997; **15:** 563-91.

Bacon K, Baggiolini M, Broxmeyer H, Horuk R, Lindley I, Mantovani A, Maysushima K, Murphy P, Nomiyama H, Oppenheim J, Rot A, Schall T, Tsang M, Thorpe R, Van Damme J, Wadhwa M, Yoshie O, Zlotnik A, Zoon K. Chemokine/chemokine receptor nomenclature. J Interferon Cytokine Res 2002; **22:** 1067-8.

Barton BE. The biological effects of interleukin 6. Med Res Rev 1996; **16:** 87-109.

Bedoui S, Lechner S, Gebhardt T, Nave H, Beck-Sickinger AG, Straub RH, Pabst R, von Horsten S. NPY modulates epinephrine-induced leukocytosis via Y-1 and Y-5 receptor activation in vivo: sympathetic co-transmission during leukocyte mobilization. J Neuroimmunol 2002; **132:** 25-33.

Beilin B, Grinevich G, Yardeni IZ, Bessler H. Tramadol does not impair the phagocytic capacity of human peripheral blood cells. Can.J Anaesth. 2005; **52:** 1035-39.

Beilin B, Shavit Y, Hart J, Mordashov B, Cohn S, Notti I, Bessler H. Effects of anesthesia based on large versus small doses of fentanyl on natural killer cell cytotoxicity in the perioperative period. Anesth Analg 1996; **82:** 492-7.
Ben-Eliyahu S, Page GG, Schleifer SJ. Stress, NK cells, and cancer: Still a promissory note. Brain Behav Immun 2007; **21:** 881-7.
Ben-Eliyahu S, Page GG, Yirmiya R, Taylor AN. Acute alcohol intoxication suppresses natural killer cell activity and promotes tumor metastasis. Nat Med 1996; **2:** 457-60.
Benschop RJ, Rodriguez-Feuerhahn M, Schedlowski M. Catecholamine-induced leukocytosis: early observations, current research, and future directions. Brain Behav Immun 1996; **10:** 77-91.
Benschop RJ, Schedlowski M, Wienecke H, Jacobs R, Schmidt RE. Adrenergic control of natural killer cell circulation and adhesion. Brain Behav Immun 1997; **11:** 321-32.
Bhargava HN, Thomas PT, Thorat S, House RV. Effects of morphine tolerance and abstinence on cellular immune function. Brain Res. 1994; **642:** 1-10.
Bhaskaran M, Kapasi AA, Reddy K, Singhal PC. Morphine priming rescues high-dose morphine-induced biological perturbations. J Infect Dis 2007; **195:** 1860-9.
Bianchi M, Panerai AE. Anti-hyperalgesic effects of tramadol in the rat. Brain Res 1998; **797:** 163-6.
Bidlack JM, Khimich M, Parkhill AL, Sumagin S, Sun B, Tipton CM. Opioid receptors and signaling on cells from the immune system. J Neuroimmune.Pharmacol 2006; **1:** 260-69.
Biki B, Mascha E, Moriarty DC, Fitzpatrick JM, Sessler DI, Buggy DJ. Anesthetic technique for radical prostatectomy surgery affects cancer recurrence: a retrospective analysis. Anesthesiology 2008; **109:** 180-7.
Bird L. Antiviral immunity: Ageing immune systems work too hard. Nat Rev Immunol 2010; **10:** 6-7.
Biron CA, Gazzinelli RT. Effects of IL-12 on immune responses to microbial infections: a key mediator in regulating disease outcome. Curr Opin Immunol 1995; **7:** 485-96.
Bonnet MP, Beloeil H, Benhamou D, Mazoit JX, Asehnoune K. The mu opioid receptor mediates morphine-induced tumor necrosis factor and interleukin-6 inhibition in toll-like receptor 2-stimulated monocytes. Anesth.Analg. 2008; **106:** 1142-9, table.
Booth V, Keizer DW, Kamphuis MB, Clark-Lewis I, Sykes BD. The CXCR3 binding chemokine IP-10/CXCL10: structure and receptor interactions. Biochemistry 2002; **41:** 10418-25.
Borgland SL. Acute opioid receptor desensitization and tolerance: is there a link? Clin Exp Pharmacol Physiol 2001; **28:** 147-54.
Borman A, Ciepielewski Z, Wrona D, Stojek W, Glac W, Leszkowicz E, Tokarski J. Small doses of morphine can enhance NK cell cytotoxicity in pigs. Int.Immunopharmacol. 2009; **9:** 277-83.
Borner C, Hollt V, Kraus J. Involvement of activator protein-1 in transcriptional regulation of the human mu-opioid receptor gene. Mol.Pharmacol 2002; **61:** 800-05.
Borner C, Hollt V, Kraus J. Cannabinoid receptor type 2 agonists induce transcription of the mu-opioid receptor gene in Jurkat T cells. Mol.Pharmacol 2006; **69:** 1486-91.
Borner C, Kraus J, Bedini A, Schraven B, Hollt V. T-cell receptor/CD28-mediated activation of human T lymphocytes induces expression of functional mu-opioid receptors. Mol.Pharmacol 2008; **74:** 496-504.
Borner C, Kraus J, Schroder H, Ammer H, Hollt V. Transcriptional regulation of the human mu-opioid receptor gene by interleukin-6. Mol.Pharmacol. 2004; **66:** 1719-26.
Borner C, Stumm R, Hollt V, Kraus J. Comparative analysis of mu-opioid receptor expression in immune and neuronal cells. J.Neuroimmunol. 2007; **188:** 56-63.
Borner C, Warnick B, Smida M, Hartig R, Lindquist JA, Schraven B, Hollt V, Kraus J. Mechanisms of opioid-mediated inhibition of human T cell receptor signaling. J Immunol. 2009; **183:** 882-89.
Borst SE, Hui KK, Conolly ME. Beta-adrenergic receptors on human lymphocytes: comparison of down-regulation in vivo and in vitro. Pharmacology 1990; **40:** 325-9.
Bosshart H. Morphine-mediated suppression of phagocytosis. Int Immunopharmacol 2009.
Bots M, Medema JP. Granzymes at a glance. J Cell Sci 2006; **119:** 5011-4.
Bower S. The uptake of fentanyl by erythrocytes. J Pharm Pharmacol 1982; **34:** 181-5.
Brack A, Rittner HL, Stein C. Immunosuppressive Effects of Opioids-Clinical Relevance. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology 2011.

Brand JM, Kirchner H, Poppe C, Schmucker P. The effects of general anesthesia on human peripheral immune cell distribution and cytokine production. Clin Immunol Immunopathol 1997; **83:** 190-4.

Bryant HU, Bernton EW, Holaday JW. Immunosuppressive effects of chronic morphine treatment in mice. Life Sci. 1987; **41:** 1731-38.

Bryant HU, Bernton EW, Holaday JW. Morphine pellet-induced immunomodulation in mice: temporal relationships. J.Pharmacol.Exp.Ther. 1988; **245:** 913-20.

Bubenik J. Tumour MHC class I downregulation and immunotherapy (Review). Oncol Rep 2003; **10:** 2005-8.

Budd K. Pain management: is opioid immunosuppression a clinical problem? Biomed.Pharmacother. 2006; **60:** 310-17.

Cadet P, Mantione KJ, Stefano GB. Molecular identification and functional expression of mu 3, a novel alternatively spliced variant of the human mu opiate receptor gene. J Immunol. 2003; **170:** 5118-23.

Callahan RJ, Au JD, Paul M, Liu C, Yost CS. Functional inhibition by methadone of N-methyl-D-aspartate receptors expressed in Xenopus oocytes: stereospecific and subunit effects. Anesth Analg 2004; **98:** 653-9, table of contents.

Campa D, Gioia A, Tomei A, Poli P, Barale R. Association of ABCB1/MDR1 and OPRM1 gene polymorphisms with morphine pain relief. Clin Pharmacol Ther 2008; **83:** 559-66.

Cantacuzene J. Nouvelles recherches sur le mode de destruction des vibrions dans l'organisme. Ann. Inst. Pasteur 1898; **12:** 274–300.

Carpenter GW, Breeden L, Carr DJ. Acute exposure to morphine suppresses cytotoxic T-lymphocyte activity. Int.J Immunopharmacol. 1995; **17:** 1001-06.

Carpenter GW, Garza HH, Jr., Gebhardt BM, Carr DJ. Chronic morphine treatment suppresses CTL-mediated cytolysis, granulation, and cAMP responses to alloantigen. Brain Behav.Immun. 1994; **8:** 185-203.

Carrigan KA, Lysle DT. Morphine-6 beta-glucuronide induces potent immunomodulation. Int Immunopharmacol 2001; **1:** 821-31.

Casellas AM, Guardiola H, Renaud FL. Inhibition by opioids of phagocytosis in peritoneal macrophages. Neuropeptides 1991; **18:** 35-40.

Castriconi R, Dondero A, Cilli M, Ognio E, Pezzolo A, De Giovanni B, Gambini C, Pistoia V, Moretta L, Moretta A, Corrias MV. Human NK cell infusions prolong survival of metastatic human neuroblastoma-bearing NOD/scid mice. Cancer Immunol Immunother 2007; **56:** 1733-42.

Chakrabarti S, Liu NJ, Gintzler AR. Formation of {micro}-/{kappa}-opioid receptor heterodimer is sex-dependent and mediates female-specific opioid analgesia. Proc Natl Acad Sci U S A 2010.

Chambers WH, Vujanovic NL, DeLeo AB, Olszowy MW, Herberman RB, Hiserodt JC. Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine-activated killer cells. J Exp Med 1989; **169:** 1373-89.

Chandra RK. Nutrition and immunology: from the clinic to cellular biology and back again. Proc Nutr Soc 1999; **58:** 681-3.

Chao CC, Molitor TW, Close K, Hu S, Peterson PK. Morphine inhibits the release of tumor necrosis factor in human peripheral blood mononuclear cell cultures. Int J Immunopharmacol 1993; **15:** 447-53.

Chiou LC, Liao YY, Fan PC, Kuo PH, Wang CH, Riemer C, Prinssen EP. Nociceptin/orphanin FQ peptide receptors: pharmacology and clinical implications. Curr Drug Targets 2007; **8:** 117-35.

Christopherson R, James KE, Tableman M, Marshall P, Johnson FE. Long-term survival after colon cancer surgery: a variation associated with choice of anesthesia. Anesthesia and analgesia 2008; **107:** 325-32.

Christrup LL. Morphine metabolites. Acta Anaesthesiol Scand 1997; **41:** 116-22.

Clancy J, Jr., Petrovic LM, Gordon BH, Handa RJ, Campbell DB, Lorens SA. Effects of subchronic d-fenfluramine on splenic immune functions in young and old male and female Fischer 344 rats. Int J Immunopharmacol 1991; **13:** 1203-12.

Clark AJ, Ahmedzai SH, Allan LG, Camacho F, Horbay GL, Richarz U, Simpson K. Efficacy and safety of transdermal fentanyl and sustained-release oral morphine in patients with cancer and chronic non-cancer pain. Curr Med Res Opin 2004; **20:** 1419-28.

Clark JD. Understanding methadone metabolism: a foundation for safer use. Anesthesiology 2008; **108:** 351-2.

Clarke RW, Ward RE. The role of 5-HT(1A)-receptors in fentanyl-induced bulbospinal inhibition of a spinal withdrawal reflex in the rabbit. Pain 2000; **85:** 239-45.

Codd EE, Shank RP, Schupsky JJ, Raffa RB. Serotonin and norepinephrine uptake inhibiting activity of centrally acting analgesics: structural determinants and role in antinociception. J Pharmacol Exp Ther 1995; **274:** 1263-70.

Coller JK, Barratt DT, Dahlen K, Loennechen MH, Somogyi AA. ABCB1 genetic variability and methadone dosage requirements in opioid-dependent individuals. Clin Pharmacol Ther 2006; **80:** 682-90.

Coller JK, Christrup LL, Somogyi AA. Role of active metabolites in the use of opioids. Eur J Clin Pharmacol 2009; **65:** 121-39.

Condevaux F, Guichard J, Forichon A, Aujoulat M, Descotes J. Compared effects of morphine and nickel chloride on NK cell activity in vitro in rats and monkeys. J Appl Toxicol 2001; **21:** 431-4.

Cone EJ, Holicky BA, Grant TM, Darwin WD, Goldberger BA. Pharmacokinetics and pharmacodynamics of intranasal "snorted" heroin. J Anal Toxicol 1993; **17:** 327-37.

Costigan M, Moss A, Latremoliere A, Johnston C, Verma-Gandhu M, Herbert TA, Barrett L, Brenner GJ, Vardeh D, Woolf CJ, Fitzgerald M. T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. J Neurosci 2009; **29:** 14415-22.

Cotran RS, Kumar V, Robbins SL. *Robbins and Cotran pathologic basis of disease*. 8th edition ed. Philadelphia, Pa: Saunders Elsevier, 2010.

Cox WI, Holbrook NJ, Grasso RJ, Specter S, Friedman H. Suppression of the natural killer cell activity of murine spleen cell cultures by dexamethasone (41489). Proc Soc Exp Biol Med 1982; **171:** 146-50.

Crary B, Borysenko M, Sutherland DC, Kutz I, Borysenko JZ, Benson H. Decrease in mitogen responsiveness of mononuclear cells from peripheral blood after epinephrine administration in humans. J Immunol 1983; **130:** 694-7.

Croker BA, Kiu H, Pellegrini M, Toe J, Preston S, Metcalf D, O'Donnell JA, Cengia LH, McArthur K, Nicola NA, Alexander WS, Roberts AW. IL-6 promotes acute and chronic inflammatory disease in the absence of SOCS3. Immunology and cell biology 2011.

Cullen SP, Brunet M, Martin SJ. Granzymes in cancer and immunity. Cell Death Differ 2010; **17:** 616-23.

Curfs JH, Meis JF, Hoogkamp-Korstanje JA. A primer on cytokines: sources, receptors, effects, and inducers. Clin Microbiol Rev 1997; **10:** 742-80.

Cutolo M, Sulli A, Villaggio B, Seriolo B, Accardo S. Relations between steroid hormones and cytokines in rheumatoid arthritis and systemic lupus erythematosus. Ann Rheum Dis 1998; **57:** 573-7.

D'Elia M, Patenaude J, Hamelin C, Garrel DR, Bernier J. No detrimental effect from chronic exposure to buprenorphine on corticosteroid-binding globulin and corticosensitive immune parameters. Clin Immunol 2003; **109:** 179-87.

Dahan A, Yassen A, Bijl H, Romberg R, Sarton E, Teppema L, Olofsen E, Danhof M. Comparison of the respiratory effects of intravenous buprenorphine and fentanyl in humans and rats. Br J Anaesth 2005; **94:** 825-34.

Dahan A, Yassen A, Romberg R, Sarton E, Teppema L, Olofsen E, Danhof M. Buprenorphine induces ceiling in respiratory depression but not in analgesia. Br J Anaesth 2006; **96:** 627-32.

Dalen P, Frengell C, Dahl ML, Sjoqvist F. Quick onset of severe abdominal pain after codeine in an ultrarapid metabolizer of debrisoquine. Ther Drug Monit 1997; **19:** 543-4.

Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, Mitsdoerffer M, Strom TB, Elyaman W, Ho IC, Khoury S, Oukka M, Kuchroo VK. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. Nature immunology 2008; **9:** 1347-55.

Darwish M, Kirby M, Robertson P, Jr., Tracewell W, Jiang JG. Pharmacokinetic properties of fentanyl effervescent buccal tablets: a phase I, open-label, crossover study of single-dose 100, 200, 400, and 800 microg in healthy adult volunteers. Clin.Ther. 2006; **28:** 707-14.

Daynes RA, Dowell T, Araneo BA. Platelet-derived growth factor is a potent biologic response modifier of T cells. The Journal of experimental medicine 1991; **174:** 1323-33.

de Leon J, Dinsmore L, Wedlund P. Adverse drug reactions to oxycodone and hydrocodone in CYP2D6 ultrarapid metabolizers. J Clin Psychopharmacol 2003; **23:** 420-1.

Delgado-Velez M, Lugo-Chinchilla A, Lizardo L, Morales I, Robles Y, Bruno N, Rodriguez JW, Rios-Olivares E, Correa M, Renaud FL. Chronic exposure of human macrophages in vitro to morphine and methadone induces a putative tolerant/dependent state. J Neuroimmunol 2008; **196:** 94-100.

Desai A, Grolleau-Julius A, Yung R. Leukocyte function in the aging immune system. J Leukoc Biol 2010.

Dietis N, Rowbotham DJ, Lambert DG. Opioid receptor subtypes: fact or artifact? British journal of anaesthesia 2011; **107:** 8-18.

Dinarello CA. The biological properties of interleukin-1. Eur Cytokine Netw 1994; **5:** 517-31.

Dinauer MC. Disorders of neutrophil function: an overview. Methods Mol Biol 2007; **412:** 489-504.

Djaldetti M, Salman H, Bergman M, Djaldetti R, Bessler H. Phagocytosis--the mighty weapon of the silent warriors. Microsc Res Tech 2002; **57:** 421-31.

Driessen B, Reimann W. Interaction of the central analgesic, tramadol, with the uptake and release of 5-hydroxytryptamine in the rat brain in vitro. Br J Pharmacol 1992; **105:** 147-51.

Driessen B, Reimann W, Giertz H. Effects of the central analgesic tramadol on the uptake and release of noradrenaline and dopamine in vitro. Br J Pharmacol 1993; **108:** 806-11.

Droney J, Riley J, Ross JR. Evolving knowledge of opioid genetics in cancer pain. Clinical oncology 2011; **23:** 418-28.

Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. Journal of immunology 2002; **168:** 3195-204.

Dyer KR, White JM, Foster DJ, Bochner F, Menelaou A, Somogyi AA. The relationship between mood state and plasma methadone concentration in maintenance patients. J Clin Psychopharmacol 2001; **21:** 78-84.

Ebert B, Andersen S, Krogsgaard-Larsen P. Ketobemidone, methadone and pethidine are non-competitive N-methyl-D-aspartate (NMDA) antagonists in the rat cortex and spinal cord. Neurosci Lett 1995; **187:** 165-8.

Elftman MD, Hunzeker JT, Mellinger JC, Bonneau RH, Norbury CC, Truckenmiller ME. Stress-Induced Glucocorticoids at the Earliest Stages of Herpes Simplex Virus-1 Infection Suppress Subsequent Antiviral Immunity, Implicating Impaired Dendritic Cell Function. J Immunol 2010; **184:** 1867-75.

Elshal MF, McCoy JP. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. Methods 2006; **38:** 317-23.

Eriksen CG, Olsen H, Husted LB, Sorensen L, Carstens M, Soballe K, Langdahl BL. The expression of IL-6 by osteoblasts is increased in healthy elderly individuals: stimulated proliferation and differentiation are unaffected by age. Calcif Tissue Int 2010; **87:** 414-23.

Escher M, Daali Y, Chabert J, Hopfgartner G, Dayer P, Desmeules J. Pharmacokinetic and pharmacodynamic properties of buprenorphine after a single intravenous administration in healthy volunteers: a randomized, double-blind, placebo-controlled, crossover study. Clin.Ther. 2007; **29:** 1620-31.

Esplugues E, Vega-Ramos J, Cartoixa D, Vazquez BN, Salaet I, Engel P, Lauzurica P. Induction of tumor NK-cell immunity by anti-CD69 antibody therapy. Blood 2005; **105:** 4399-406.

Evans HC, Easthope SE. Transdermal buprenorphine. Drugs 2003; **63:** 1999-2010; discussion 11-2.

Everhart ET, Cheung P, Shwonek P, Zabel K, Tisdale EC, Jacob P, 3rd, Mendelson J, Jones RT. Subnanogram-concentration measurement of buprenorphine in human plasma by electron-capture capillary gas chromatography: application to pharmacokinetics of sublingual buprenorphine. Clin Chem 1997; **43:** 2292-302.

Exadaktylos AK, Buggy DJ, Moriarty DC, Mascha E, Sessler DI. Can anesthetic technique for primary breast cancer surgery affect recurrence or metastasis? Anesthesiology 2006; **105:** 660-4.

Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, Cianfarani F, Odorisio T, Traidl-Hoffmann C, Behrendt H, Durham SR, Schmidt-Weber CB, Cavani A. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. The Journal of clinical investigation 2009; **119:** 3573-85.

Faras-Melainis K, Gomes I, Rozenfeld R, Zachariou V, Devi L. Modulation of opioid receptor function by protein-protein interactions. Front Biosci. 2009; **14:** 3594-607.
Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol 1993; **11:** 571-611.
Fecho K, Dykstra LA, Lysle DT. Evidence for beta adrenergic receptor involvement in the immunomodulatory effects of morphine. J Pharmacol Exp Ther 1993; **265:** 1079-87.
Fecho K, Maslonek KA, Dykstra LA, Lysle DT. Evidence for sympathetic and adrenal involvement in the immunomodulatory effects of acute morphine treatment in rats. J Pharmacol Exp Ther 1996; **277:** 633-45.
Feierman DE, Lasker JM. Metabolism of fentanyl, a synthetic opioid analgesic, by human liver microsomes. Role of CYP3A4. Drug Metab Dispos 1996; **24:** 932-9.
Felten DL, Felten SY, Carlson SL, Olschowka JA, Livnat S. Noradrenergic and peptidergic innervation of lymphoid tissue. J Immunol 1985; **135:** 755s-65s.
Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 2001; **53:** 1-24.
Ferraccioli G, Zizzo G. The potential role of Th17 in mediating the transition from acute to chronic autoimmune inflammation: rheumatoid arthritis as a model. Discovery medicine 2011; **11:** 413-24.
Ferrari A, Coccia CP, Bertolini A, Sternieri E. Methadone--metabolism, pharmacokinetics and interactions. Pharmacol Res 2004; **50:** 551-9.
Findlay JW, Jones EC, Butz RF, Welch RM. Plasma codeine and morphine concentrations after therapeutic oral doses of codeine-containing analgesics. Clin Pharmacol Ther 1978; **24:** 60-8.
Finn AK, Whistler JL. Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. Neuron 2001; **32:** 829-39.
Fouser LA, Wright JF, Dunussi-Joannopoulos K, Collins M. Th17 cytokines and their emerging roles in inflammation and autoimmunity. Immunol Rev 2008; **226:** 87-102.
Friedman H, Newton C, Klein TW. Microbial infections, immunomodulation, and drugs of abuse. Clin.Microbiol.Rev. 2003; **16:** 209-19.
Frink MC, Hennies HH, Englberger W, Haurand M, Wilffert B. Influence of tramadol on neurotransmitter systems of the rat brain. Arzneimittelforschung 1996; **46:** 1029-36.
Fuchs EJ, Matzinger P. Is cancer dangerous to the immune system? Semin Immunol 1996; **8:** 271-80.
Fuggetta MP, Di FP, Falchetti R, Cottarelli A, Rossi L, Tricarico M, Lanzilli G. Effect of morphine on cell-mediated immune responses of human lymphocytes against allogeneic malignant cells. J.Exp.Clin.Cancer Res. 2005; **24:** 255-63.
Fujioka N, Nguyen J, Chen C, Li Y, Pasrija T, Niehans G, Johnson KN, Gupta V, Kratzke RA, Gupta K. Morphine-Induced Epidermal Growth Factor Pathway Activation in Non-Small Cell Lung Cancer. Anesthesia and analgesia 2011.
Gabay E, Wolf G, Shavit Y, Yirmiya R, Tal M. Chronic blockade of interleukin-1 (IL-1) prevents and attenuates neuropathic pain behavior and spontaneous ectopic neuronal activity following nerve injury. Eur J Pain 2010.
Gabrilovac J, Marotti T. Gender-related differences in murine T- and B-lymphocyte proliferative ability in response to in vivo [Met(5)]enkephalin administration. Eur.J.Pharmacol. 2000; **392:** 101-08.
Gaffen SL. Structure and signalling in the IL-17 receptor family. Nat Rev Immunol 2009; **9:** 556-67.
Gaffen SL, Kramer JM, Yu JJ, Shen F. The IL-17 cytokine family. Vitam Horm 2006; **74:** 255-82.
Galley HF, DiMatteo MA, Webster NR. Immunomodulation by anaesthetic, sedative and analgesic agents: does it matter? Intensive Care Med 2000; **26:** 267-74.
Gan SH, Ismail R, Wan Adnan WA, Wan Z. Correlation of tramadol pharmacokinetics and CYP2D6*10 genotype in Malaysian subjects. J Pharm.Biomed.Anal. 2002; **30:** 189-95.
Garcia-Quetglas E, Azanza JR, Sadaba B, Munoz MJ, Gil I, Campanero MA. Pharmacokinetics of tramadol enantiomers and their respective phase I metabolites in relation to CYP2D6 phenotype. Pharmacol Res. 2007; **55:** 122-30.
Garrido F, Algarra I. MHC antigens and tumor escape from immune surveillance. Adv Cancer Res 2001; **83:** 117-58.
Gaspani L, Bianchi M, Limiroli E, Panerai AE, Sacerdote P. The analgesic drug tramadol prevents the effect of surgery on natural killer cell activity and metastatic colonization in rats. J.Neuroimmunol. 2002; **129:** 18-24.

Gaveriaux-Ruff C, Matthes HW, Peluso J, Kieffer BL. Abolition of morphine-immunosuppression in mice lacking the mu-opioid receptor gene. Proc.Natl.Acad.Sci.U.S.A 1998; **95:** 6326-30.

George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, O'Dowd BF. Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. J Biol Chem 2000; **275:** 26128-35.

Gerosa F, Tommasi M, Benati C, Gandini G, Libonati M, Tridente G, Carra G, Trinchieri G. Differential effects of tyrosine kinase inhibition in CD69 antigen expression and lytic activity induced by rIL-2, rIL-12, and rIFN-alpha in human NK cells. Cell Immunol 1993; **150:** 382-90.

Gilron I, Wajsbrot D, Therrien F, Lemay J. Pregabalin for peripheral neuropathic pain: a multicenter, enriched enrollment randomized withdrawal placebo-controlled trial. The Clinical journal of pain 2011; **27:** 185-93.

Glattard E, Welters ID, Lavaux T, Muller AH, Laux A, Zhang D, Schmidt AR, Delalande F, Laventie BJ, Dirrig-Grosch S, Colin DA, Van Dorsselaer A, Aunis D, Metz-Boutigue MH, Schneider F, Goumon Y. Endogenous morphine levels are increased in sepsis: a partial implication of neutrophils. PLoS One 2010; **5:** e8791.

Gleeson M. Immune function in sport and exercise. J Appl Physiol 2007; **103:** 693-9.

Gomes I, Jordan BA, Gupta A, Trapaidze N, Nagy V, Devi LA. Heterodimerization of mu and delta opioid receptors: A role in opiate synergy. J Neurosci 2000; **20:** RC110.

Gomez-Flores R, Weber RJ. Differential effects of buprenorphine and morphine on immune and neuroendocrine functions following acute administration in the rat mesencephalon periaqueductal gray. Immunopharmacology 2000; **48:** 145-56.

Gonzalez FM, Vargas JA, Lopez-Cortijo C, Castejon R, Gorriz C, Ramirez-Camacho R, Millan I, Durantez A. Prognostic significance of natural killer cell activity in patients with laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1998; **124:** 852-6.

Gordin V. The role of cytokines in chronic pain processing and opioid tolerance: an Australian study. Pain Med 2010; **11:** 562-3.

Gottschalk A, Ford JG, Regelin CC, You J, Mascha EJ, Sessler DI, Durieux ME, Nemergut EC. Association between epidural analgesia and cancer recurrence after colorectal cancer surgery. Anesthesiology 2010; **113:** 27-34.

Greeneltch KM, Kelly-Welch AE, Shi Y, Keegan AD. Chronic morphine treatment promotes specific Th2 cytokine production by murine T cells in vitro via a Fas/Fas ligand-dependent mechanism. J.Immunol. 2005; **175:** 4999-5005.

Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 1982; **155:** 1823-41.

Grond S, Meuser T, Uragg H, Stahlberg HJ, Lehmann KA. Serum concentrations of tramadol enantiomers during patient-controlled analgesia. Br J Clin Pharmacol 1999; **48:** 254-7.

Grond S, Sablotzki A. Clinical pharmacology of tramadol. Clin.Pharmacokinet. 2004; **43:** 879-923.

Grutz G. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. Journal of leukocyte biology 2005; **77:** 3-15.

Guan L, Townsend R, Eisenstein TK, Adler MW, Rogers TJ. Both T cells and macrophages are targets of kappa-opioid-induced immunosuppression. Brain Behav.Immun. 1994; **8:** 229-40.

Hale M, Khan A, Kutch M, Li S. Once-daily OROS hydromorphone ER compared with placebo in opioid-tolerant patients with chronic low back pain. Current medical research and opinion 2010; **26:** 1505-18.

Handa K, Suzuki R, Matsui H, Shimizu Y, Kumagai K. Natural killer (NK) cells as a responder to interleukin 2 (IL 2). II. IL 2-induced interferon gamma production. J Immunol 1983; **130:** 988-92.

Hatsukari I, Hitosugi N, Ohno R, Hashimoto K, Nakamura S, Satoh K, Nagasaka H, Matsumoto I, Sakagami H. Induction of apoptosis by morphine in human tumor cell lines in vitro. Anticancer Res 2007; **27:** 857-64.

He SQ, Zhang ZN, Guan JS, Liu HR, Zhao B, Wang HB, Li Q, Yang H, Luo J, Li ZY, Wang Q, Lu YJ, Bao L, Zhang X. Facilitation of mu-opioid receptor activity by preventing delta-opioid receptor-mediated codegradation. Neuron 2011; **69:** 120-31.

Heeren T, D'Agostino R. Robustness of the two independent samples t-test when applied to ordinal scaled data. Stat Med 1987; **6:** 79-90.

Heikkila K, Ebrahim S, Rumley A, Lowe G, Lawlor DA. Associations of circulating C-reactive protein and interleukin-6 with survival in women with and without cancer: findings from the British Women's Heart and Health Study. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2007; **16:** 1155-9.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 2003a; **374:** 1-20.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem.J 2003b; **374:** 1-20.

Henney CS, Kuribayashi K, Kern DE, Gillis S. Interleukin-2 augments natural killer cell activity. Nature 1981; **291:** 335-38.

Henry DJ, Grandy DK, Lester HA, Davidson N, Chavkin C. Kappa-opioid receptors couple to inwardly rectifying potassium channels when coexpressed by Xenopus oocytes. Mol Pharmacol 1995; **47:** 551-7.

Hernandez MC, Flores LR, Bayer BM. Immunosuppression by morphine is mediated by central pathways. J.Pharmacol.Exp.Ther. 1993; **267:** 1336-41.

Hilburger ME, Adler MW, Truant AL, Meissler JJ, Jr., Satishchandran V, Rogers TJ, Eisenstein TK. Morphine induces sepsis in mice. J Infect Dis 1997; **176:** 183-8.

Hjelm Skog AL, Wadhwa M, Hassan M, Gharizadeh B, Bird C, Ragnhammar P, Thorpe R, Mellstedt H. Alteration of interleukin 2 (IL-2) pharmacokinetics and function by IL-2 antibodies induced after treatment of colorectal carcinoma patients with a combination of monoclonal antibody 17-1A, granulocyte macrophage colony-stimulating factor, and IL-2. Clin Cancer Res 2001; **7:** 1163-70.

Hoehe MR, Kopke K, Wendel B, Rohde K, Flachmeier C, Kidd KK, Berrettini WH, Church GM. Sequence variability and candidate gene analysis in complex disease: association of mu opioid receptor gene variation with substance dependence. Hum.Mol.Genet. 2000; **9:** 2895-908.

Hopkinson K, Williams EA, Fairburn B, Forster S, Flower DJ, Saxton JM, Pockley AG. A MitoTracker Green-based flow cytometric assay for natural killer cell activity: variability, the influence of platelets and a comparison of analytical approaches. Exp Hematol 2007; **35:** 350-7.

Hou J, Schindler U, Henzel WJ, Ho TC, Brasseur M, McKnight SL. An interleukin-4-induced transcription factor: IL-4 Stat. Science 1994; **265:** 1701-06.

House RV, Thomas PT, Bhargava HN. In vitro evaluation of fentanyl and meperidine for immunomodulatory activity. Immunol.Lett. 1995; **46:** 117-24.

Huang P, Kehner GB, Cowan A, Liu-Chen LY. Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. J Pharmacol Exp Ther 2001; **297:** 688-95.

Hutchinson MR, Coats BD, Lewis SS, Zhang Y, Sprunger DB, Rezvani N, Baker EM, Jekich BM, Wieseler JL, Somogyi AA, Martin D, Poole S, Judd CM, Maier SF, Watkins LR. Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. Brain Behav.Immun. 2008.

Hutchinson MR, Somogyi AA. Diacetylmorphine degradation to 6-monoacetylmorphine and morphine in cell culture: implications for in vitro studies. Eur J Pharmacol 2002; **453:** 27-32.

Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, Zhao TX, Slivka PF, Coats BD, Rezvani N, Wieseler J, Hughes TS, Landgraf KE, Chan S, Fong S, Phipps S, Falke JJ, Leinwand LA, Maier SF, Yin H, Rice KC, Watkins LR. Evidence that opioids may have toll-like receptor 4 and MD-2 effects. Brain Behav Immun 2010; **24:** 83-95.

Ihnatko R, Kubes M. TNF signaling: early events and phosphorylation. Gen Physiol Biophys 2007; **26:** 159-67.

Ikeda K, Kobayashi T, Kumanishi T, Niki H, Yano R. Involvement of G-protein-activated inwardly rectifying K (GIRK) channels in opioid-induced analgesia. Neurosci Res 2000; **38:** 113-6.

Inturrisi CE. Pharmacology of methadone and its isomers. Minerva Anestesiol 2005; **71:** 435-7.

Inturrisi CE, Max MB, Foley KM, Schultz M, Shin SU, Houde RW. The pharmacokinetics of heroin in patients with chronic pain. N Engl J Med 1984; **310:** 1213-7.
Irwin M, Hauger RL, Brown M, Britton KT. CRF activates autonomic nervous system and reduces natural killer cytotoxicity. Am J Physiol 1988; **255:** R744-7.
Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, Aridome K, Hokita S, Aikou T. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer 2000; **88:** 577-83.
Ismail H, Ho KM, Narayan K, Kondalsamy-Chennakesavan S. Effect of neuraxial anaesthesia on tumour progression in cervical cancer patients treated with brachytherapy: a retrospective cohort study. British journal of anaesthesia 2010; **105:** 145-9.
Jacobs R, Karst M, Scheinichen D, Bevilacqua C, Schneider U, Heine J, Schedlowski M, Schmidt RE. Effects of fentanyl on cellular immune functions in man. Int.J Immunopharmacol. 1999; **21:** 445-54.
Jaeger K, Scheinichen D, Heine J, Andre M, Bund M, Piepenbrock S, Leuwer M. Remifentanil, fentanyl, and alfentanil have no influence on the respiratory burst of human neutrophils in vitro. Acta Anaesthesiol Scand 1998; **42:** 1110-3.
Jager MJ, Hurks HM, Levitskaya J, Kiessling R. HLA expression in uveal melanoma: there is no rule without some exception. Human immunology 2002; **63:** 444-51.
Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. J Immunol 2009; **182:** 4572-80.
Kadiev E, Patel V, Rad P, Thankachan L, Tram A, Weinlein M, Woodfin K, Raffa RB, Nagar S. Role of pharmacogenetics in variable response to drugs: focus on opioids. Expert Opin Drug Metab Toxicol 2008; **4:** 77-91.
Kambur O, Mannisto PT. Catechol-o-methyltransferase and pain. Int Rev Neurobiol 2010; **95:** 227-79.
Kambur O, Talka R, Ansah O, Kontinen V, Pertovaara A, Kalso E, Mannisto P. Inhibitors of catechol-O-methyltransferase sensitize mice to pain. Br J Pharmacol 2010; **161:** 1553-65.
Kampen AH, Tollersrud T, Larsen S, Roth JA, Frank DE, Lund A. Repeatability of flow cytometric and classical measurement of phagocytosis and respiratory burst in bovine polymorphonuclear leukocytes. Veterinary immunology and immunopathology 2004; **97:** 105-14.
Kane KL, Ashton FA, Schmitz JL, Folds JD. Determination of natural killer cell function by flow cytometry. Clinical and diagnostic laboratory immunology 1996; **3:** 295-300.
Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol 2003; **3:** 984-93.
Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010; **11:** 373-84.
Kawasaki T, Ogata M, Kawasaki C, Okamoto K, Sata T. Effects of epidural anaesthesia on surgical stress-induced immunosuppression during upper abdominal surgery. Br.J.Anaesth. 2007; **98:** 196-203.
Keiser MJ, Roth BL, Armbruster BN, Ernsberger P, Irwin JJ, Shoichet BK. Relating protein pharmacology by ligand chemistry. Nat Biotechnol 2007; **25:** 197-206.
Keiser MJ, Setola V, Irwin JJ, Laggner C, Abbas AI, Hufeisen SJ, Jensen NH, Kuijer MB, Matos RC, Tran TB, Whaley R, Glennon RA, Hert J, Thomas KL, Edwards DD, Shoichet BK, Roth BL. Predicting new molecular targets for known drugs. Nature 2009; **462:** 175-81.
Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ, von Zastrow M. mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. Mol Pharmacol 1998; **53:** 377-84.
Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, Kramer A, Maier B. A circadian clock in macrophages controls inflammatory immune responses. Proc Natl Acad Sci U S A 2009; **106:** 21407-12.
Kelly E, Bailey CP, Henderson G. Agonist-selective mechanisms of GPCR desensitization. Br J Pharmacol 2008; **153 Suppl 1:** S379-88.
Kennedy BC, Panksepp JB, Wong JC, Krause EJ, Lahvis GP. Age-dependent and strain-dependent influences of morphine on mouse social investigation behavior. Behavioural pharmacology 2011.

Khan MM, Sansoni P, Silverman ED, Engleman EG, Melmon KL. Beta-adrenergic receptors on human suppressor, helper, and cytolytic lymphocytes. Biochem Pharmacol 1986; **35:** 1137-42.

Kidd S, Brennan S, Stephen R, Minns R, Beattie T. Comparison of morphine concentration-time profiles following intravenous and intranasal diamorphine in children. Arch Dis Child 2009; **94:** 974-8.

Kilpatrick GJ, Smith TW. Morphine-6-glucuronide: actions and mechanisms. Med Res Rev 2005; **25:** 521-44.

Kim I, Barnes AJ, Oyler JM, Schepers R, Joseph RE, Jr., Cone EJ, Lafko D, Moolchan ET, Huestis MA. Plasma and oral fluid pharmacokinetics and pharmacodynamics after oral codeine administration. Clin Chem 2002; **48:** 1486-96.

Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology 2007; **121:** 1-14.

Kin NW, Sanders VM. It takes nerve to tell T and B cells what to do. J Leukoc Biol 2006; **79:** 1093-104.

Kirchheiner J, Schmidt H, Tzvetkov M, Keulen JT, Lotsch J, Roots I, Brockmoller J. Pharmacokinetics of codeine and its metabolite morphine in ultra-rapid metabolizers due to CYP2D6 duplication. Pharmacogenomics J 2007; **7:** 257-65.

Klein TW, Newton C, Larsen K, Chou J, Perkins I, Lu L, Nong L, Friedman H. Cannabinoid receptors and T helper cells. J Neuroimmunol. 2004; **147:** 91-94.

Klepstad P, Fladvad T, Skorpen F, Bjordal K, Caraceni A, Dale O, Davies A, Kloke M, Lundstrom S, Maltoni M, Radbruch L, Sabatowski R, Sigurdardottir V, Strasser F, Fayers PM, Kaasa S. Influence from genetic variability on opioid use for cancer pain: a European genetic association study of 2294 cancer pain patients. Pain 2011.

Klepstad P, Rakvag TT, Kaasa S, Holthe M, Dale O, Borchgrevink PC, Baar C, Vikan T, Krokan HE, Skorpen F. The 118 A > G polymorphism in the human mu-opioid receptor gene may increase morphine requirements in patients with pain caused by malignant disease. Acta Anaesthesiol Scand 2004; **48:** 1232-9.

Koodie L, Ramakrishnan S, Roy S. Morphine suppresses tumor angiogenesis through a HIF-1alpha/p38MAPK pathway. The American journal of pathology 2010; **177:** 984-97.

Kraus J. Regulation of mu-opioid receptors by cytokines. Front Biosci.(Schol.Ed) 2009; **1:** 164-70.

Kraus J, Borner C, Giannini E, Hickfang K, Braun H, Mayer P, Hoehe MR, Ambrosch A, Konig W, Hollt V. Regulation of mu-opioid receptor gene transcription by interleukin-4 and influence of an allelic variation within a STAT6 transcription factor binding site. J Biol Chem. 2001; **276:** 43901-08.

Kraus J, Borner C, Giannini E, Hollt V. The role of nuclear factor kappaB in tumor necrosis factor-regulated transcription of the human mu-opioid receptor gene. Mol.Pharmacol 2003; **64:** 876-84.

Kraus J, Borner C, Lendeckel U, Hollt V. Interferon-gamma down-regulates transcription of the mu-opioid receptor gene in neuronal and immune cells. J Neuroimmunol 2006; **181:** 13-8.

Kraus J, Horn G, Zimprich A, Simon T, Mayer P, Hollt V. Molecular cloning and functional analysis of the rat mu opioid receptor gene promoter. Biochem.Biophys.Res.Commun. 1995; **215:** 591-97.

Kristensen K, Christensen CB, Christrup LL. The mu1, mu2, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. Life Sci 1995; **56:** PL45-50.

Kuno K, Matsushima K. The IL-1 receptor signaling pathway. J Leukoc Biol 1994; **56:** 542-7.

Labuz D, Schmidt Y, Schreiter A, Rittner HL, Mousa SA, Machelska H. Immune cell-derived opioids protect against neuropathic pain in mice. J Clin.Invest 2009; **119:** 278-86.

Labuz D, Schreiter A, Schmidt Y, Brack A, Machelska H. T lymphocytes containing beta-endorphin ameliorate mechanical hypersensitivity following nerve injury. Brain Behav Immun 2010.

Lacroix-Fralish ML, Mogil JS. Progress in genetic studies of pain and analgesia. Annual review of pharmacology and toxicology 2009; **49:** 97-121.

Laird BJ, Scott AC, Colvin LA, McKeon AL, Murray GD, Fearon KC, Fallon MT. Cancer pain and its relationship to systemic inflammation: an exploratory study. Pain 2011; **152:** 460-3.

Langford DJ, Tuttle AH, Briscoe C, Harvey-Lewis C, Baran I, Gleeson P, Fischer DB, Buonora M, Sternberg WF, Mogil JS. Varying perceived social threat modulates pain behavior in

male mice. The journal of pain : official journal of the American Pain Society 2011; **12:** 125-32.
Lariviere WR, Mogil JS. The genetics of pain and analgesia in laboratory animals. Methods in molecular biology 2010; **617:** 261-78.
Larsen B, Hoff G, Wilhelm W, Buchinger H, Wanner GA, Bauer M. Effect of intravenous anesthetics on spontaneous and endotoxin-stimulated cytokine response in cultured human whole blood. Anesthesiology 1998; **89:** 1218-27.
Law PY, Wong YH, Loh HH. Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 2000; **40:** 389-430.
Lawson KP, Nag S, Thompson AD, Mokha SS. Sex-specificity and estrogen-dependence of kappa opioid receptor-mediated antinociception and antihyperalgesia. Pain 2010; **151:** 806-15.
Lazaro MI, Tomassini N, Gonzalez I, Renaud FL. Reversibility of morphine effects on phagocytosis by murine macrophages. Drug Alcohol Depend. 2000; **58:** 159-64.
Lee JD, Rhoades K, Economou JS. Interleukin-4 inhibits the expression of tumour necrosis factors alpha and beta, interleukins-1 beta and -6 and interferon-gamma. Immunol Cell Biol 1995a; **73:** 57-61.
Lee PJ, Delaney P, Keogh J, Sleeman D, Shorten GD. Catecholamine-o-methyltransferase polymorphisms are associated with postoperative pain intensity. The Clinical journal of pain 2011; **27:** 93-101.
Lee SC, Kress Y, Zhao ML, Dickson DW, Casadevall A. Cryptococcus neoformans survive and replicate in human microglia. Lab Invest 1995b; **73:** 871-9.
Lehmann KA, Kratzenberg U, Schroeder-Bark B, Horrichs-Haermeyer G. Postoperative patient-controlled analgesia with tramadol: analgesic efficacy and minimum effective concentrations. Clin J Pain 1990; **6:** 212-20.
Lenz H, Sandvik L, Qvigstad E, Bjerkelund CE, Raeder J. A comparison of intravenous oxycodone and intravenous morphine in patient-controlled postoperative analgesia after laparoscopic hysterectomy. Anesth Analg 2009; **109:** 1279-83.
Lewis SS, Hutchinson MR, Rezvani N, Loram LC, Zhang Y, Maier SF, Rice KC, Watkins LR. Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1beta. Neuroscience 2010; **165:** 569-83.
Li D, Xu XN. NKT cells in HIV-1 infection. Cell Res. 2008; **18:** 817-22.
Liang D, Shi X, Qiao Y, Angst MS, Yeomans DC, Clark JD. Chronic morphine administration enhances nociceptive sensitivity and local cytokine production after incision. Mol Pain 2008; **4:** 7.
Liew FY. T(H)1 and T(H)2 cells: a historical perspective. Nat Rev Immunol 2002; **2:** 55-60.
Liljefors M, Nilsson B, Hjelm Skog AL, Ragnhammar P, Mellstedt H, Frodin JE. Natural killer (NK) cell function is a strong prognostic factor in colorectal carcinoma patients treated with the monoclonal antibody 17-1A. Int J Cancer 2003; **105:** 717-23.
Lipovsky MM, Gekker G, Hu S, Hoepelman AI, Peterson PK. Morphine enhances complement receptor-mediated phagocytosis of Cryptococcus neoformans by human microglia. Clin Immunol Immunopathol 1998; **87:** 163-7.
Liu Y, Blackbourn DJ, Chuang LF, Killam KF, Jr., Chuang RY. Effects of in vivo and in vitro administration of morphine sulfate upon rhesus macaque polymorphonuclear cell phagocytosis and chemotaxis. J Pharmacol Exp Ther 1992; **263:** 533-9.
Liu Z, Gao F, Tian Y. Effects of morphine, fentanyl and tramadol on human immune response. J Huazhong Univ Sci Technolog Med Sci 2006; **26:** 478-81.
Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunology today 1990; **11:** 237-44.
Logani MK, Alekseev S, Bhopale MK, Slovinsky WS, Ziskin MC. Effect of millimeter waves and cyclophosphamide on cytokine regulation. Immunopharmacology and immunotoxicology 2011.
Lopez-Gimenez JF, Milligan G. Opioid regulation of mu receptor internalisation: relevance to the development of tolerance and dependence. CNS & neurological disorders drug targets 2010; **9:** 616-26.
Lotsch J, Klepstad P, Doehring A, Dale O. A GTP cyclohydrolase 1 genetic variant delays cancer pain. Pain 2010; **148:** 103-6.
Lotsch J, Skarke C, Schmidt H, Rohrbacher M, Hofmann U, Schwab M, Geisslinger G. Evidence for morphine-independent central nervous opioid effects after administration

of codeine: contribution of other codeine metabolites. Clin Pharmacol Ther 2006; **79:** 35-48.
Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clin Microbiol Rev 1996; **9:** 532-62.
Lund FE, Randall TD. Effector and regulatory B cells: modulators of CD4(+) T cell immunity. Nat Rev Immunol 2010; **10:** 236-47.
Luster AD, Unkeless JC, Ravetch JV. Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature 1985; **315:** 672-6.
Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 2002; **115:** 455-65.
Lysle DT, Coussons ME, Watts VJ, Bennett EH, Dykstra LA. Morphine-induced alterations of immune status: dose dependency, compartment specificity and antagonism by naltrexone. J Pharmacol Exp Ther 1993; **265:** 1071-8.
Lysle DT, Hoffman KE, Dykstra LA. Evidence for the involvement of the caudal region of the periaqueductal gray in a subset of morphine-induced alterations of immune status. J Pharmacol Exp Ther 1996; **277:** 1533-40.
Ma CS, Tangye SG, Deenick EK. Human Th9 cells: inflammatory cytokines modulate IL-9 production through the induction of IL-21. Immunology and cell biology 2010; **88:** 621-3.
Machelska H, Stein C. Leukocyte-derived opioid peptides and inhibition of pain. J.Neuroimmune.Pharmacol. 2006; **1:** 90-97.
Maddocks I, Somogyi A, Abbott F, Hayball P, Parker D. Attenuation of morphine-induced delirium in palliative care by substitution with infusion of oxycodone. J Pain Symptom Manage 1996; **12:** 182-9.
Madjd Z, Parsons T, Watson NF, Spendlove I, Ellis I, Durrant LG. High expression of Lewis y/b antigens is associated with decreased survival in lymph node negative breast carcinomas. Breast cancer research : BCR 2005; **7:** R780-7.
Magazine HI, Chang J, Goumon Y, Stefano GB. Rebound from nitric oxide inhibition triggers enhanced monocyte activation and chemotaxis. J Immunol 2000; **165:** 102-7.
Magazine HI, Liu Y, Bilfinger TV, Fricchione GL, Stefano GB. Morphine-induced conformational changes in human monocytes, granulocytes, and endothelial cells and in invertebrate immunocytes and microglia are mediated by nitric oxide. J Immunol 1996; **156:** 4845-50.
Maguire P, Tsai N, Kamal J, Cometta-Morini C, Upton C, Loew G. Pharmacological profiles of fentanyl analogs at mu, delta and kappa opiate receptors. European journal of pharmacology 1992; **213:** 219-25.
Maino VC, Suni MA, Ruitenberg JJ. Rapid flow cytometric method for measuring lymphocyte subset activation. Cytometry 1995; **20:** 127-33.
Makman MH, Dvorkin B, Stefano GB. Murine macrophage cell lines contain mu 3-opiate receptors. Eur J Pharmacol 1995; **273:** R5-6.
Manchikanti L, Vallejo R, Manchikanti KN, Benyamin RM, Datta S, Christo PJ. Effectiveness of long-term opioid therapy for chronic non-cancer pain. Pain physician 2011; **14:** E133-56.
Manson JE. Pain: sex differences and implications for treatment. Metabolism 2010; **59 Suppl 1:** S16-20.
Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nature reviews. Immunology 2011; **11:** 519-31.
Mardiney M, 3rd, Brown MR, Fleisher TA. Measurement of T-cell CD69 expression: a rapid and efficient means to assess mitogen- or antigen-induced proliferative capacity in normals. Cytometry 1996; **26:** 305-10.
Martin JL, Charboneau R, Barke RA, Roy S. Chronic morphine treatment inhibits LPS-induced angiogenesis: implications in wound healing. Cellular immunology 2010; **265:** 139-45.
Martini L, Whistler JL. The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. Curr.Opin.Neurobiol. 2007; **17:** 556-64.
Martucci C, Franchi S, Lattuada D, Panerai AE, Sacerdote P. Differential involvement of RelB in morphine-induced modulation of chemotaxis, NO, and cytokine production in murine macrophages and lymphocytes. J.Leukoc.Biol. 2007; **81:** 344-54.
Martucci C, Panerai AE, Sacerdote P. Chronic fentanyl or buprenorphine infusion in the mouse: similar analgesic profile but different effects on immune responses. Pain 2004; **110:** 385-92.

Mazoit JX, Butscher K, Samii K. Morphine in postoperative patients: pharmacokinetics and pharmacodynamics of metabolites. Anesth.Analg. 2007; **105:** 70-78.
Mazzardo-Martins L, Martins DF, Marcon R, Dos Santos UD, Speckhann B, Gadotti VM, Sigwalt AR, Guglielmo LG, Soares Santos AR. High-Intensity Extended Swimming Exercise Reduces Pain-Related Behavior in Mice: Involvement of Endogenous Opioids and the Serotonergic System. J Pain 2010.
Meert TF, Vermeirsch HA. A preclinical comparison between different opioids: antinociceptive versus adverse effects. Pharmacol Biochem Behav 2005; **80:** 309-26.
Mellon RD, Bayer BM. Role of central opioid receptor subtypes in morphine-induced alterations in peripheral lymphocyte activity. Brain Res. 1998; **789:** 56-67.
Menon AG, Janssen-van Rhijn CM, Morreau H, Putter H, Tollenaar RA, van de Velde CJ, Fleuren GJ, Kuppen PJ. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. Lab Invest 2004; **84:** 493-501.
Menon AG, Morreau H, Tollenaar RA, Alphenaar E, Van Puijenbroek M, Putter H, Janssen-Van Rhijn CM, Van De Velde CJ, Fleuren GJ, Kuppen PJ. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. Lab Invest 2002; **82:** 1725-33.
Mercadante S, Bianchi M, Villari P, Ferrera P, Casuccio A, Fulfaro F, Gebbia V. Opioid plasma concentration during switching from morphine to methadone: preliminary data. Support Care Cancer 2003; **11:** 326-31.
Mercadante S, Villari P, Ferrera P, Casuccio A, Gambaro V. Opioid plasma concentrations during a switch from transdermal fentanyl to methadone. J Palliat.Med. 2007; **10:** 338-44.
Mercer SL, Coop A. Opioid Analgesics and P-glycoprotein Efflux Transporters: A Potential Systems-Level Contribution to Analgesic Tolerance. Curr Top Med Chem 2010.
Meuldermans WE, Hurkmans RM, Heykants JJ. Plasma protein binding and distribution of fentanyl, sufentanil, alfentanil and lofentanil in blood. Arch Int Pharmacodyn Ther 1982; **257:** 4-19.
Mignat C, Wille U, Ziegler A. Affinity profiles of morphine, codeine, dihydrocodeine and their glucuronides at opioid receptor subtypes. Life sciences 1995; **56:** 793-9.
Mika J. Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. Pharmacol.Rep. 2008; **60:** 297-307.
Mitchell TB, Lintzeris N, Bond A, Strang J. Feasibility and acceptability of an intranasal diamorphine spray as an alternative to injectable diamorphine for maintenance treatment. Eur Addict Res 2006; **12:** 91-5.
Mocellin S, Marincola F, Rossi CR, Nitti D, Lise M. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. Cytokine & growth factor reviews 2004; **15:** 61-76.
Mody M, Lazarus AH, Semple JW, Freedman J. Preanalytical requirements for flow cytometric evaluation of platelet activation: choice of anticoagulant. Transfusion medicine 1999; **9:** 147-54.
Mogil JS, Bailey AL. Sex and gender differences in pain and analgesia. Progress in brain research 2010; **186:** 141-57.
Mohan S, Davis RL, DeSilva U, Stevens CW. Dual regulation of mu opioid receptors in SK-N-SH neuroblastoma cells by morphine and interleukin-1beta: evidence for opioid-immune crosstalk. Journal of neuroimmunology 2010; **227:** 26-34.
Molina PE, Hashiguchi Y, Meijerink WJ, Naukam RJ, Boxer R, Abumrad NN. Modulation of endogenous opiate production: effect of fasting. Biochem Biophys Res Commun 1995; **207:** 312-7.
Molitor TW, Murtaugh MP, Click RE, Gekker G, Chao C, Peterson PK. Functional alterations of swine peripheral blood mononuclear cells by methadone. J Leukoc Biol 1992; **51:** 124-8.
Molloy S. Innate immunity: The stress connection. Nat Rev Micro 2010; **8:** 387-87.
Morita CT, Mariuzza RA, Brenner MB. Antigen recognition by human gamma delta T cells: pattern recognition by the adaptive immune system. Springer seminars in immunopathology 2000; **22:** 191-217.
Mossner R, Lesch KP. Role of serotonin in the immune system and in neuroimmune interactions. Brain Behav Immun 1998; **12:** 249-71.

Motzer SA, Tsuji J, Hertig V, Johnston SK, Scanlan J. Natural killer cell cytotoxicity: a methods analysis of 51chromium release versus flow cytometry. Biological research for nursing 2003; **5:** 142-52.

Mucci-LoRusso P, Berman BS, Silberstein PT, Citron ML, Bressler L, Weinstein SM, Kaiko RF, Buckley BJ, Reder RF. Controlled-release oxycodone compared with controlled-release morphine in the treatment of cancer pain: a randomized, double-blind, parallel-group study. Eur.J Pain 1998; **2:** 239-49.

Myles PS, Peyton P, Silbert B, Hunt J, Rigg JR, Sessler DI. Perioperative epidural analgesia for major abdominal surgery for cancer and recurrence-free survival: randomised trial. BMJ 2011; **342:** d1491.

Nair MP, Schwartz SA, Polasani R, Hou J, Sweet A, Chadha KC. Immunoregulatory effects of morphine on human lymphocytes. Clin Diagn Lab Immunol 1997; **4:** 127-32.

Nair MP, Schwartz SA, Wu K, Kronfol Z. Effect of neuropeptide Y on natural killer activity of normal human lymphocytes. Brain Behav Immun 1993; **7:** 70-8.

Nakamura H, Kawasaki N, Hagiwara M, Saito M, Konaka C, Kato H. Cellular immunologic parameters related to age, gender, and stage in lung cancer patients. Lung Cancer 2000; **28:** 139-45.

Negus S, Woods J. Reinforcing effects, discriminative stimulus effects and physical dependence liability of buprenorphine. In: Cowan A, Lewis J, eds. *Buprenorphine; Combatting Drug Abuse with a Unique Opioid*. New York: Wiley-Liss, Inc; 1995:71–101.

Nelson CJ, Dykstra LA, Lysle DT. Comparison of the time course of morphine's analgesic and immunologic effects. Anesth Analg 1997; **85:** 620-6.

Nelson CJ, Schneider GM, Lysle DT. Involvement of central mu- but not delta- or kappa-opioid receptors in immunomodulation. Brain Behav.Immun. 2000; **14:** 170-84.

Nestler EJ. Historical review: Molecular and cellular mechanisms of opiate and cocaine addiction. Trends Pharmacol Sci 2004; **25:** 210-8.

Nielsen DA, Yuferov V, Hamon S, Jackson C, Ho A, Ott J, Kreek MJ. Increased OPRM1 DNA methylation in lymphocytes of methadone-maintained former heroin addicts. Neuropsychopharmacology 2009; **34:** 867-73.

Nikiteas NI, Tzanakis N, Gazouli M, Rallis G, Daniilidis K, Theodoropoulos G, Kostakis A, Peros G. Serum IL-6, TNFalpha and CRP levels in Greek colorectal cancer patients: prognostic implications. World journal of gastroenterology : WJG 2005; **11:** 1639-43.

Nikolarakis KE, Pfeiffer A, Stalla GK, Herz A. Facilitation of ACTH secretion by morphine is mediated by activation of CRF releasing neurons and sympathetic neuronal pathways. Brain Res 1989; **498:** 385-8.

Novick DM, Ochshorn M, Ghali V, Croxson TS, Mercer WD, Chiorazzi N, Kreek MJ. Natural killer cell activity and lymphocyte subsets in parenteral heroin abusers and long-term methadone maintenance patients. J Pharmacol Exp Ther 1989; **250:** 606-10.

Nüssler NC, Stange BJ, Petzold M, Nussler AK, Glanemann M, Guckelberger O. Reduced NK-Cell Activity in Patients with Metastatic Colon Cancer. EXCLI Journal 2007**:** 1-9.

O'Garra A, Arai N. The molecular basis of T helper 1 and T helper 2 cell differentiation. Trends Cell Biol 2000; **10:** 542-50.

O'Neill LA. Interleukin-1 signal transduction. International journal of clinical & laboratory research 1995; **25:** 169-77.

Obar JJ, Molloy MJ, Jellison ER, Stoklasek TA, Zhang W, Usherwood EJ, Lefrancois L. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. Proc Natl Acad Sci U S A 2010; **107:** 193-8.

Oda Y, Kharasch ED. Metabolism of methadone and levo-alpha-acetylmethadol (LAAM) by human intestinal cytochrome P450 3A4 (CYP3A4): potential contribution of intestinal metabolism to presystemic clearance and bioactivation. J Pharmacol Exp Ther 2001; **298:** 1021-32.

Oertel BG, Schmidt R, Schneider A, Geisslinger G, Lotsch J. The mu-opioid receptor gene polymorphism 118A>G depletes alfentanil-induced analgesia and protects against respiratory depression in homozygous carriers. Pharmacogenet Genomics 2006; **16:** 625-36.

Orditura M, Romano C, De Vita F, Galizia G, Lieto E, Infusino S, De Cataldis G, Catalano G. Behaviour of interleukin-2 serum levels in advanced non-small-cell lung cancer patients: relationship with response to therapy and survival. Cancer Immunol Immunother 2000; **49:** 530-6.

Ordonez Gallego A, Gonzalez Baron M, Espinosa Arranz E. Oxycodone: a pharmacological and clinical review. Clin Transl Oncol 2007; **9:** 298-307.
Pacifici R, di Carlo S, Bacosi A, Pichini S, Zuccaro P. Pharmacokinetics and cytokine production in heroin and morphine-treated mice. Int J Immunopharmacol 2000; **22:** 603-14.
Pacifici R, Patrini G, Venier I, Parolaro D, Zuccaro P, Gori E. Effect of morphine and methadone acute treatment on immunological activity in mice: pharmacokinetic and pharmacodynamic correlates. J Pharmacol Exp Ther 1994; **269:** 1112-6.
Page GG. The immune-suppressive effects of pain. Adv.Exp.Med.Biol. 2003; **521:** 117-25.
Page GG. Immunologic effects of opioids in the presence or absence of pain. J.Pain Symptom.Manage. 2005; **29:** S25-S31.
Page GG, Blakely WP, Ben-Eliyahu S. Evidence that postoperative pain is a mediator of the tumor-promoting effects of surgery in rats. Pain 2001; **90:** 191-99.
Paldyova E, Bereczki E, Santha M, Wenger T, Borsodi A, Benyhe S. Altered gene expression and functional activity of opioid receptors in the cerebellum of CB1 cannabinoid receptor knockout mice after acute treatments with cannabinoids. Acta biologica Hungarica 2007; **58 Suppl:** 113-29.
Paller CJ, Campbell CM, Edwards RR, Dobs AS. Sex-based differences in pain perception and treatment. Pain Med 2009; **10:** 289-99.
Palm S, Mignat C, Kuhn K, Umbreit H, Barth J, Stuber F, Maier C. Phytohemagglutinin-dependent T-cell proliferation is not impaired by morphine. Methods Find Exp Clin Pharmacol 1996; **18:** 159-65.
Pan YX. Diversity and complexity of the mu opioid receptor gene: alternative pre-mRNA splicing and promoters. DNA Cell Biol 2005; **24:** 736-50.
Pan YX, Xu J, Mahurter L, Bolan E, Xu M, Pasternak GW. Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene. Proc Natl Acad Sci U S A 2001; **98:** 14084-9.
Park HJ, Shinn HK, Ryu SH, Lee HS, Park CS, Kang JH. Genetic polymorphisms in the ABCB1 gene and the effects of fentanyl in Koreans. Clin Pharmacol Ther 2007; **81:** 539-46.
Patrini G, Massi P, Ricevuti G, Mazzone A, Fossati G, Mazzucchelli I, Gori E, Parolaro D. Changes in opioid receptor density on murine splenocytes induced by in vivo treatment with morphine and methadone. J Pharmacol Exp Ther 1996; **279:** 172-6.
Paul WE. Interleukin-4: a prototypic immunoregulatory lymphokine. Blood 1991; **77:** 1859-70.
Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? Nat Rev Immunol 2010; **10:** 225-35.
Paust S, Senman B, Andrian UHv. Adaptive immune responses mediated by natural killer cells. Immunological Reviews 2010; **235:** 286-96.
Pawelec G. Tumour escape: antitumour effectors too much of a good thing? Cancer Immunol Immunother 2004; **53:** 262-74.
Pedersen BK. Muscular IL-6 and Its Role as an Energy Sensor. Medicine and science in sports and exercise 2011.
Peiro AM, Margarit C, Gimenez P, Horga JF. Pharmacogenetic testing is of limited utility for predicting analgesic response to morphine. J Pain Symptom Manage 2010; **39:** e8-e11.
Peng X, Mosser DM, Adler MW, Rogers TJ, Meissler JJ, Jr., Eisenstein TK. Morphine enhances interleukin-12 and the production of other pro-inflammatory cytokines in mouse peritoneal macrophages. J Leukoc Biol 2000; **68:** 723-8.
Pergolizzi J, Boger RH, Budd K, Dahan A, Erdine S, Hans G, Kress HG, Langford R, Likar R, Raffa RB, Sacerdote P. Opioids and the management of chronic severe pain in the elderly: consensus statement of an International Expert Panel with focus on the six clinically most often used World Health Organization step III opioids (buprenorphine, fentanyl, hydromorphone, methadone, morphine, oxycodone). Pain Pract. 2008; **8:** 287-313.
Peroutka SJ, Howell TA. The molecular evolution of G protein-coupled receptors: focus on 5-hydroxytryptamine receptors. Neuropharmacology 1994; **33:** 319-24.
Pesonen A, Suojaranta-Ylinen R, Hammaren E, Tarkkila P, Seppala T, Rosenberg PH. Comparison of effects and plasma concentrations of opioids between elderly and middle-aged patients after cardiac surgery. Acta Anaesthesiol Scand 2009; **53:** 101-8.
Peterson PK, Gekker G, Brummitt C, Pentel P, Bullock M, Simpson M, Hitt J, Sharp B. Suppression of human peripheral blood mononuclear cell function by methadone and morphine. J.Infect.Dis. 1989; **159:** 480-87.

Peterson PK, Gekker G, Hu S, Sheng WS, Molitor TW, Chao CC. Morphine stimulates phagocytosis of Mycobacterium tuberculosis by human microglial cells: involvement of a G protein-coupled opiate receptor. Adv Neuroimmunol 1995; **5:** 299-309.

Peterson PK, Molitor TW, Chao CC. The opioid-cytokine connection. J.Neuroimmunol. 1998; **83:** 63-69.

Peterson PK, Sharp B, Gekker G, Brummitt C, Keane WF. Opioid-mediated suppression of cultured peripheral blood mononuclear cell respiratory burst activity. J Immunol 1987; **138:** 3907-12.

Pine SR, Mechanic LE, Enewold L, Chaturvedi AK, Katki HA, Zheng YL, Bowman ED, Engels EA, Caporaso NE, Harris CC. Increased levels of circulating interleukin 6, interleukin 8, C-reactive protein, and risk of lung cancer. Journal of the National Cancer Institute 2011; **103:** 1112-22.

Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 2005; **5:** 375-86.

Qian YN, Jin WJ, Wang L, Wang HJ. Effect of different concentrations of morphine and tramadol on the differentiation of human helper T cells in vitro. Br.J Anaesth. 2005; **95:** 277.

Quinones-Jenab V, Jenab S, Ogawa S, Inturrisi C, Pfaff DW. Estrogen regulation of mu-opioid receptor mRNA in the forebrain of female rats. Brain Res.Mol.Brain Res. 1997; **47:** 134-38.

Rabquer BJ, Tsou PS, Hou Y, Thirunavukkarasu E, Haines GK, 3rd, Impens AJ, Phillips K, Kahaleh B, Seibold JR, Koch AE. Dysregulated expression of MIG/CXCL9, IP-10/CXCL10 and CXCL16 and their receptors in systemic sclerosis. Arthritis research & therapy 2011; **13:** R18.

Raffa RB. A novel approach to the pharmacology of analgesics. Am J Med 1996; **101:** 40S-46S.

Raffa RB, Friderichs E, Reimann W, Shank RP, Codd EE, Vaught JL, Jacoby HI, Selve N. Complementary and synergistic antinociceptive interaction between the enantiomers of tramadol. J Pharmacol Exp Ther 1993; **267:** 331-40.

Rakvag TT, Klepstad P, Baar C, Kvam TM, Dale O, Kaasa S, Krokan HE, Skorpen F. The Val158Met polymorphism of the human catechol-O-methyltransferase (COMT) gene may influence morphine requirements in cancer pain patients. Pain 2005; **116:** 73-8.

Rakvag TT, Ross JR, Sato H, Skorpen F, Kaasa S, Klepstad P. Genetic variation in the catechol-O-methyltransferase (COMT) gene and morphine requirements in cancer patients with pain. Mol Pain 2008; **4:** 64.

Ramnath N, Tan D, Li Q, Hylander BL, Bogner P, Ryes L, Ferrone S. Is downregulation of MHC class I antigen expression in human non-small cell lung cancer associated with prolonged survival? Cancer immunology, immunotherapy : CII 2006; **55:** 891-9.

Rao Y, Wang YL, Li JG, Ke JJ. Effects of morphine and fentanyl on tumor necrosis factor-alpha and interleukin-6 concentrations in human whole blood in vitro. Chin Med J (Engl) 2004; **117:** 303-4.

Reiman JM, Kmieciak M, Manjili MH, Knutson KL. Tumor immunoediting and immunosculpting pathways to cancer progression. Semin.Cancer Biol 2007; **17:** 275-87.

Rentsch KM, Kullak-Ublick GA, Reichel C, Meier PJ, Fattinger K. Arterial and venous pharmacokinetics of intravenous heroin in subjects who are addicted to narcotics. Clin Pharmacol Ther 2001; **70:** 237-46.

Rittner HL, Brack A, Stein C. Pain and the immune system. Br.J.Anaesth. 2008; **101:** 40-44.

Robinson SL, Rowbotham DJ, Smith G. Morphine compared with diamorphine. A comparison of dose requirements and side-effects after hip surgery. Anaesthesia 1991; **46:** 538-40.

Rojavin M, Szabo I, Bussiere JL, Rogers TJ, Adler MW, Eisenstein TK. Morphine treatment in vitro or in vivo decreases phagocytic functions of murine macrophages. Life Sci 1993; **53:** 997-1006.

Rook EJ, Huitema AD, van den Brink W, van Ree JM, Beijnen JH. Pharmacokinetics and pharmacokinetic variability of heroin and its metabolites: review of the literature. Curr Clin Pharmacol 2006; **1:** 109-18.

Roozafzoon R, Goodarzi A, Vousooghi N, Sedaghati M, Yaghmaei P, Zarrindast MR. Expression of NMDA receptor subunits in human peripheral blood lymphocytes in opioid addiction. Eur J Pharmacol 2010; **638:** 29-32.

Ross JR, Riley J, Quigley C, Welsh KI. Clinical pharmacology and pharmacotherapy of opioid switching in cancer patients. Oncologist 2006; **11:** 765-73.

Rossi GC, Brown GP, Leventhal L, Yang K, Pasternak GW. Novel receptor mechanisms for heroin and morphine-6 beta-glucuronide analgesia. Neurosci Lett 1996; **216:** 1-4.

Rossi S, Furlan R, De Chiara V, Muzio L, Musella A, Motta C, Studer V, Cavasinni F, Bernardi G, Martino G, Cravatt BF, Lutz B, Maccarrone M, Centonze D. Cannabinoid CB1 receptors regulate neuronal TNF-alpha effects in experimental autoimmune encephalomyelitis. Brain, behavior, and immunity 2011.

Roth BL, Willins DL. What's all the RAVE about receptor internalization? Neuron 1999; **23:** 629-31.

Rothe G, Valet G. Flow cytometric assays of oxidative burst activity in phagocytes. Methods Enzymol 1994; **233:** 539-48.

Roy S, Balasubramanian S, Sumandeep S, Charboneau R, Wang J, Melnyk D, Beilman GJ, Vatassery R, Barke RA. Morphine directs T cells toward T(H2) differentiation. Surgery 2001; **130:** 304-9.

Roy S, Barke RA, Loh HH. MU-opioid receptor-knockout mice: role of mu-opioid receptor in morphine mediated immune functions. Brain Res.Mol.Brain Res. 1998a; **61:** 190-94.

Roy S, Cain KJ, Chapin RB, Charboneau RG, Barke RA. Morphine modulates NF kappa B activation in macrophages. Biochem Biophys Res Commun 1998b; **245:** 392-6.

Roy S, Ninkovic J, Banerjee S, Charboneau RG, Das S, Dutta R, Kirchner VA, Koodie L, Ma J, Meng J, Barke RA. Opioid Drug Abuse and Modulation of Immune Function: Consequences in the Susceptibility to Opportunistic Infections. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology 2011.

Roy S, Wang J, Charboneau R, Loh HH, Barke RA. Morphine induces CD4+ T cell IL-4 expression through an adenylyl cyclase mechanism independent of the protein kinase A pathway. J Immunol 2005; **175:** 6361-7.

Rozenfeld R, Devi LA. Receptor heterodimerization leads to a switch in signaling: beta-arrestin2-mediated ERK activation by mu-delta opioid receptor heterodimers. FASEB J 2007; **21:** 2455-65.

Rubovitch V, Gafni M, Sarne Y. The mu opioid agonist DAMGO stimulates cAMP production in SK-N-SH cells through a PLC-PKC-Ca++ pathway. Brain Res Mol Brain Res 2003; **110:** 261-6.

Sabat R, Grutz G, Warszawska K, Kirsch S, Witte E, Wolk K, Geginat J. Biology of interleukin-10. Cytokine & growth factor reviews 2010; **21:** 331-44.

Sacerdote P. Opioid-induced immunosuppression. Curr.Opin.Support.Palliat.Care 2008; **2:** 14-18.

Sacerdote P, Bianchi M, Gaspani L, Manfredi B, Maucione A, Terno G, Ammatuna M, Panerai AE. The effects of tramadol and morphine on immune responses and pain after surgery in cancer patients. Anesth.Analg. 2000; **90:** 1411-14.

Sacerdote P, Bianchi M, Gaspani L, Panerai AE. Effects of tramadol and its enantiomers on Concanavalin-A induced-proliferation and NK activity of mouse splenocytes: involvement of serotonin. Int.J Immunopharmacol. 1999; **21:** 727-34.

Sacerdote P, Franchi S, Gerra G, Leccese V, Panerai AE, Somaini L. Buprenorphine and methadone maintenance treatment of heroin addicts preserves immune function. Brain Behav.Immun. 2008; **22:** 606-13.

Sacerdote P, Manfredi B, Mantegazza P, Panerai AE. Antinociceptive and immunosuppressive effects of opiate drugs: a structure-related activity study. Br.J.Pharmacol. 1997; **121:** 834-40.

Said EA, Dupuy FP, Trautmann L, Zhang Y, Shi Y, El-Far M, Hill BJ, Noto A, Ancuta P, Peretz Y, Fonseca SG, Van Grevenynghe J, Boulassel MR, Bruneau J, Shoukry NH, Routy JP, Douek DC, Haddad EK, Sekaly RP. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. Nature medicine 2010; **16:** 452-9.

Saito H, Osaki T, Ikeguchi M. Decreased NKG2D expression on NK cells correlates with impaired NK cell function in patients with gastric cancer. Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association 2011.

Salmon AY, Goren Z, Avissar Y, Soreq H. Human erythrocyte but not brain acetylcholinesterase hydrolyses heroin to morphine. Clin Exp Pharmacol Physiol 1999; **26:** 596-600.

Samer CF, Daali Y, Wagner M, Hopfgartner G, Eap CB, Rebsamen MC, Rossier MF, Hochstrasser D, Dayer P, Desmeules JA. Genetic polymorphisms and drug interactions

modulating CYP2D6 and CYP3A activities have a major effect on oxycodone analgesic efficacy and safety. Br J Pharmacol 2010; **160:** 919-30.
Sancho D, Gomez M, Sanchez-Madrid F. CD69 is an immunoregulatory molecule induced following activation. Trends Immunol 2005; **26:** 136-40.
Sancho D, Gomez M, Viedma F, Esplugues E, Gordon-Alonso M, Garcia-Lopez MA, de la Fuente H, Martinez AC, Lauzurica P, Sanchez-Madrid F. CD69 downregulates autoimmune reactivity through active transforming growth factor-beta production in collagen-induced arthritis. J Clin Invest 2003; **112:** 872-82.
Sandel MH, Speetjens FM, Menon AG, Albertsson PA, Basse PH, Hokland M, Nagelkerke JF, Tollenaar RA, van de Velde CJ, Kuppen PJ. Natural killer cells infiltrating colorectal cancer and MHC class I expression. Mol Immunol 2005; **42:** 541-6.
Santos V, Lopez KJ, Santos LM, Yonamine M, Carmona MJ, Santos SR. Determining plasma morphine levels using GC-MS after solid phase extraction to monitor drug levels in the postoperative period. Clinics. 2008; **63:** 307-14.
Saurer TB, Carrigan KA, Ijames SG, Lysle DT. Suppression of natural killer cell activity by morphine is mediated by the nucleus accumbens shell. J.Neuroimmunol. 2006a; **173:** 3-11.
Saurer TB, Ijames SG, Lysle DT. Neuropeptide Y Y1 receptors mediate morphine-induced reductions of natural killer cell activity. J.Neuroimmunol. 2006b; **177:** 18-26.
Schantz SP, Ordonez NG. Quantitation of natural killer cell function and risk of metastatic poorly differentiated head and neck cancer. Nat Immun Cell Growth Regul 1991; **10:** 278-88.
Schedlowski M, Hosch W, Oberbeck R, Benschop RJ, Jacobs R, Raab HR, Schmidt RE. Catecholamines modulate human NK cell circulation and function via spleen-independent beta 2-adrenergic mechanisms. J Immunol 1996; **156:** 93-9.
Schneemilch CE, Hachenberg T, Ansorge S, Ittenson A, Bank U. Effects of different anaesthetic agents on immune cell function in vitro. Eur.J Anaesthesiol. 2005; **22:** 616-23.
Schramm CL, Honda CN. Co-administration of delta- and mu-opioid receptor agonists promotes peripheral opioid receptor function. Pain 2010; **151:** 763-70.
Schutze GE, Hall MA, Baker CJ, Edwards MS. Role of neutrophil receptors in opsonophagocytosis of coagulase-negative staphylococci. Infect Immun 1991; **59:** 2573-8.
Schwartz S, Etropolski M, Shapiro DY, Okamoto A, Lange R, Haeussler J, Rauschkolb C. Safety and efficacy of tapentadol ER in patients with painful diabetic peripheral neuropathy: results of a randomized-withdrawal, placebo-controlled trial. Current medical research and opinion 2011; **27:** 151-62.
Schwartz Y, Avraham R, Benish M, Rosenne E, Ben-Eliyahu S. Prophylactic IL-12 treatment reduces postoperative metastasis: mediation by increased numbers but not cytotoxicity of NK cells. Breast Cancer Res Treat 2008; **107:** 211-23.
Sergeeva MG, Grishina ZV, Varfolomeyev SD. Morphine effect on proliferation of normal and tumor cells of immune origin. Immunology letters 1993; **36:** 215-8.
Serreze DV, Chapman HD, Post CM, Johnson EA, Suarez-Pinzon WL, Rabinovitch A. Th1 to Th2 cytokine shifts in nonobese diabetic mice: sometimes an outcome, rather than the cause, of diabetes resistance elicited by immunostimulation. Journal of immunology 2001; **166:** 1352-9.
Shah JC, Mason WD. Plasma codeine and morphine concentrations after a single oral dose of codeine phosphate. J Clin Pharmacol 1990; **30:** 764-6.
Shalev I, Schmelzle M, Robson SC, Levy G. Making sense of regulatory T cell suppressive function. Seminars in immunology 2011.
Shavit Y, Ben-Eliyahu S, Zeidel A, Beilin B. Effects of fentanyl on natural killer cell activity and on resistance to tumor metastasis in rats. Dose and timing study. Neuroimmunomodulation. 2004; **11:** 255-60.
Shavit Y, Depaulis A, Martin FC, Terman GW, Pechnick RN, Zane CJ, Gale RP, Liebeskind JC. Involvement of brain opiate receptors in the immune-suppressive effect of morphine. Proc Natl Acad Sci U S A 1986; **83:** 7114-7.
Shavit Y, Lewis JW, Terman GW, Gale RP, Liebeskind JC. Opioid peptides mediate the suppressive effect of stress on natural killer cell cytotoxicity. Science 1984; **223:** 188-90.
Sheen CH, Schleimer RP, Kulka M. Codeine induces human mast cell chemokine and cytokine production: involvement of G-protein activation. Allergy 2007; **62:** 532-8.

Sheeran TP, Jackson FR, Dawes PT, Collins M, Shadforth MF. Measurement of natural killer cell cytotoxicity by area under a cytotoxic curve. A method suitable for rheumatoid arthritis. J Immunol Methods 1988; **115:** 95-8.

Shen CH, Tsai RY, Shih MS, Lin SL, Tai YH, Chien CC, Wong CS. Etanercept restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in morphine-tolerant rats. Anesthesia and analgesia 2011; **112:** 454-9.

Shephard RJ, Shek PN. Exercise, immunity, and susceptibility to infection: a j-shaped relationship? Phys Sportsmed 1999; **27:** 47-71.

Shi TX, Tong MJ, Bohman R. The application of flow cytometry in the study of natural killer cell cytotoxicity. Clinical immunology and immunopathology 1987; **45:** 356-65.

Shiow LR, Rosen DB, Brdickova N, Xu Y, An J, Lanier LL, Cyster JG, Matloubian M. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature 2006; **440:** 540-4.

Shirzad H, Shahrani M, Rafieian-Kopaei M. Comparison of morphine and tramadol effects on phagocytic activity of mice peritoneal phagocytes in vivo. Int.Immunopharmacol. 2009; **9:** 968-70.

Simms PE, Ellis TM. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. Clin Diagn Lab Immunol 1996; **3:** 301-4.

Singhal PC, Sharma P, Kapasi AA, Reddy K, Franki N, Gibbons N. Morphine enhances macrophage apoptosis. J Immunol 1998; **160:** 1886-93.

Skov Madsen P, Hokland P, Hokland M. Secretory products from thrombin-stimulated human platelets exert an inhibitory effect on NK-cytotoxic activity. Acta Pathol Microbiol Immunol Scand C 1986; **94:** 193-200.

Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annual review of immunology 2009; **27:** 591-619.

Smith TW, Binning AR, Dahan A. Efficacy and safety of morphine-6-glucuronide (M6G) for postoperative pain relief: a randomized, double-blind study. Eur J Pain 2009; **13:** 293-9.

Sokol CL, Medzhitov R. Emerging functions of basophils in protective and allergic immune responses. Mucosal Immunol 2010; **3:** 129-37.

Somogyi AA, Barratt DT, Coller JK. Pharmacogenetics of opioids. Clin Pharmacol Ther 2007; **81:** 429-44.

Sowa G, Gekker G, Lipovsky MM, Hu S, Chao CC, Molitor TW, Peterson PK. Inhibition of swine microglial cell phagocytosis of Cryptococcus neoformans by femtomolar concentrations of morphine. Biochem Pharmacol 1997; **53:** 823-8.

Stamer UM, Musshoff F, Kobilay M, Madea B, Hoeft A, Stuber F. Concentrations of tramadol and O-desmethyltramadol enantiomers in different CYP2D6 genotypes. Clin.Pharmacol Ther. 2007; **82:** 41-47.

Stefano GB, Cadet P, Kream RM, Zhu W. The presence of endogenous morphine signaling in animals. Neurochem.Res. 2008a; **33:** 1933-39.

Stefano GB, Digenis A, Spector S, Leung MK, Bilfinger TV, Makman MH, Scharrer B, Abumrad NN. Opiate-like substances in an invertebrate, an opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression. Proc.Natl.Acad.Sci.U.S.A 1993; **90:** 11099-103.

Stefano GB, Kream RM, Mantione KJ, Sheehan M, Cadet P, Zhu W, Bilfinger TV, Esch T. Endogenous morphine/nitric oxide-coupled regulation of cellular physiology and gene expression: implications for cancer biology. Semin Cancer Biol 2008b; **18:** 199-210.

Stein C, Lang LJ. Peripheral mechanisms of opioid analgesia. Curr Opin Pharmacol 2009; **9:** 3-8.

Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melian A, Bogdan C, Porcelli SA, Bloom BR, Krensky AM, Modlin RL. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 1998; **282:** 121-5.

Suzuki S, Chuang LF, Yau P, Doi RH, Chuang RY. Interactions of opioid and chemokine receptors: oligomerization of mu, kappa, and delta with CCR5 on immune cells. Exp.Cell Res. 2002; **280:** 192-200.

Suzuki S, Miyagi T, Chuang TK, Chuang LF, Doi RH, Chuang RY. Morphine upregulates mu opioid receptors of human and monkey lymphocytes. Biochem Biophys Res Commun 2000; **279:** 621-8.

Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, Figueiredo JL, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R, Pittet MJ. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science 2009; **325:** 612-6.

Szabo I, Rojavin M, Bussiere JL, Eisenstein TK, Adler MW, Rogers TJ. Suppression of peritoneal macrophage phagocytosis of Candida albicans by opioids. J Pharmacol Exp Ther 1993; **267:** 703-6.

Szkaradkiewicz A, Karpinski TM, Drews M, Borejsza-Wysocki M, Majewski P, Andrzejewska E. Natural killer cell cytotoxicity and immunosuppressive cytokines (IL-10, TGF-beta1) in patients with gastric cancer. J Biomed Biotechnol 2010; **2010:** 901564.

Taketomi A, Shimada M, Shirabe K, Kajiyama K, Gion T, Sugimachi K. Natural killer cell activity in patients with hepatocellular carcinoma: a new prognostic indicator after hepatectomy. Cancer 1998; **83:** 58-63.

Tan EC, Lim EC, Teo YY, Lim Y, Law HY, Sia AT. Ethnicity and OPRM variant independently predict pain perception and patient-controlled analgesia usage for post-operative pain. Mol Pain 2009; **5:** 32.

Tandonnet F, Bourgain JL, McGee K, Comoy E, Truffa-Bachi J. Hemodynamic and catecholamine response to isoflurane versus droperidol in complement to fentanyl anaesthesia. Acta Anaesthesiol Scand 1991; **35:** 123-8.

Tassinari D, Sartori S, Tamburini E, Scarpi E, Raffaeli W, Tombesi P, Maltoni M. Adverse effects of transdermal opiates treating moderate-severe cancer pain in comparison to long-acting morphine: a meta-analysis and systematic review of the literature. J Palliat Med 2008; **11:** 492-501.

Taub DD, Eisenstein TK, Geller EB, Adler MW, Rogers TJ. Immunomodulatory activity of mu- and kappa-selective opioid agonists. Proc.Natl.Acad.Sci.U.S.A 1991; **88:** 360-64.

Teixeira LK, Fonseca BP, Barboza BA, Viola JP. The role of interferon-gamma on immune and allergic responses. Mem Inst Oswaldo Cruz 2005; **100 Suppl 1:** 137-44.

Testi R, D'Ambrosio D, De Maria R, Santoni A. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. Immunol Today 1994; **15:** 479-83.

Thomas PT, Bhargava HN, House RV. Immunomodulatory effects of in vitro exposure to morphine and its metabolites. Pharmacology 1995a; **50:** 51-62.

Thomas PT, House RV, Bhargava HN. Direct cellular immunomodulation produced by diacetylmorphine (heroin) or methadone. Gen.Pharmacol. 1995b; **26:** 123-30.

Thomson IR, Harding G, Hudson RJ. A comparison of fentanyl and sufentanil in patients undergoing coronary artery bypass graft surgery. J Cardiothorac.Vasc.Anesth. 2000; **14:** 652-56.

Tomassini N, Renaud F, Roy S, Loh HH. Morphine inhibits Fc-mediated phagocytosis through mu and delta opioid receptors. J Neuroimmunol. 2004; **147:** 131-33.

Tomassini N, Renaud FL, Roy S, Loh HH. Mu and delta receptors mediate morphine effects on phagocytosis by murine peritoneal macrophages. J Neuroimmunol 2003; **136:** 9-16.

Tomei EZ, Renaud FL. Effect of morphine on Fc-mediated phagocytosis by murine macrophages in vitro. J Neuroimmunol 1997; **74:** 111-6.

Toskulkao T, Pornchai R, Akkarapatumwong V, Vatanatunyakum S, Govitrapong P. Alteration of lymphocyte opioid receptors in methadone maintenance subjects. Neurochem Int 2010; **56:** 285-90.

Trabold B, Gruber M, Frohlich D. Functional and phenotypic changes in polymorphonuclear neutrophils induced by catecholamines. Scandinavian cardiovascular journal : SCJ 2007; **41:** 59-64.

Trafton JA, Abbadie C, Marek K, Basbaum AI. Postsynaptic signaling via the [mu]-opioid receptor: responses of dorsal horn neurons to exogenous opioids and noxious stimulation. J Neurosci. 2000; **20:** 8578-84.

Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 1994; **84:** 4008-27.

Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003; **3:** 133-46.

Tso PH, Wong YH. Molecular basis of opioid dependence: role of signal regulation by G-proteins. Clin Exp Pharmacol Physiol 2003; **30:** 307-16.

Tsui BC, Rashiq S, Schopflocher D, Murtha A, Broemling S, Pillay J, Finucane BT. Epidural anesthesia and cancer recurrence rates after radical prostatectomy. Canadian journal of anaesthesia = Journal canadien d'anesthesie 2010; **57:** 107-12.

Tsujikawa H, Shoda T, Mizota T, Fukuda K. Morphine induces DNA damage and P53 activation in CD3(+) T cells. Biochim.Biophys.Acta 2009.

Tubaro E, Avico U, Santiangeli C, Zuccaro P, Cavallo G, Pacifici R, Croce C, Borelli G. Morphine and methadone impact on human phagocytic physiology. Int J Immunopharmacol 1985; **7:** 865-74.

Tubaro E, Santiangeli C, Belogi L, Borelli G, Cavallo G, Croce C, Avico U. Methadone vs morphine: comparison of their effect on phagocytic functions. Int J Immunopharmacol 1987; **9:** 79-88.

Twycross RG. *Symptom management in advanced cancer*. Abingdon, Oxon, UK: Radcliffe Medical Press, 1997.

Vallejo R, Barkin RL, Wang VC. Pharmacology of opioids in the treatment of chronic pain syndromes. Pain physician 2011; **14:** E343-60.

Vallejo R, de Leon-Casasola O, Benyamin R. Opioid therapy and immunosuppression: a review. Am.J.Ther. 2004; **11:** 354-65.

van de Veerdonk FL, Gresnigt MS, Kullberg BJ, van der Meer JW, Joosten LA, Netea MG. Th17 responses and host defense against microorganisms: an overview. BMB reports 2009; **42:** 776-87.

Van Der Laan JW, Timmerman H, Van Loveren H. Comparison of the in vivo effects of morphine and methadone on natural killer cell activity in spleen, peritoneal cavity, and lungs in rats. International Journal of Immunopharmacology 1996; **18:** 401-07.

van Leeuwen MT, Webster AC, McCredie MR, Stewart JH, McDonald SP, Amin J, Kaldor JM, Chapman JR, Vajdic CM, Grulich AE. Effect of reduced immunosuppression after kidney transplant failure on risk of cancer: population based retrospective cohort study. BMJ 2010; **340:** c570.

Van Loon GR, Appel NM, Ho D. beta-Endorphin-induced stimulation of central sympathetic outflow: beta-endorphin increases plasma concentrations of epinephrine, norepinephrine, and dopamine in rats. Endocrinology 1981; **109:** 46-53.

van Pelt LJ, van Zwieten R, Weening RS, Roos D, Verhoeven AJ, Bolscher BG. Limitations on the use of dihydrorhodamine 123 for flow cytometric analysis of the neutrophil respiratory burst. J Immunol Methods 1996; **191:** 187-96.

van Rijn RM, Whistler JL, Waldhoer M. Opioid-receptor-heteromer-specific trafficking and pharmacology. Current opinion in pharmacology 2010; **10:** 73-9.

Vidal EL, Patel NA, Wu G, Fiala M, Chang SL. Interleukin-1 induces the expression of mu opioid receptors in endothelial cells. Immunopharmacology 1998; **38:** 261-66.

Villegas FR, Coca S, Villarrubia VG, Jimenez R, Chillon MJ, Jareno J, Zuil M, Callol L. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer 2002; **35:** 23-8.

Von Hoff DD, Kuhn JG, Burris HA, III, Miller LJ. Does intraosseous equal intravenous? A pharmacokinetic study. Am.J Emerg.Med. 2008; **26:** 31-38.

von Zastrow M, Svingos A, Haberstock-Debic H, Evans C. Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. Curr Opin Neurobiol 2003; **13:** 348-53.

Vousooghi N, Goodarzi A, Roushanzamir F, Sedaghati T, Zarrindast MR, Noori-Daloii MR. Expression of mu opioid receptor splice variants mRNA in human blood lymphocytes: A peripheral marker for opioid addiction studies. Int.Immunopharmacol. 2009.

Walter C, Lotsch J. Meta-analysis of the relevance of the OPRM1 118A>G genetic variant for pain treatment. Pain 2009; **146:** 270-5.

Wan YY, Flavell RA. How diverse--CD4 effector T cells and their functions. Journal of molecular cell biology 2009; **1:** 20-36.

Wang G, Weng Y, Ishiguro Y, Sakamoto H, Morita S. The effect of tramadol on serum cytokine response in patients undergoing pulmonary lobectomy. J.Clin.Anesth. 2005a; **17:** 444-50.

Wang J, Barke RA, Charboneau R, Roy S. Morphine impairs host innate immune response and increases susceptibility to Streptococcus pneumoniae lung infection. J.Immunol. 2005b; **174:** 426-34.

Wang J, Barke RA, Ma J, Charboneau R, Roy S. Opiate abuse, innate immunity, and bacterial infectious diseases. Arch.Immunol.Ther.Exp.(Warsz.) 2008; **56:** 299-309.

Wang J, Charboneau R, Balasubramanian S, Barke RA, Loh HH, Roy S. Morphine modulates lymph node-derived T lymphocyte function: role of caspase-3, -8, and nitric oxide. J Leukoc Biol 2001; **70:** 527-36.

Wang J, Charboneau R, Balasubramanian S, Barke RA, Loh HH, Roy S. The immunosuppressive effects of chronic morphine treatment are partially dependent on corticosterone and mediated by the mu-opioid receptor. J.Leukoc.Biol. 2002; **71:** 782-90.

Wang X, Douglas SD, Peng JS, Zhou DJ, Wan Q, Ho WZ. An in vitro model of morphine withdrawal manifests the enhancing effect on human immunodeficiency virus infection of human T lymphocytes through the induction of substance P. Am.J.Pathol. 2006; **169:** 1663-70.

Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 2003; **14:** 361-8.

Watkins LR, Hutchinson MR, Rice KC, Maier SF. The "toll" of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia. Trends in pharmacological sciences 2009; **30:** 581-91.

Watkins LR, Maier SF, Goehler LE. Immune activation: the role of pro-inflammatory cytokines in inflammation, illness responses and pathological pain states. Pain 1995; **63:** 289-302.

Watson B, Meng F, Akil H. A chimeric analysis of the opioid receptor domains critical for the binding selectivity of mu opioid ligands. Neurobiol Dis 1996; **3:** 87-96.

Weber RJ, Gomez-Flores R, Sora I, Uhl GR. Loss of Morphine-induced Suppression of NK Cell activity and T-cell Functions in mu Opioid Receptor Knockout Mice. American Journal of Immunology 2006; **2:** 35-39.

Weber RJ, Pert A. The periaqueductal gray matter mediates opiate-induced immunosuppression. Science 1989; **245:** 188-90.

Wei G, Moss J, Yuan CS. Opioid-induced immunosuppression: is it centrally mediated or peripherally mediated? Biochem.Pharmacol. 2003; **65:** 1761-66.

Welters ID, Menzebach A, Goumon Y, Cadet P, Menges T, Hughes TK, Hempelmann G, Stefano GB. Morphine inhibits NF-kappaB nuclear binding in human neutrophils and monocytes by a nitric oxide-dependent mechanism. Anesthesiology 2000a; **92:** 1677-84.

Welters ID, Menzebach A, Goumon Y, Langefeld TW, Teschemacher H, Hempelmann G, Stefano GB. Morphine suppresses complement receptor expression, phagocytosis, and respiratory burst in neutrophils by a nitric oxide and mu(3) opiate receptor-dependent mechanism. J Neuroimmunol 2000b; **111:** 139-45.

West JP, Lysle DT, Dykstra LA. Tolerance development to morphine-induced alterations of immune status. Drug Alcohol Depend 1997; **46:** 147-57.

Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 1999; **23:** 737-46.

Williams JP, Thompson JP, McDonald J, Barnes TA, Cote T, Rowbotham DJ, Lambert DG. Human peripheral blood mononuclear cells express nociceptin/orphanin FQ, but not mu, delta, or kappa opioid receptors. Anesth.Analg. 2007; **105:** 998-1005, table.

Williams LT, Snyderman R, Lefkowitz RJ. Identification of beta-adrenergic receptors in human lymphocytes by (-) (3H) alprenolol binding. J Clin Invest 1976; **57:** 149-55.

Wong MT, Ye JJ, Alonso MN, Landrigan A, Cheung RK, Engleman E, Utz PJ. Regulation of human Th9 differentiation by type I interferons and IL-21. Immunology and cell biology 2010; **88:** 624-31.

Wrona D. Neural-immune interactions: an integrative view of the bidirectional relationship between the brain and immune systems. J Neuroimmunol 2006; **172:** 38-58.

Wu Y, Wang Y, Zhan J. Effects of remifentanyl and fentanyl on LPS-induced cytokine release in human whole blood in vitro. Mol Biol Rep 2009; **36:** 1113-7.

Wullaert A, Heyninck K, Beyaert R. Mechanisms of crosstalk between TNF-induced NF-kappaB and JNK activation in hepatocytes. Biochem Pharmacol 2006; **72:** 1090-101.

Wybran J, Appelboom T, Famaey JP, Govaerts A. Suggestive evidence for receptors for morphine and methionine-enkephalin on normal human blood T lymphocytes. J Immunol 1979; **123:** 1068-70.

Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. The Journal of clinical investigation 1998; **101:** 311-20.

Yaksh TL. Pharmacology and mechanisms of opioid analgesic activity. Acta Anaesthesiol Scand 1997; **41:** 94-111.

Yates A, Bergmann C, Van Hemmen JL, Stark J, Callard R. Cytokine-modulated regulation of helper T cell populations. Journal of theoretical biology 2000; **206:** 539-60.

Yeager MP, Colacchio TA, Yu CT, Hildebrandt L, Howell AL, Weiss J, Guyre PM. Morphine inhibits spontaneous and cytokine-enhanced natural killer cell cytotoxicity in volunteers. Anesthesiology 1995; **83:** 500-08.

Yeager MP, Procopio MA, DeLeo JA, Arruda JL, Hildebrandt L, Howell AL. Intravenous fentanyl increases natural killer cell cytotoxicity and circulating CD16(+) lymphocytes in humans. Anesth.Analg. 2002; **94:** 94-9.

Yeager MP, Yu CT, Campbell AS, Moschella M, Guyre PM. Effect of morphine and beta-endorphin on human Fc receptor-dependent and natural killer cell functions. Clin Immunol Immunopathol 1992; **62:** 336-43.

Yoshida O, Akbar SM, Chen S, Miyake T, Abe M, Murakami H, Hiasa Y, Onji M. Regulatory natural killer cells in murine liver and their immunosuppressive capacity. Liver Int 2010.

Zanger UM, Klein K, Saussele T, Blievernicht J, Hofmann MH, Schwab M. Polymorphic CYP2B6: molecular mechanisms and emerging clinical significance. Pharmacogenomics 2007; **8:** 743-59.

Zarbock A, Ley K. Mechanisms and consequences of neutrophil interaction with the endothelium. Am J Pathol 2008; **172:** 1-7.

Zhang H, Cui Y, Voong N, Sabatino M, Stroncek DF, Morisot S, Civin CI, Wayne AS, Levine BL, Mackall CL. Activating signals dominate inhibitory signals in CD137L/IL-15 activated natural killer cells. Journal of immunotherapy 2011; **34:** 187-95.

Zhang JL, Simeonowa I, Wang Y, Sebald W. The high-affinity interaction of human IL-4 and the receptor alpha chain is constituted by two independent binding clusters. J Mol Biol 2002; **315:** 399-407.

Zhang Y, Du LN, Wu GC, Cao XD. Modulation of intrathecal morphine-induced immunosuppression by microinjection of naloxone into periaqueductal gray. Zhongguo Yao Li Xue.Bao. 1998; **19:** 519-22.

Zhu W, Cadet P, Baggerman G, Mantione KJ, Stefano GB. Human white blood cells synthesize morphine: CYP2D6 modulation. J.Immunol. 2005; **175:** 7357-62.

Zubieta JK, Smith YR, Bueller JA, Xu Y, Kilbourn MR, Jewett DM, Meyer CR, Koeppe RA, Stohler CS. Regional mu opioid receptor regulation of sensory and affective dimensions of pain. Science 2001; **293:** 311-5.