Immunomodulation in Patients Receiving Systemic Anti-Cancer Therapy

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

The use of immunotherapy to treat cancer is a rapidly expanding field. Over the last ten years, the number of clinical trials of immunotherapy, both alone and in combination with other agents, including cytotoxic chemotherapy, has grown exponentially. While the use of immunotherapy has revolutionised the management of cancers such as melanoma, renal cell carcinoma and lung cancer, other diseases, including colorectal cancer, have fared less well. In going forward, an increased understanding of the mechanism of immunotherapy in cancer patients is a vital step in being able to exploit these drugs in cancers where clinical efficacy has previously been limited. Better understanding of immune responses in these patients may identify potential responders to novel immunotherapy.

To this end, we developed and validated a panel of immune functional assays, which can be used in clinical trials to assess the immune response in peripheral blood. This panel can therefore be used to gather translational data from clinical trials of anti-cancer therapy. We used these assays to analyse the peripheral blood immune response in patients with metastatic colorectal cancer undergoing first-line chemotherapy, with reference to the neutrophil lymphocyte ratio (NLR). Analysis of samples from 29 patients revealed that, in patients with a high NLR prior to treatment, there was evidence of an attenuated immune response, with increased levels of certain immunosuppressive cytokines and depressed NK cell function. Over the course of treatment, we demonstrated that chemotherapy could partially reverse this phenomenon, potentially enhancing anti-tumour immunity.

Similarly, we interrogated blood samples of patients with metastatic melanoma receiving immune therapy with checkpoint inhibitors. Here, we observed evidence of a more active immune response, highlighting the differences between the two patient groups (those with colorectal cancer and those with melanoma).

In conclusion, we confirmed that our functional immune assay panel can be used effectively in different groups of cancer patients undergoing a variety of treatments, with the aim of an improved understanding of the immune system in cancer, response to therapy and how this may be exploited in the development of novel treatment strategies.

Aims and Objectives

This project set out to develop and validate a panel of immune assays, which could be performed on peripheral blood, with a high degree of quality and reproducibility. As such, we aimed to show that clinical samples could be collected from patients receiving systemic anti-cancer therapy (including those partaking in clinical trials of novel therapies), cryopreserved and batch analysed in order to produce robust results.
Using this immune assay panel, we sought to interrogate the response to first-line chemotherapy in patients with metastatic colorectal cancer. Patients were stratified according to baseline peripheral blood neutrophil lymphocyte ratio (NLR). We hypothesised that patients with a high baseline NLR have suppressed immune responses, which could be reversed by chemotherapy and could contribute to differences in overall survival (OS) between those with high NLR and low NLR prior to starting treatment.

Finally, in order to confirm that these assays can yield meaningful results in different patient groups, we analysed peripheral blood samples from those receiving immunotherapy for metastatic melanoma. This allowed us to compare immune responses in patients with different cancers undergoing different systemic therapies and put this data into the context of what we already understand about the immune system in cancer.

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Abbreviations

µCi	Micro Curie
^{51}Cr	Chromium-51
5-FU	5-Flurouracil
ACK	Ammonium Chloride Potassium
ADCC	Antibody-Dependent Cellular Cytotoxicity
AE	Adverse Event
AF	Alexa Fluor®
AJCC	American Joint Committee on Cancer
ANC	Absolute Neutrophil Count
APC	Allophycocyanin
APC	Adenomatous Polyposis Coli (Gene)
APCs	Antigen Presenting Cells
BCG	Bacillus Calmette-Guérin
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium
BRAF	B-Rapidly Accelerated Fibrosarcoma (gene)
B-Raf	B-Rapidly Accelerated Fibrosarcoma (protein)
BRAFi	BRAF inhibitor
BTLA	B and T Lymphocyte Attenuator
BV	Brilliant Violet
CAF	Cancer-Associated Fibroblast
CAPOX	Capecitabine and Oxaliplatin
CCL	Chemokine (C-C Motif) Ligand
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
cDCs	Conventional/Classical Dendritic Cells
CEA	Carcinoembryonic Antigen
CEF	Cytomegalovirus/Epstein-Barr Virus/Influenza
CI	Confidence Interval
CLL	Chronic Lymphocytic Leukaemia
CMV	Cytomegalovirus
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
cpm	Counts Per Minute
CR	Complete Response
CRC	Colorectal Cancer
CRF	Controlled Rate Freezer
CRP	C-Reactive Protein
CSS	Cancer-Specific Survival
CT	Core of the Tumour
CTACK	Cutaneous T-cell-Attracting Chemokine
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein-4
CXCL-10	C-X-C motif Chemokine 10
DAMPs	Danger-Associated Molecular Patterns
DC	Dendritic Cell
ddH_20	Double-Distilled Water
DFS	Disease-Free Survival

DMEM	Dulbecco's Modified Eagle Medium
dMMR	Deficient Mismatch Repair
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNLR	Derived Neutrophil Lymphocyte Ratio
DR	Death Receptor
dsRNA	Double Stranded Ribonucleic Acid
DTIC	Dacarbazine
E:T	Effector:Target
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked ImmunoSpot
ERK	Extracellular-Signal-Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FcγR	Fc-Gamma Receptor
FFPE	Formalin-Fixed Paraffin-Embedded
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FM	Freezing Mixture
FOLFIRI	5-FU and Irinotecan
FOLFOX	5-FU and Oxaliplatin
FoxP3	Forkhead Box P3
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
gp100	Glycoprotein 100
HBSS	Hank's Buffered Salt Solution
HCC	Hepatocellular Carcinoma
HD	Healthy Donor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HER	Human Epidermal Growth Factor
HGF	Hepatocyte Growth Factor
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HR	Hazard Ratio
HRP	Horseradish Peroxidase
HS	Human Serum
HSV	Herpes Simplex Virus
i.t.	Intratumoural
i.v.	Intravenous
ICOS	Inducible T Cell Co-Stimulator
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
ILP	Isolated Limb Perfusion
IM	Invasive Margin
IP-10	Interferon γ-Induced Protein 10
irRC	Immune-related Response Criteria
ISG	IFN-stimulated gene

JAK	Janus Kinase
JAK-STAT	Janus Kinase-Signal Transducer and Activator of Transcription
KIRs	Killer-Cell Immunoglobulin-Like Receptors
L	Ligand
LAG-3	Lymphocyte-Activation Gene-3
LAMP	Lysosomal-Associated Membrane Protein
LDH	Lactate Dehydrogenase
LIF	Leukaemia Inhibitory Factor
LLN	Lower Limit of Normal
LN	Lymph Node
LN2	Liquid Nitrogen
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MART-1	Melanoma-Associated Antigen Recognised by T cells 1
MCP	Monocyte Chemoattractant Protein
mCRC	Metastatic Colorectal Cancer
M-CSF	Macrophage-Colony Stimulating Factor
mDCs	Myeloid Dendritic Cells
MDSC	Myeloid-Derived Suppressor Cells
MEK	Mitogen-activated protein kinase kinase (MAPK/ERK kinase)
MEKi	MEK inhibitor
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIC	MHC Class I Chain-Related Protein
MIF	Macrophage Migration Inhibitory Factor
MIG	Monokine Induced by Gamma Interferon
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinases
MMR	Mismatch Repair
MSI	Microsatellite Instability/Instable
MSS	Microsatellite Stability/Stable
NAb	Neutralising Antibody
$NaHCO_3$	Sodium Bicarbonate
NCRs	Natural Cytotoxicity Receptors
NETs	Neutrophil Extracellular Traps
NGF	Nerve Growth Factor
NICE	National Institute for Health and Care Excellence
NK Cell	Natural Killer Cell
NKT Cells	Natural Killer T Cell
NLR	Neutrophil Lymphocyte Ratio
NO	Nitric Oxide
NSCLC	Non Small-Cell Lung Cancer
OS	Overall Survival
OV	Oncolytic Virus
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PBS-T	PBS-Tween
PD-1	Programmed Cell Death Protein-1
pDCs	Plasmacytoid Dendritic Cells

PD-L1	Programmed Death Ligand-1
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll Protein
PFA	Paraformaldehyde
PFS	Progression-Free Survival
pfu	Plaque-Forming Unit
pg	Picogram
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-Kinase
PKR	Protein Kinase R
PLR	Platelet Lymphocyte Ratio
PMA	Phorbol Myristate Acetate
pMMR	Proficient Mismatch Repair
pNPP	p-Nitrophenyl Phosphate
PR	Partial Response
PRRs	Pattern Recognition Receptors
PVDF	Polyvinylidene Difluoride
RAF	Rapidly Accelerated Fibrosarcoma (Gene)
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RAS	Rat Sarcoma (Gene)
RCC	Renal Cell Carcinoma
RECIST	Response Evaluation Criteria in Solid Tumours
reoTCM	Reovirus Tumour-Conditioned Media
Reovirus	Respiratory and Enteric Orphan Virus
RET	Rearranged During Transfection (Gene)
RFS	Recurrence-Free Survival
RMS	Relative Median Shift
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RR	Response Rate
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
s.c.	Subcutaneous
SACT	Systemic Anti-Cancer Therapy
SCC	Squamous Cell Carcinoma
SCF	Stem Cell Factor
SCGF	Stem Cell Growth Factor
SDF	Stromal Cell-Derived Factor
SEM	Standard Error of the Mean
SFU	Spot Forming Units
SNB	Sentinel Node Biopsy
SOP	Standard Operating Procedure
TAA	Tumour-Associated Antigen
TAMs	Tumour-Associated Macrophages
TANs	Tumour-Associated Neutrophils
T_{CM}	Central Memory T Cells
TCR	T Cell Receptor

T_{EM}	Effector Memory T Cells
TGF	Transforming Growth Factor
T_H	T Helper
T_H0	Naïve T Cells
TILs	Tumour-Infiltrating Lymphocytes
TIM	T cell Immunoglobulin and Mucin Domain Containing
TKI	Tyrosine Kinase Inhibitor
TLRs	Toll-Like Receptors
TMB	Tetramethylbenzidine
TME	Tumour Microenvironment
TMZ	Temozolomide
TNF	Tumour Necrosis Factor
TNM	Tumour Node Metastasis
TRAIL	TNF Related Apoptosis-Inducing Ligand
Treg	Regulatory T Cell
TRMs	Tissue-Resident Macrophages
TS	Thymidilate Synthase
TTP	Time To Progression
T-VEC	Talimogene Laherparepvec
ULN	Upper Limit of Normal
UV	Ultraviolet
v/v	Volume per Volume
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VGP	Vertical Growth Phase
vs.	Versus
VSV	Vesicular Stomatitis Virus
w/v	Weight per Volume
WBC	White Blood Cell
WHO	World Health Organisation
WT	Wild Type
γδ T Cell	Gamma Delta T Cell

Chapter 1 Introduction

1.1 The Immune Response in Cancer

In order to place into context the effects of anti-cancer therapy on host immunity, we first need to have an understanding of the complex role of the immune system in the pathogenesis and development of cancer. Broadly, immune activation involves a carefully regulated system of checks and balances that first initiate and then attenuate a response. This is initially through rapid activation of the innate immune system, followed by the development of specific immunity against a particular pathogen. It is now well recognised that these mechanisms do not function appropriately in patients with cancer and may be dysfunctional in a way that supports tumour growth and metastasis. The ability of cancer to evade the immune response has now been incorporated into the 'Hallmarks of Cancer' (1), along with genetic instability, resistance to cell death, ongoing cellular proliferation, and metastasis. Other features of malignant cells have been targeted therapeutically and current interest in anti-cancer immunotherapy is increasing exponentially. The ability to manipulate the immune system to target cancer has changed the field of oncology in the last 10 years and produced results that are truly paradigm changing.

1.1.1 Adaptive and Innate Immune Response

The innate immune response is non-specific but has the ability to control and manage pathogens until the development of an adaptive response. One of the primary effector cells of the innate immune system is the natural killer (NK) cell, which exerts cytotoxic effects against abnormal/infected cells and releases cytokines (which assist in priming T cells against specific antigens) and chemokines (which recruit immune cells to the site of infection or inflammation) (2). In parallel, the detection of pathogens by phagocytic cells such neutrophils and macrophages through pattern recognition receptors (PRRs) on the cell surface initiate phagocytosis, whereby foreign organisms are engulfed and destroyed (3). NK cells also contribute to the maturation of antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages, which process and present antigens. Tumour-associated antigens (TAAs) are molecules which can be specific to (or over-expressed in) malignant cells, such as carcinoembryonic antigen (CEA) in colorectal cancer (CRC) and melanoma-associated antigen recognized by T cells-1 (MART-1). Presentation of antigens on APCs to T cells stimulates

proliferation of a clonal population of T cells recognising specific antigens as part of a specific adaptive response. Further T cell differentiation is dependent on the immune environment and the presence of immunosuppressive or stimulatory cytokines (discussed in more detail in section 1.2.2). Activation of B cells, the other component of adaptive immunity, forms the humoural immune response and the secretion of antibodies (immunoglobulin) against specific antigens. Binding of these antibodies to antigen contributes to their recognition by phagocytes. Alongside this is the complement cascade, comprised of three functional systems: the classical, alternative and lectin pathways (reviewed in (4)). Their main functions are to complement the cellular and humoural immune responses by contributing to the destruction of pathogenic organisms through opsonisation (attaching to the surface of damaged cells), thereby recruiting macrophages and neutrophils to initiate phagocytosis and directly lysing the cell membrane of abnormal cells through the membrane attack complex. These systems are interwoven and complex and both the innate and adaptive arms of the immune response can influence and regulate one another, depending on the situation, whether that be infection, inflammation or cancer.

1.1.2 Immunosurveillance and Immunoediting

Through a process of selection, cancerous cells that are naturally more susceptible to immune-mediated cytotoxicity are eradicated earlier on in the development of a tumour (immunosurveillance). What remains is a clone of relatively resistant malignant cells. These are not only impervious to immune-mediated cell death but can manipulate the tumour microenvironment (TME) to support tumour growth, with increased levels of immunosuppressive cytokines. These cytokines then push infiltrating immune cells towards a phenotype which perpetuates this process. The three stages of tumour 'escape' from immune system control (immunoediting) are known as the 'three E's':

Elimination

The immunosurveillance and elimination of cell populations which are susceptible to immune-mediated cell death involves interferon (IFN)-γ and perforin release from lymphocyte populations. To illustrate this simply, it has been demonstrated in *in vivo* models that mice insensitive to IFN-γ are much more susceptible to the development of carcinogen-induced tumours (5,6). Similarly, mice deficient in perforin (which is essential to both T cell- and NK cell-mediated degranulation and cytotoxicity) developed tumours more readily following injections of carcinogen (7).

Immune responses against damaged cells are triggered by the presence of molecules in the TME. Pathogen-associated molecular patterns (PAMPs) are molecules, such as lipopolysaccharide (LPS) from bacterial cell membranes, which are recognised by PRRs on innate immune cells, including toll-like receptors (TLRs). Danger theory (6) expands this to include molecules that are not related specifically to pathogens but rather to danger signals derived from damaged or dying cells (termed danger-associated molecular patterns, DAMPs). When cells are killed through the immune-silent process of apoptosis, cellular contents are compartmentalised and destroyed in a controlled way that does not stimulate an immune response. In contrast, cells that undergo necrosis have a disorganised cell death resulting in the release of molecules including fragments of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and other cellular proteins such as reactive oxygen species (ROS). Elements of the immune response have also been postulated to act as DAMPs, such as the T cell stimulatory ligand CD40-L and inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1 (6).

Equilibrium

If the immune system does not completely eradicate the tumour, there may be a period of latency (immune regulation), where clones of malignant cells (not clinically detectable) are held in check. This period can last for many years and is illustrated by case reports of malignant disease developing in recipients of transplanted organs (8). Similarly, there is also evidence that an immune insult could contribute to relapse of cancer at a later date, following initial treatment. Raised serum levels of C-reactive protein (CRP) are associated with decreased disease-free survival (DFS) and overall survival (OS) in patients who have received treatment for breast cancer with curative intent (9). In contrast, a number of studies and meta-analysis have found no link between cancer recurrence and immunosuppressive therapies for unrelated auto-immune conditions (10), suggesting that the processes controlling dormancy of quiescent cancer cells is multi-factorial.

Escape

During the equilibrium phase, new clones of cells can develop due to selection pressures, with new genetic mutations or variants, which then subvert maintenance by the immune system. This leads to the development of clinically detectable disease. There are a number of mechanisms aside from genetic instability which can contribute to tumour escape. Recurrent tumours in murine models of myeloma were found to have developed due to downregulation

of major histocompatibility complex (MHC) class-I and/or co-stimulatory molecules on the surface of tumour cells, which would be one mechanism of escaping an anti-tumour cytotoxic T lymphocytes (CTL) response (8). In addition to the loss of molecules involved in efficient antigen presentation, the expression of TAAs may also change over the course of disease. Examination of formalin-fixed paraffin-embedded (FFPE) sections of melanoma specimens demonstrated that positivity for MART-1 decreased according to the stage of disease (8).

1.2 Immune Cells

1.2.1 Natural Killer Cells

NK cells (CD3-/CD56+) and Natural Killer T (NKT) cells (CD3+/CD56+) are a vital part of the innate immune response, acting against virally-infected cells or tumour cells and releasing immune-stimulatory cytokines which, in turn, trigger T cell activation (11). Variable expression of CD56 can further characterise NK cells into cytotoxic ($CD56^{dim}$) and cytokine-producing ($CD56^{bright}$) (2,12). NK and NKT cells are activated by cytokines including IL-12 (also known as NK cell-stimulating factor) and can produce a range of both stimulatory and suppressive cytokines, including IFN-γ, TNF-α, TNF-β, IL-10 and IL-13 (12).

Unlike T cells, activation of NK cells is not dependent on either priming by antigen recognition or a secondary activating signal. Instead, NK cell activation is controlled by a balance of activating and inhibitory surface receptors engaging with appropriate ligands on the target cell surface. NK cell-activating receptors include NKG2D and the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 (13). Infected or malignant cells express ligands to these activating receptors and recognition of such initiates NK cell-mediated cytotoxicity and target cell lysis (14). As well as viral proteins, NCRs recognise ligands such as B7-H6, which binds to NKp30 and has been found to be over-expressed on malignant cells, including in breast cancer (15). Downregulation of these activating NK receptors is associated with decreased immune-surveillance and development of tumours (16). Activating receptors can be down-regulated on NK cells of patients with metastatic disease (16,17) due to the effect of transforming growth factor (TGF)-β or indoleamine 2,3-dioxygenase (IDO) (18). Soluble NK receptor ligands in the TME can also competitively compete with tumour-associated ligands, further impairing the anti-tumour NK cell-mediated response (19). NKG2D ligands include MHC (also known as human leukocyte antigen, HLA in humans) class I chain-related proteins A and B (MICA and MICB).

Inhibitory receptors, such as some killer-cell immunoglobulin-like receptors (KIRs) and NKG2A, recognise MHC class-I (MHC-I) molecules on target cells; engagement of these prevents NK cell-mediated cytotoxicity. This is particularly relevant in the development of self-tolerance: inhibitory ligands recognise self MHC-I molecules on the surface of target cells and cytotoxicity is prevented. In target cells which do not express MHC-I, the absence of an inhibitory signal initiates NK cell-mediated death (the concept of 'missing-self' (11)). As these inhibitory receptors are extremely polymorphic, populations of NK cells develop which can discriminate between a wide variety of 'self' and 'non-self' targets. NK cells therefore preferentially pursue cells which downregulate MHC-I, presumably as a mechanism for abrogating T cell-mediated lysis, such as virally-infected or tumour cells (20).

NK cells employ a number of mechanisms by which they can exert a cytolytic effect. Upon activation, NK cells release cytoplasmic granules containing perforin (which perforates cell membranes) and granzyme, a proteolytic enzyme which then triggers target cell apoptosis. They also express lysosomal-associated membrane protein (LAMP)-1 and LAMP-2 (also known as CD107a and CD107b) on their inner surface (21). As the granules merge with the NK cell membrane these molecules are expressed on the cell surface (22). They can then be used to identify activated (degranulating) NK cells. Target cell apoptosis can also be induced through the engagement of Fas-ligand (Fas-L, CD95-L) or TNF-related apoptosis-inducing ligand (TRAIL) (expressed on NK cells) and target cell Fas (CD95) and TRAIL-receptor, respectively. NK cells also play a role in antibody-dependent cellular cytotoxicity (ADCC). Following the binding of monoclonal antibodies to target cell receptors (such as rituximab to CD20), the predominant Fc receptor (FcR) expressed by NK cells (FcγRIII/CD16; the others being FcγRI/CD64 and FcγRII/CD32) binds to the Fc portion of the antibodies, triggering the release of cytolytic granules. Cell death can, in turn, promote an adaptive response through the release of TAAs. ADCC has been shown to contribute to the therapeutic effect of monoclonal antibodies such as rituximab and trastuzumab, which binds to the human epidermal growth factor (HER) -2 receptor (23).

There are a number of ways in which malignant cells can subvert NK-mediated cytotoxicity. In addition to NK-specific inhibitory receptors, activated NK cells can upregulate checkpoint inhibitors including programmed cell death protein-1 (PD-1, CD279), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4, CD152) and T-cell immunoglobulin and mucin domain containing (TIM) -3 (section 1.5.2), which can inhibit NK cell function following engagement with their ligands on target tumour cells (24). As such, therapy with checkpoint inhibitors,

which were primarily thought to enhance an adaptive anti-tumour T cell response, may also enhance NK cell responses and have been shown to heighten anti-tumour responses in models of melanoma (25).

1.2.2 T Cells

The major classes of T cell are the T helper (T_H) cells (CD3+/CD4+) and cytotoxic T cells (CD3+/CD8+, CTLs), the main effector cell of the adaptive immune response. CTLs are effective against intracellular pathogens such as viruses and bacteria. Cell surface T cell receptors (TCRs) recognise antigen presented on MHC-I, which is present on all nucleated cells. For MHC- I, there are 3 HLA molecules: HLA-A, HLA-B and HLA-C. T_H cells recognise exogenous antigens presented on MHC-II molecules (MHC-II: HLA-DR, HLA-DP, HLA-DM, HLA-DOA, HLA-DOB and HLA-DQ), found on professional APCs such as DCs.

In addition to the engagement between the TCR and the MHC/antigen complex, another costimulatory signal is required to induce T cells to proliferate and become effector cells against a specific antigen. The most well-defined of these is engagement of the CD28 receptor, expressed on the T cell surface and it's ligands B7-1 (CD80) and B7-2 (CD86) on the APC. There is, however, a complex network of other co-stimulatory signals at the immune synapse, including CD40-L, CD278 (inducible T cell co-stimulator, ICOS) and TIM-1. Furthermore, not only is T cell function controlled by activating receptors, it is also governed by inhibitory molecules (such as CTLA-4, PD-1, TIM-3 and lymphocyte-activation gene (LAG)-3), the immune checkpoints. In the case of CTLA-4, for example, T cell activation causes upregulation of the molecule, which binds with greater affinity than CD28 to CD80 and CD86, abrogating cellular activity (26)(Figure 1.1).

Once T cells have been stimulated, they will proliferate into effector cells, each with characteristic functions. A cohort will remain, however, as memory T cells that can proliferate rapidly should the same antigen be encountered in the future. These memory cells are further differentiated into central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). Memory T cells express CD45RO, differentiating them from naïve T cells, which express the CD45RA isoform. As T_{CM} cells also express the lymph node (LN) homing receptor C-C chemokine receptor (CCR) 7, they are found in LN as well as peripheral blood. In contrast, T_{EM} cells are CCR7-negative and hence reside in peripheral blood and tissues (27). CCR7 is expressed on T cells, regulatory T cells (Tregs) and DCs and regulates the migration of these cells to LNs where chemokine (C-C motif) ligand (CCL) 19 and CCL21 are expressed. As such, expression of CCR7

on gastric carcinoma cells is associated with a significantly higher incidence of LN metastases and poorer survival (28).

1.2.2.1 T Helper Cells

Naïve CD4+ cells (T_H0) can differentiate into a number of effector T cells under different environmental conditions, each with specific functions. For example, under the influence of IL-12 and IFN-γ, T_H0 cells differentiate into T_H1 cells, which in turn produce IL-2, TNF-α and IFN-γ, activating CTLs and macrophages (29). The fact that these cytokines can be more directly cytotoxic to tumour cells, contribute to DC maturation, activity of macrophages and proliferation of CTLs means that T_H1 responses are generally thought to be weighted towards anti-tumour immunity. Conversely, T_H0 cells differentiate into T_H2 cells under the influence of IL-2 and IL-4 and produce IL-4, IL-5, IL-10 and IL-13 (30). Although T_H2 cells can activate B cells to produce antibodies, cytokines including IL-10 and IL-13 can exert more of an immunosuppressive effect and favour a pro-tumour environment. Clinical studies have borne this out, with sera or peripheral blood lymphocytes from patients with melanoma (31) and colon cancer (32) showing elevated levels of T_H2 cytokines, specifically IL-4 and IL-10. In contrast, when infiltrating immune cells from CRC specimens were examined, higher T_H1 gene expression was associated with prolonged DFS (33).

IL-6 and TGF-β induce differentiation into a T_H17 phenotype. T_H17 cells produce IL-17 and are implicated in hypersensitivity reactions, autoimmune disease and inflammation (34). The role of this population of CD4+ cells in tumour immunology is unclear. Notably, although patients with advanced gastric cancer have increased levels of T_H17 cells in peripheral blood (35), the reverse has been demonstrated in patients with ovarian cancer (36).

Under the influence of TGF-β (without IL-6), naïve CD4+ T cells differentiate into Tregs which express the transcription factor Forkhead box P3 (Fox-P3) (37). As the name suggests, Tregs are involved in the regulation of the immune response (and pertinently, the anti-tumour immune response) through the production of IL-10. Tregs are discussed in detail in section 1.2.3.

1.2.2.2 Cytotoxic T Cells

Activated CTLs can kill target cells (infected cells or tumour cells) in a number of ways. In a mechanism similar to NK cells, CTLs kill target cells via release of granzyme/perforin granules

(38). In addition, CTLs can induce apoptosis of target cells through engagement of Fas-L on the T cell and Fas receptor of the tumour cell, triggering apoptotic pathways and cell death (39). Activated T cells also produce cytokines which boost the immune response; for example, IFN-γ increases MHC expression on DCs (and thus further antigen presentation) (40).

The presence of cytotoxic tumour-infiltrating lymphocytes (TILs) within the TME is associated with an improved prognosis in many different cancers including CRC (41,42), breast cancer (43) and melanoma (44).

Some viruses cause down-regulation of host cell MHC to prevent CD8+ T cell-mediated cell death and subsequent destruction of the virus. Tumour cells employ similar mechanisms to avoid immune-mediated cytotoxicity by upregulating inhibitory receptors such as programmed cell death ligand 1 (PD-L1), the ligand for PD-1, in response to IFN-γ produced by activated CTLs (45). Downregulation of MHC-I by malignant cells (and hence decreased antigen presentation to CTLs) also contributes to immune evasion by tumours (20).

Prolonged stimulation of the CTL population can eventually lead to tolerance to tumour antigens (46) with upregulation of immune checkpoints including PD-1 on T cells (47), decreased production of immune-stimulatory cytokines such as IFN-γ, reduced ability to control tumour growth *in vivo* and diminished T cell proliferation (48).

1.2.3 Regulatory T Cells

Tregs are thought to play a major role in the suppression of anti-tumour immunity and the subversion of T cell responses against tumour cells (49). They can be phenotypically defined as CD3+/CD4+/$CD25^{high}$/$CD127^{low}$/Fox-P3+.

Patient-derived Tregs, analysed *in vitro*, produce both IL-10 and TGF-β (50) and prevent the proliferation and function (IFN-γ production) of both effector CD4+ (Fox-P3-negative) and CD8+ T cells (51,52). Immunosuppressive cytokines can themselves stimulate the differentiation of naïve T_H0 cells into Tregs in peripheral blood. CD4+/CD25- T cells can be induced to differentiate into Tregs by TGF-β and TCR stimulation (53). In *in vivo* models of melanoma, Tregs accumulated at the site of B16 melanoma tumours (54). While CD8+ TILs demonstrated a specific anti-tumour response, the CD4+ TILs produced high levels of both IL-10 and TGF-β but low levels of IFN-γ (suggesting a Treg rather than a T_H1 phenotype) (54). Interestingly, inhibition of IL-10 in tumour-bearing mice led to a reduction in the proportion of CD4+ Tregs in splenocytes and decreased tumour growth. This was associated with diminished

TGF-β and elevated IFN-γ production, suggesting a further role for IL-10 (as well as TGF-β) in the development of Tregs and the suppression of anti-tumour immunity (54).

Elevated populations of Tregs have been noted in both the peripheral blood and TILs of patients with many other cancers, including gastric (51), breast cancer (55) and CRC (56). As such, an increased proportion of Tregs has also been linked to poorer disease-specific survival (50,57). Antibodies against CD25 (daclizumab and basiliximab) are currently being investigated in the clinical setting (glioblastoma, NCT00626483 and melanoma, NCT00847106) with some evidence that they can deplete Treg populations and enhance anti-tumour responses (58).

Tregs can act in a number of ways to suppress the anti-tumour immune response. CTLs and NK cells both exert direct cytolytic effects through secretion of granules containing perforin and granzyme. Tregs can induce apoptosis of CD8+ effector cells through engagement of Fas, which is upregulated on CD4+ and CD8+ T cells in response to activation by anti-CD3 antibody and IL-2 (59). Interestingly, upregulation of Fas-L expression is proportionately higher on Tregs from cancer patients, prompting the suggestion that Tregs can mediate apoptosis of tumour-specific CTLs (59). In addition, Tregs have an adverse effect on DC function. Not only can Tregs can downregulate CD80/CD86 on DCs, thereby inhibiting DC-mediated stimulation of T cells (60) but engagement of CTLA-4 on Tregs with CD80/CD86 expressed on DCs can also suppress the function of these APCs (60).

1.2.4 γδT Cells

On average, γδT cells (CD3+/γδTCR+) constitute up to 10 % of the CD3+ T cell population. Unlike CD4+ and CD8+ T cells, the TCR of γδT cells is formed of one γ and one δ chain, rather than the α and β chains which make up the TCR of other T cell subsets. As they are activated independently of MHC molecules, they produce a rapid (non-specific) immune response, more similar to that of innate effector cells (61). As with the differentiation of CD4+ T helper cells, γδT cells can also show a degree of plasticity in their functional capacity, acting in both immune-stimulatory and regulatory ways (62).

Although their activation is largely independent of MHC, γδT cells actually have the capacity to be professional APCs: upregulating MHC-I in response to antigen stimulation, enhancing proliferation of effector CD8+ T cells and cytotoxicity of antigen-specific target tumour cells *in vitro* (63). Similar antigen-priming has also been observed by stimulation of CD4+ cells with γδT cells, enhancing their differentiation into a T_H1 phenotype (64). γδT cells also exhibit

direct cytotoxic effects when expressing CD56. These CD56+ γδT cells have cytotoxic activity against tumour cells *ex vivo*: increased expression of CD107a suggests that cell death is mediated by perforin and granzyme release (65). Finally, in another mechanism similar to NK cells, γδT cells have ADCC capability (66).

Contrary to these immune-stimulatory effects, γδT cells also exert regulatory, or immunosuppressive, properties. γδT cells stimulated with monoclonal antibody against the δTCR can be induced to express the transcription factor Fox-P3 as well as CD25 (a similar phenotype to 'classical' Tregs) (67). Furthermore, these regulatory γδT cells produce TGF-β and suppress T cell proliferation (67).

1.2.5 Dendritic Cells

DCs are a population of bone marrow progenitor-derived cells which can be broadly classified as either myeloid DCs (mDCs, also known as conventional/classical DCs, cDCs) or plasmacytoid DCs (pDCs). While pDCs produce high levels of type I interferons (IFN-α and -β) in response to activation of TLR-7 and TLR-9 (68) by PAMPs, they are very much in the minority, constituting less than 0.5 % of all peripheral blood mononuclear cells (PBMCs). Although pDCs can present antigen to T cells (as they express MHC-II and co-stimulatory molecules such as CD80/86 upon activation), they are much less potent than mDCs. Unlike mDCs, they do not express CD11c.

Although a small proportion of circulating PBMCs, pDCs have shown a role in priming both an innate and adaptive immune response. Through production of type 1 IFNs, pDCs can stimulate activation of NK cells (68). Similarly, IFN production from pDCs can initiate differentiation of B cells into plasma cells and production of specific antibodies following stimulation with influenza (69). Despite mDCs being the more effective APC, pDCs are capable of antigen cross-presentation (presentation of exogenous peptides to CD8+ cells on MHC-I through the phagocytosis of peptide antigens), triggering an IFN-γ response from antigen specific CD8+ T cells (70). Conversely, pDCs can initiate the differentiation of naïve CD4+ T cells to IL-10-producing Tregs (71).

mDCs are essential to the development of an antigen-specific adaptive immune response. They classically present endogenous antigens on MHC-I to CD8+ T cells and exogenous antigens on MHC-II to CD4+ T cells. Recognition of antigen on MHC molecules by TCRs initiate antigen-specific T cell activation and proliferation in the presence of a co-stimulatory signal (such as binding of CD28 on the T cell to CD80/86 on the APC). mDCs commonly express TLRs

2-6 and 8, plus CD11c (3) and can produce cytokines such as IL-12 upon activation. Although exposure and processing of antigens can take place in peripheral blood, tissue or tumour, upregulation of the chemokine receptor CCR7 on activated mDCs initiates their migration to LNs expressing the CCR7 ligands CCL19 and CCL21 (72). It is in the LNs that DCs encounter and prime naïve T cells.

In the same way that pDCs can influence the differentiation of naïve T cells, mDCs can also direct polarisation of T cells. Activation of T cells is dependent on three signals from DCs: (i) engagement of MHC-antigen complex with the TCR; (ii) an additional co-stimulatory signal and (iii) the production of cytokines. Release of cytokines from DCs is modulated by stimulation through different signals: for example, interaction of OX40 on the T cell (also known as CD134, a member of the TNF family) with its ligand OX40-L, another co-stimulatory signal, stimulates T_H2 differentiation (73). Upon activation of specific TLRs by pathogens, DCs will express various immunomodulatory cytokines which, in turn, exert effects on T cells. Activation of TLR-4 on human DCs *in vitro*, for example, has been demonstrated to initiate release of interferon-γ-induced protein-10 (IP-10, also known as CXCL-10) favouring T_H1 differentiation, whereas TLR-2 stimulation provokes release of IL-12p40 and T_H2 differentiation (74).

In summary, DCs act to bridge elements of both the innate and adaptive immune system and are themselves influenced by their environment in how they are activated and how they, in turn, affect the priming and differentiation of naïve T cells.

1.2.6 Monocytes and Macrophages

Aside from the phagocytosis of pathogens, macrophages can act as APCs and produce a range of immune-modulatory cytokines. Mature macrophages differentiate from monocytes under the influence of macrophage-colony stimulating factor (M-CSF) and are resident in tissue and tumour sites. Monocytes can also differentiate into mDCs when stimulated *in vitro* by IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF). Usually identified by their expression of CD14, monocytes can also differentially express CD16: 'classical' monocytes highly express CD14, whereas the other subset of monocytes express proportionately lower CD14 but also CD16. These CD14+CD16+ monocytes (constituting 15-20 % of the monocyte population) also express high level MHC-II but exhibit decreased phagocytic potential than CD16- monocytes (75) and therefore are similar phenotypically to tissue-resident macrophages (TRMs) (76). TRMs maintain tissue homeostasis and so differentiate and

function in a plethora of ways dependent on the tissues they reside in: these include Kupffer cells (liver), alveolar macrophages (lung) and Langerhans cells (skin).

Chemotaxis of classical monocytes to the tumour environment is controlled by CCR2 and its ligand CCL2, also known as monocyte chemoattractant protein (MCP)-1, which is over-expressed in various malignancies and is associated with inflammation (77).

Similar to DCs, activation of classical monocytes though TLRs, for example TLR-4, stimulates an effector immune response. As IFN-γ and IL-2 upregulate monocyte expression of TLR-4, this illustrates the role of classical monocytes in the host response to bacterial pathogens (78,79).

As with other cell types, variations in the immune environment can initiate differentiation of macrophages into cells with different functional capabilities. Ergo, IFN-γ can polarise macrophages into the T_H1-like 'M1' phenotype, producing IFN-γ, TNF, IL-12 and IL-2. In contrast, cytokines such as IL-4, IL-10 and IL-13, produced in the TME (80), facilitate differentiation into the 'M2' phenotype, which are not only poor APCs but have similar properties to T_H2 cells and produce cytokines such as IL-4 and IL-10 (81). Tumour-associated macrophages (TAMs, which exhibit the M2 phenotype) produce cytokines which potentiate the 'pro-tumourigenic' environment, such as CCL22, which induces Treg migration into tumour *in vivo* (57).

Moreover, M2 TAMs can stimulate angiogenesis in the TME, further supporting tumour growth and survival by contributing to the 'angiogenic switch' which initiates vascular development in hypoxic tumours. This is associated with the production of pro-angiogenic factors such as IL-8 (also known as neutrophil chemotactic factor or CXCL-8) (82) and vascular endothelial growth factor (VEGF) (83). As such, increased TAM infiltration is associated with increased tumour neo-vascularisation. Other mechanisms by which TAMs encourage tumour growth and progression include expression of PD-L1, the engagement of which by PD-1 on T cells inhibits adaptive immune responses against tumour antigens (84).

High TAM infiltration is poorly prognostic and/or associated with distant metastasis in a variety of tumours, including bladder (85,86), gastric (86) and ovarian cancers (86). Interestingly, an increased macrophage population in the TME of patients with prostate cancer (87) and CRC (86) is associated with an improved prognosis, although this may be skewed by the phenotype of infiltrating cells being predominately M1. This further highlights the complexities of the immune milieu in the TME, which determines the phenotype of TAMs and their clinical effect.

1.2.7 Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogenous population of immature cells of myeloid lineage which are increased in states of infection, inflammation and cancer where they exert an immunosuppressive effect on anti-tumour immune responses. MDSCs express the myeloid markers CD11b and CD33 but are negative for lineage markers including CD3, CD4, CD19 and CD56, in addition to HLA-DR. Differentiation of this population of immunosuppressive cells is in response to various cytokines and chemokines, including GM-CSF (88), IL-13 (89), IL-10 and TGF-β (90) and they are recruited into tumour sites by chemokines such as CCR2 (91). MDSCs have been associated with poorer prognosis in a number of different malignancies, likely due to their suppressive effects on the anti-tumour response (92).

MDSCs can exert an immunosuppressive action through the interruption of IFN signalling pathways (section 1.3.1.1) and responsiveness of immune cells to T_H1 signalling by nitric oxide (NO), as demonstrated in murine *in vivo* melanoma models (93). They can also, through production of IL-10 and TGF-β, induce the differentiation of Tregs in the TME and, as such, ongoing immunosuppression at the tumour site. In addition to suppressing T cell activation and proliferation in response to peptide antigens (94), there is also evidence that the production of ROS and NO by MDSCs can affect the integrity of TCR-MHC complexes involved in T cell priming (95).

Antibody-depletion of the MDSC population enhances both innate and adaptive immune responses in murine models of lung cancer (92). Furthermore, chemotherapeutic agents, such as gemcitabine and 5-fluorouracil (5-FU), can exert immunomodulatory effects by decreasing the number of MDSCs *in situ* (96,97).

1.2.8 Neutrophils

As an important part of the innate immune response, neutrophils (CD15+/CD16+) react rapidly to bacterial pathogens, migrating to areas of inflammation in response to chemokines such as IL-8 and CXCL-2 (98). They constitute approximately 70 % of circulating white blood cells (WBCs) and with basophils and eosinophils form the granulocyte population of WBCs. Basophils and eosinophils are involved in allergic reactions and host defence against parasites.

One of the main functions of the neutrophil is to engulf microbes, bacteria and other extracellular pathogens through phagocytosis: PRRs on the surface of the cell recognise PAMPs or antibodies bound directly to the pathogen. Such pathogens are then engulfed by the neutrophil and incorporated into the cell as a phagosome (98). The contents of the phagosome can then be destroyed by merging with enzyme-containing lysosomes within the cell or by the production of ROS (98). Neutrophils can also function through degranulation and through neutrophil extracellular traps (NETs): extracellular networks of DNA which can bind to trap pathogens and contain anti-microbial molecules such as proteolytic enzymes.

In the same way that the TME can manipulate the phenotype of TAMs, tumour-associated neutrophils (TANs) exhibit pro-tumour characteristics (99). TGF-β blockade in *in vivo* models of mesothelioma increased infiltration of neutrophils into the TME, with associated ROS-mediated tumour cell cytotoxicity (100). Interestingly, depletion of neutrophils in the absence of TGF-β blockade also enhanced CD8+ T cell activation, suggesting different phenotypes of neutrophils in the TME: 'N1' (anti-tumour) TANs, in the absence of TGF-β and 'N2' (pro-tumour) TANs, in the presence of TGF-β (100). Assessment of the peripheral blood of patients with melanoma reveals that an increasing neutrophil count corresponds with stage of disease. These neutrophils inhibit proliferation of melanoma-antigen specific CD8+ T cells through IL-10 production (101). The 'N2' phenotype is associated with the progression of liver metastases in mouse models of breast cancer (102).

The role of neutrophils in CRC is discussed in further detail in section 4.1.6.2.

1.2.9 B Cells

B cells (CD19+) form the cornerstone of the humoural adaptive immune response and, as such, there is growing recognition of - and investigation into - their role in anti-tumour immunity. Following binding of antigen to the B cell receptor, internalisation and processing leads to its presentation on MHC class II molecules, which initiates an immune response in a number of ways. Firstly, engagement of the MHC-II complex by TCR and co-stimulatory signals initiates antigen-specific T cell proliferation and activation. Secondary, stimulatory signalling by production of cytokines, such as IL-4, from the T cell initiates differentiation of the B cell into a mature plasma cell, capable of producing antigen-specific antibodies. In addition, secretion of cytokines such as IFN-γ, TNF-α, IL-2 and IL-12 continue to perpetuate an immune response though mechanisms such as further differentiation of T_H1 cells. Once tumour cells

are opsonised by antibody (bound to specific surface antigens), death can be mediated through either phagocytosis or ADCC.

Effector functions of B cells may also differ as a consequence of alternative stimulation. B cells co-cultured with T_H1 cells, for example, produce higher levels of IFN-γ and IL-2, whereas those cultured with T_H2 cells produce greater levels of IL-2, IL-6 and IL-10 (103). Thus, in a similar fashion to other immune cells (T helper cells, neutrophils, macrophages), B cells can exhibit both pro- and anti-tumourigenic properties. Given that tumour-specific antibodies can be detected in the blood of cancer patients (104), suppressive mechanisms must also exist, in that the presence of such antibodies does not result in spontaneous tumour rejection (or prevention of tumour development). Tumour inoculation of breast cancer cells *in vivo* has been shown to initiate a humoural response with increased antibody production (105). Despite this, the presence of B cells inhibited T cell-mediated anti-tumour responses. One possible explanation may be increased competition between B cells and APCs, which is abrogated in a B cell-depleted environment, where anti-tumour responses were restored (105).

Co-culturing human B cells and CD4+ T cells *ex vivo* has demonstrated that B cells can induce the differentiation and expansion of a potently immunosuppressive Treg population (106). There is also data supporting a regulatory B cell phenotype, which suppress IFN-γ production (and hence T cell responses to antigen) through the production of IL-10 (107). As such, B cell depletion in murine models of melanoma enhanced tumour-antigen-specific T cell responses (107).

1.3 Cytokines and Chemokines

Cytokines are proteins secreted by cells which are involved in all aspects of biological function, including cell-to-cell communication, immune activation and inhibition. They can be broadly divided into pro-inflammatory and suppressive cytokines. Other categories also exist: interleukins, involved in cellular interactions between leucocytes; haematopoietic cytokines, involved in blood cell formation (e.g. GM-CSF, G-CSF) and those cytokines involved in humoral immunity, such as IL-4. For the purposes of this thesis, only the following examples have been discussed in specific detail.

1.3.1 Pro-Inflammatory Cytokines

1.3.1.1 Interferons

IFNs are broadly categorised into three groups: type I (including IFN-α and IFN-β), type II (IFN-γ) and type III (the IFN-λ family, including IL-28 and IL-29). They exert their individual functions through receptive IFN-stimulated genes (ISGs).

IFN production is stimulated by the engagement of PRRs (such as TLRs) with PAMPs from viruses (and other pathogens). TLR-7 and -8, for example, recognise PAMPs from RNA viruses such as vesicular stomatitis virus (VSV) and influenza, whereas TLR-9 recognises DNA viruses such as herpes simplex virus (HSV). TLR-4 initiates production of type I IFNs following activation by the LPS from bacterial membranes (108).

Type I IFNs are predominantly - but not exclusively - released as part of an anti-viral response by lymphocytes including T cells and NK cells. Immune cells produce both IFN-α and -β, while other cells such as fibroblasts and epithelial cells produce IFN-β. These bind to the type I IFN cell-surface receptor, which engages with a part of the intracellular Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway (109). Subsequent phosphorylation and activation of the JAK-STAT pathway initiates transcription of ISGs (110). As well as release of IFN-γ (the actions of which are discussed below), the transcription of these ISGs results in proteins that can inhibit various stages of viral entry, cell infection and replication. ISGs also stimulate an immune response by increasing the ability of APCs to prime adaptive immune responses through increased expression of MHC molecules (111), activation of NK cells, stimulation of antibody production by B cells (112) and upregulation of apoptosis-inducing molecules such as TRAIL (section 1.3.1.2) (113).

Viruses can manipulate type I IFN signalling pathways to subvert the anti-viral effects of IFNs: hepatitis C virus, which is implicated in the pathogenesis of hepatocellular carcinoma (HCC), can inhibit JAK-STAT signalling (114).

IFN-γ is secreted by NK cells, macrophages, APCs and T cells. Binding of IFN-γ to its receptor induces interaction with intracellular JAK1 and JAK2 (109). Hence, it also induces intracellular signalling of the JAK-STAT pathway and ISG transcription. In contrast to the production of type I IFNs, IFN-γ production from immune cells can be stimulated by IL-12 (secreted by macrophages in response to pathogens), IFN-α (as discussed above) and IL-18 (also known as IFN-γ inducing factor), secreted by monocytes and macrophages (115–117). Secretion of IFN-γ

is inhibited by cytokines such as TGF-β and IL-4 (118). The functions of IFN-γ are incredibly wide and various but include upregulation of MHC molecules to enhance antigen presentation (111), activating monocyte and macrophage activity against pathogens (79) and perpetuating a T_H1 response by activating NK cells and T cells (including through the upregulation of APC co-stimulatory molecules such as CD80 and CD86 (119)).

In malignant disease, IFNs exert multiple anti-tumour effects though similar pathways: upregulation of TRAIL and Fas by IFNs can contribute to cancer cell apoptosis (113,120). Similarly, the effects of type I IFNs on the inhibition of cell proliferation and cell cycle stasis has been demonstrated in prostate cancer cell lines (121) and melanoma (122). In the TME IFNs can induce polarisation of macrophages into an 'anti-tumour' phenotype (section 1.2.6). Type I IFNs have also been shown to increase the expression of TAAs (123) and maintain T cell populations (124), contributing to the priming of an adaptive anti-tumour response. Of note, while IFNs generally create an immune-stimulatory environment, this leads to the upregulation of checkpoint molecules such as CTLA-4 and PD-1/PD-L1 which can down-regulate immune responses (125).

Because of their immune-stimulatory properties, IFNs have been used in cancer immunotherapy, particularly in immunogenic malignancies such as renal cell carcinoma (RCC) (126) and melanoma (127,128) but their use has declined in recent years due to a combination of poor toxicity profile and newer immunotherapy agents (such as the checkpoint inhibitors) which offer an increased chance of response and improved outcomes.

1.3.1.2 TNF-Related Apoptosis-Inducing Ligand

TRAIL is a member of the TNF family which can initiate apoptosis of tumour cells through engagement with death receptors (DR) 4 (also known as TRAIL-receptor 1, TRAIL-R1) and DR5 (TRAIL-R2) (129). TRAIL is produced by T cells, NK cells, monocytes and DCs. As expected, the higher levels of DR4/5 observed in neoplastic cells (compared to normal epithelial cells) correspond to greater target cell apoptosis (130) and reduced tumour growth in *in vivo* murine models of breast cancer (129).

Although TRAIL is expressed constitutively on a range of normal tissues, immune cells may only express low levels of the molecule. Upregulation of TRAIL by IL-2 and IL-15 increases NK cell-mediated apoptosis of L929 fibrosarcoma cells *in vivo* (131). Stimulation of monocytes by IFN-γ and IFN-α can enhance TRAIL-mediated apoptosis of a range of malignant cell lines,

including ovarian, colorectal and prostate carcinoma cells through a similar mechanism (132). CD4+ T cells can also exert an apoptotic effect on melanoma cell lines through TRAIL (133).

TRAIL-mediated apoptosis of tumour cells can also be augmented in *in vivo* models of breast cancer by pre-treatment with chemotherapeutic agents including paclitaxel, vincristine, vinblastine, etoposide and doxorubicin which up-regulate DR4/5 death receptors (134). Similarly, CPT-11 (irinotecan) can increase TRAIL-mediated apoptosis of colorectal tumour cells through the same mechanism (135).

Although cell death can be initiated through interaction between TRAIL and DR4/DR5, the other TRAIL receptors, TRAIL-R3 and TRAIL-R4 (also known as DcR1 and DcR2, respectively), appear to inhibit the apoptotic effect of TRAIL, possibly through competitive binding of the ligand. As such, expression of TRAIL-R3 confers protection against TRAIL-mediated apoptosis of tumour cell lines (136).

Due to an apparent ability to preferentially target tumour cells for apoptosis, TRAIL-based therapeutic agents would be attractive. Although a range of phase I and II trials have been completed, (including those of mapatumumab, a monoclonal antibody to TRAIL-R1 (137,138)), such therapy has not yet progressed to routine clinical use due to a paucity of patient responses.

1.3.2 Immunosuppressive Cytokines

1.3.2.1 Transforming Growth Factor-β

TGF-β is an immune regulatory cytokine produced by a wide range of cells in response to stimulation by signals including mitogen-activated protein kinases (MAPK) pathway activation and cytokines such as TNF-α and TGF-β itself. Signal transduction through intracellular signalling pathways leads to TGF-β target gene transcription in the nucleus, controlling multiple different regulatory cell and immune responses, cell proliferation, differentiation and apoptosis, amongst others (139). TGF-β is usually associated with inhibition of cell proliferation, for example through inhibition of cell cycle progression through G1 by the production of proteins which block this process (reviewed in (140)). However, this mechanism seems disrupted in malignant disease. Levels of TGF-β are elevated in the serum of patients with cancer (141,142) and resistance to TGF-β-mediated cell death may be due to a number of mechanisms, including downregulation of TGF-β receptors (143). Thus, the anti-tumour effects of TGF-β may be outweighed by resistance mechanisms in the TME and the other

effects that TGF-β has in encouraging the epithelial-mesenchymal transition process, tumour invasion and metastases (144).

In contrast, TGF-β has many effects on the function of immune cells which ultimately may favour tumour growth. *In vitro* cultures with exogenous TGF-β inhibit NK cell proliferation and production of both IFN-γ and TNF-α (145). It can also downregulate activating NK cell surface receptors (146,147) and affect the mobility of DCs to the LN, both increasing the chance of metastatic LN spread but also impeding antigen presentation (and T cell expansion) (148). It also stimulates differentiation of immune cells from 'anti-tumour' to 'pro-tumour' phenotypes, such as macrophages from M1 to M2 (section 1.2.6) and neutrophils from N1 to N2 (section 1.2.8) (81,100).

Some of the effects of TGF-β on T cell functionality is mediated though it's inhibitory effects on cytokines such as IL-2 (139). It is also implicated in the impairment of effector memory T cell function: memory T cells from patients with melanoma re-stimulated *ex vivo* with antigen produced significantly lower amounts of IFN-γ than those cultured in the absence of TGF-β (149). As such, *in vivo* models of prostate cancer using TGF-β-resistant CD8+ T cells demonstrated reduced tumour growth and angiogenesis (150). Furthermore, TGF-β is responsible for inhibiting T_H1 differentiation of CD4+ naïve T cells and stimulating differentiation of this population into Tregs. Clinical trials of anti-TGF-β antibodies and small molecules are ongoing and seem to have tolerable safety profiles in early-phase trials (151).

1.3.2.2 Interleukin-10

IL-10 is produced by cells including Tregs, T_H2 T cells, monocytes, B cells and NK cells. Much like the IFNs, binding of IL-10 to its receptors (expressed on most cells) induces intracellular signalling through the JAK-STAT pathway and transcription of IL-10-dependent genes.

The exact mechanisms through which each cell type are stimulated to produce IL-10 are complex and not clearly delineated but can involve PRRs (such as TLRs) being activated by extracellular signals which are transduced into intracellular signalling, such as MAPK activation (152). IL-10 production is upregulated in response to inhibitory signals. As such, PD-1 expression on monocytes increases during chronic human immunodeficiency virus (HIV) infection and interaction between PD-1 and PD-L1 stimulates IL-10 production (153). The production of IL-10 consequently inhibits the proliferation and cytokine production (IFN-γ and IL-2) by CD4+ cells (153).

IL-10 is a potent modulator of antigen presentation and exerts many effects through DCs, such as inhibiting their production/maturation (154) or decreasing the potential of monocytes/macrophages as efficient APCs (155). Additionally, IL-10 downregulates the expression of the B7 co-stimulatory molecules on APCs (156) and MHC class II on APCs, hampering the efficient presentation of antigen and abrogating proliferation of T cells in response to antigen stimulation (157). Consequently, blocking IL-10 signalling with anti-IL-10-antibody in *in vivo* murine models of cervical cancer decreases tumour growth (158). IL-10 also mediates the differentiation of T_H2 cells and inhibits cytokines such as IL-2, IL-12 and IFN-γ and inflammatory cytokines such as TNF-α (159).

Unsurprisingly, higher IL-10 concentrations have been associated with poor prognosis in cancer. Levels of IL-10 in serum have been detected at elevated levels in patients undergoing surgery for CRC and were associated with higher stage and predicted recurrence rates following surgery (160). Similarly, pre-treatment quantities of IL-10 were lower in melanoma patients who went on to have a good response to therapy with B-rapidly-accelerated fibrosarcoma (BRAF) inhibitors (BRAFi) (161).

1.3.3 Chemokines

Chemokines are a subgroups of cytokines involved in chemotaxis: movement of specific cells towards an intended target, including migration of immune cells and the metastasis/invasion of tumour cells (reviewed in (162)). Regulated on activation, normal T cell expressed and secreted (RANTES, CCL5), for example, is a chemoattractant for T cells expressing the CCR5 ligand. Consequently, it has been found at higher levels in melanoma tumours infiltrated by T cells (163). IL-8 (CXCL-8) is a neutrophil chemotactic factor whereas IP-10 (CXCL-10) and monokine induced by gamma-interferon (MIG, CXCL-9) both attract monocytes. The receptor for both IP-10 and MIG, CXCR3, is expressed on T cells and is responsible for the chemokine-mediated migration of immune cells into TMEs where IP-10 and MIG are secreted (164).

MIG also exerts angiostatic properties and, as such, treatment with MIG has been shown to attenuate non-small cell lung cancer (NSCLC) tumour growth *in vivo* (165). Lower expression of IP-10 is associated with poorer prognosis in stage II and III CRC (166). Despite this, upregulation of IP-10 in CRC cell lines enhances factors associated with metastasis and invasion, such as matrix metalloproteinase (MMP) expression, which degrades the extracellular matrix and allows cell migration (165).

1.4 Inflammation in Cancer

Inflammation is the normal physiological process used to fight infection and other biological insults or pathogens. It primarily involves the mobilisation of phagocytic immune cells (neutrophils and macrophages) through production of chemokines/cytokines, acute phase proteins such as CRP, complement and coagulation factors, such as fibrinogen, to sites of injury. Inflammation is generally a positive response to an insult: indeed, the biological processes involved in acute inflammation have been exploited in the management of early cancers, such as in the use of intravesical Bacillus Calmette-Guérin (BCG) for the management of superficial bladder tumours. There is, however, an overlap between this normal physiological response and many of the factors that can contribute to tumourigenesis. In fact, inflammation is known to be associated with the development of tumours and is considered to be one of the hallmarks of cancer (1). The link between inflammatory bowel disease and CRC, *Helicobacter pylori* infection and gastric cancer, viral hepatitis and HCC and smoking and lung cancer all illustrate the relationship between chronic inflammation and tumourigenesis. Inflammation and cancer are linked in many complex ways but there are two pathways which broadly delineate this association. Firstly, genetic mutations lead to the development of malignant cells, which, in turn, produce cytokines and other molecules creating an inflammatory environment (intrinsic pathway). Secondly, an underlying inflammatory condition induces oncogenesis and the development of tumours (extrinsic pathway) (167). Illustrating this concept, the induction of pancreatitis can lead to the development of pancreatic ductal carcinoma in mice bearing mutations in the Rat Sarcoma proto-oncogene *KRAS* (168). Mutations in the 'rearranged during transfection' (*RET*) oncogene, which is associated with the development of thyroid cancers, can induce a pro-inflammatory environment. Expression of *RET* in normal thyroid cells upregulates the expression of genes encoding a wide variety of inflammatory mediators including the cytokines IL-1 and GM-CSF and the immune cell chemokines CXCL-10 and CXCL-12. Additionally, factors associated with tumour metastases, such as MMPs, were also upregulated (169).

The production of chemokines and cytokines, plus activation of certain transcription factors, either as the result of inflammatory disease or oncogenesis, contribute further to tumour cell development, proliferation and survival. As an example, IL-1α and IL-1β, key pro-inflammatory cytokines, have been shown to play an essential role in tumour metastases and neo-vascularisation in mouse models of melanoma (170). Another inflammatory cytokine, IL-6, which can stimulate the production of neutrophils in response to infection, supports tumour

cell growth and proliferation through activation of STAT3 (171). Production of IL-6 in this model of lung cancer is also upregulated by mutations in the epithelial growth factor receptor (EGFR) (171).

The presence of pro-inflammatory mediators can also contribute to the accumulation of genetic mutations which can increase the predilection for malignant transformation. ROS and NO released by TILs can cause DNA damage and inhibit the activity of the tumour suppressor gene p53, which has a role in the repair of DNA damage (167).

The interplay between each of these factors: activation of transcription factors which enhance cell proliferation, increased chromosomal instability and genetic damage, immune cell infiltration and possible underlying mutations in oncogenes culminate in a strongly pro-tumourgenic environment through manipulation of the normal biological processes involved in inflammation.

1.5 Immune Effects of Anti-Cancer Therapy

Systemic anti-cancer therapy (SACT) can generate an anti-tumour immune response in a number of ways, most simply by initiating tumour cell death and release of TAAs which has the potential to prime an adaptive immune response. However, there are other mechanisms through which SACT can directly and indirectly modulate the immune system, dependent on a number of factors including the type of therapeutic agent, tumour type and baseline immune functionality of the patient. Conversely, treatment-mediated tumour cell death, although leading to antigen release, subsequently generates a clone of tumour cells resistant to therapy and which have the potential to metastasise.

1.5.1 Chemotherapy

Although established dogma states that traditional cytotoxic chemotherapy regimens are immunosuppressive, it is becoming more obvious that these chemotherapy drugs can actually contribute to an anti-tumour response, rather than tumour tolerance. Therefore, in addition to specifically targeting the tumour itself, there is more of an appreciation that the immune effect of cytotoxic chemotherapy can be exploited for further clinical benefit.

1.5.1.1 Taxanes (Paclitaxel and Docetaxel)

Taxanes are anti-microtubular agents originally derived from the yew tree. They exert an anti-cancer effect by preventing cell division through their effect on microtubules, which form an integral part of the cell cytoskeleton. Docetaxel therapy can decrease the proportion of Tregs in peripheral blood of patients receiving treatment for metastatic castrate-resistant prostate cancer (172) without any significant change in either the total number of PBMCs or the CD4+ population. Similar results are seen in patients receiving docetaxel for metastatic breast cancer and those receiving cisplatin and vinorelbine for NSCLC in the adjuvant setting (172).

Taxanes have been shown to change the cytokine profile in patients receiving treatment for breast cancer. Compared to healthy controls, levels of IL-2, IFN-γ and GM-CSF are reduced in patients prior to chemotherapy, whereas levels of TNF-α and IL-6 were elevated (173). Treatment with either paclitaxel or docetaxel reversed these findings and was associated with both an increase in NK cell-mediated cytotoxicity against target tumour cells and enhanced T cell proliferation (173). Additionally, cytolytic activity of immune cells, including macrophages, against tumours is enhanced following treatment with paclitaxel (174,175).

1.5.1.2 5-Fluorouracil

The anti-metabolite 5-FU inhibits the enzyme thymidilate synthase (TS), which is involved in DNA synthesis. While taxanes can modulate the immune response by depleting Tregs, 5-FU has been shown to diminish populations of MDSCs *in vivo* (96), without affecting other immune cell populations. This may be mediated by low expression of TS by MDSCs; sensitivity of tumour cells to 5-FU has been shown previously to be related to TS expression (176,177). Depletion of these immunosuppressive cells leads to subsequent enhancement of CD8+ T cell-modulated anti-tumour immune responses (96). Both 5-FU and irinotecan (CPT-11, which disrupts DNA replication through the inhibition of the topoisomerase enzymes), also mediate the death of CRC cell lines *in vitro* by enhancing CTL-mediated tumour cell lysis (178).

Patients with oesophageal cancer have been found to have greater infiltration of immune effector cells into the TME after receiving neoadjuvant chemotherapy (179). Treatment with pre-operative 5-FU (and cisplatin) was associated with more CD4+ cells in the tumour bed and CD8+ cells in adjacent stroma. This was not associated with a concomitant increase in Tregs (179). Infiltration of these immune cells in both the tumour mass and tumour stoma have been associated with improved prognosis (180).

1.5.1.3 Cyclophosphamide

Cyclophosphamide is an alkylating agent, which causes DNA damage by cross-linking DNA strands and preventing DNA replication. It can deplete Tregs (independent of the total CD4+ population) and slow the progression of murine colon cancer tumours *in vivo* (181). This effect is mirrored in patients with advanced cancer, who are shown to have higher numbers of circulating Tregs compared to healthy volunteers. Low dose cyclophosphamide reduces the number of circulating Tregs, without affecting other immune-cell subsets; this is associated with an increase in both NK cell-mediated tumour cell lysis and T cell proliferation *ex vivo* (182). Aside from a reduction in the number of Tregs, thought to be attributable to increased apoptosis of cells induced by the drug (183), cyclophosphamide can also reverse Treg-mediated suppression of CD8+ and CD4+CD25- T cell proliferation (183). Unfortunately, this effect has not been borne out in early phase trials of cyclophosphamide given in combination with an oncolytic virus: populations of Tregs were not modified with combination therapy and there was no suppression in levels of anti-viral titres (184).

1.5.1.4 Anthracyclines

Anthracyclines such as doxorubicin and epirubicin exert anti-cancer effects through a range of mechanisms, including cross-linking of DNA strands (like alkylating agents), generation of ROS that damage DNA, proteins and cell membranes and activation of p53. Doxorubicin has long been known to have immunomodulatory effects. It has been observed *in vivo* that splenocytes treated with doxorubicin have greater cytotoxicity against target tumour cell lines compared to untreated splenocytes (185). Similarly, PBMCs from patients treated with doxorubicin exhibit enhanced cytotoxicity against tumour targets, which is likely mediated by increased IL-2 and greater numbers of CD8+ T cells (186). Doxorubicin can also activate TLR-3, initiating a type I interferon response similar to the anti-viral response, to stimulate the production of IP-10 (187).

1.5.1.5 Gemcitabine

Gemcitabine causes tumour cell death by being incorporated into DNA, preventing cell replication and division. As well as having the potential to diminish populations of immunosuppressive MDSCs like 5-FU (97), gemcitabine can have differential effects on various elements of the immune system. Whilst *in vivo* treatment with gemcitabine caused the expected depletion of both lymphocyte and B cell populations, T cell responses to TAAs were

enhanced by therapy (188). Conversely, B cell-mediated antibody production was abrogated (188).

1.5.2 Checkpoint Inhibitors

In addition to stimulatory signals such as engagement of CD80/CD86, there are a wide variety of inhibitory 'checkpoint' molecules which act at the immunological synapse to regulate immune responses against TAAs. These include the immune checkpoints CTLA-4 (which binds to CD80/CD86) and PD-1 and its ligand PD-L1. However, with the expanding interest in immunotherapy and immune checkpoint blockade, more attention is being paid to the potential of other receptors including LAG-3 and TIM-3. Targeting these molecules effectively inhibits the negative-feedback response to immune stimulation, thereby increasing the effect against tumours. Use of antibodies against CTLA-4, PD-1 and PD-L1 (and combination therapies) have been established in the clinic in diseases including melanoma (189), lung cancer (190,191) and RCC (192) and other targeted antibodies therapies are currently making their way through clinical trials. The clinical efficacy of checkpoint inhibitors in melanoma will be discussed in more detail in section 5.1.4.3.1.

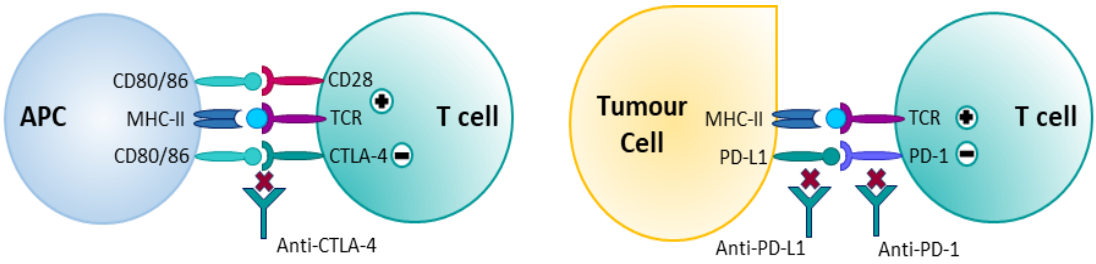


Figure 1.1 Checkpoint inhibition by anti-CTLA-4 and anti-PD-1/-L1 antibodies

T cells are activated following engagement between the TCR and the MHC/antigen complex, as well as co-stimulatory signalling through binding of CD28 and CD80/86. CTLA-4 competitively binds with CD80/86 to switch off T cell activation. This process is blocked by CTLA-4 antibodies **(a)**. Similarly, anti-PD-1 and anti-PD-L1 antibodies block PD-1/PD-L1, respectively, at the interface between tumour cells and T cells **(b)**.

1.5.2.1 CTLA-4

Following T cell activation, CTLA-4 expression on the cell surface is upregulated and will competitively bind with CD80/86 co-stimulatory molecules on the surface of APCs. As this is with higher affinity than CD28, T cell activation is effectively switched off. Anti-CTLA-4 antibodies, therefore, block this mechanism and the T cell remains activated, with ongoing proliferation (193) and amplification of antigen-specific responses (Figure 1.1a). In a small number of heavily-pretreated patients with metastatic melanoma receiving the fully humanised anti-CTLA-4 antibody ipilimumab, a higher proportion of CD4+/ICOS+ or CD8+/ICOS+ cells by week 7 of therapy correlated with disease control. An increase in these populations of activated T cells were also associated with greater OS (194). Similarly, activation of CD4+ and CD8+T cells was enhanced in patients with melanoma and RCC receiving anti-CTLA-4 antibody (195).

As well as the enhancement of T cell responses, checkpoint inhibition can also affect the immunosuppressive action of Tregs, which not only constitutively express CTLA-4 but also PD-1, PD-L1, TIM-3 and LAG-3. Although there is conflicting data in both animal and human models, there is evidence that the suppressive effect of Tregs is dependent, to some extent, on the presence of CTLA-4 (60,196). In patients with advanced prostate cancer, treatment with ipilimumab increased the proportion of both activated CD4+ effector cells and Tregs (193). Similar findings have been seen *ex vivo* from samples of patients treated with metastatic melanoma and RCC: anti-CTLA-4 blockade seem to exert an effect through enhanced activation of T cell populations rather than decreased Treg activity (195). Indeed, if Treg populations are depleted prior to treatment with anti-CTLA-4 antibody (on this occasion with the humanised antibody tremelimumab), there is evidence of increased effector T cell proliferation *in vitro* and *in vivo*. This corresponds to elevated cytotoxicity against specific target cell lines (197). Presumably, in the absence of Treg suppression, activation of CD4+ cells triggers increased expression of CTLA-4 as part of normal feedback and thus making target tumour cells more susceptible to CTLA-4 blockade (197). This is supported by further *in vivo* data which demonstrates that, in murine models of melanoma, blocking CTLA-4 on both Treg and effector T cells prolonged survival and significantly decreased tumour growth (198).

1.5.2.2 PD-1/PD-L1

In contrast, the interaction between PD-1 and PD-L1 is at the interface between immune cells and tissues/tumour (rather than CTLA-4 on the T cells and APCs in the LN). T cells, which have already been primed against antigens, travel to antigen-positive tissue to exert their effect. Following activation, IFN-γ and other pro-immune inflammatory signals upregulate PD-1 and PD-L1 (199), thereby abrogating the T cell response at the site of infection/tumour (Figure 1.1b) . In contrast to CTLA-4, PD-1 is also expressed on B cells and NK cells and so PD-1 blockade with anti-PD-1 (or PD-L1 antibodies) may have multi-factorial effects including antibody production and innate NK cell-mediated cytotoxicity. Expression of PD-1 on tumour-infiltrating immune cells (200) and PD-L1 on tumour cells are both increased in malignancy (199). Indeed, higher PD-L1 expression on tumour cells has been associated with poorer prognosis (201).

In addition to abrogating T cell activity, the engagement of PD-1 on antigen specific T cells and PD-L1 on target tumour cells has been shown to amplify apoptosis of CTLs (199). Interestingly, the efficacy of anti-PD-1 therapy does not seem to be wholly dependent on PD-L1 expression on tumour cells: in *in vivo* models of CRC, mice with PD-L1 knockout tumours still responded to anti-PD-L1 therapy (202). This is particularly pertinent given that current guidance for using the humanised IgG4 anti-PD-1 antibody pembrolizumab in patients with NSCLC is based on PD-L1 expression in the tumour. As PD-L1 is also expressed on DCs, blocking PD-1 also leads to increased T cell activation and cytokine production though stimulation of the TCR by activated DCs (203). Constant stimulation of T cells, in either chronic infection or cancer, can lead to ongoing PD-1 expression and T cell anergy. Blocking PD-1 or PD-L1 in this context can boost proliferation, cytotoxicity and IFN-γ production of this population of previously 'exhausted' T cells (204), highlighting the potential for anti-PD-1/-L1 therapy in malignant disease.

1.5.2.3 TIM-3, BTLA and LAG-3

TIM-3 binds to galectin-9, one of a number of proteins which are responsible for transmembrane signalling and cell-cell interactions. It is expressed constitutively on NK cells (especially the $CD56^{dim}$ phenotype) (205). As seen with other inhibitory immune checkpoints, TIM-3 is upregulated on activated antigen positive CD8+ T cells (206) and CD4+ T_H1 cells (207). Engagement of TIM-3 and galectin-9 decreases T_H1 effector cell proliferation and IFN-γ production (207) and therapy with anti-TIM-3 antibody can reduce tumour burden in an *in*

vivo model of murine sarcoma (208). Phase I clinical trials of the anti-TIM-3 antibodies TSR-022 and LY3321367 are ongoing (NCT02817633, NCT03099109).

B and T lymphocyte attenuator (BTLA, also known as CD272) is another T cell-associated inhibitory molecule. It exerts its inhibitory properties through binding with the TNF receptor superfamily member 14, which is expressed on tumour cells such as melanoma (209). Like PD-1 and CTLA-4, it is expressed following T cell activation but upregulation on T_H1 (rather than T_H2) effector cells is prolonged (210). Similarly to other checkpoint molecules, BTLA-deficient T cells exhibit increased activation and proliferation following stimulation with the TCR and anti-CD3 antibody (210) and BTLA-deficient mice exhibit a greater frequency of autoimmune diseases.

As well as its effect on T cell-mediated immunity through engagement with MHC-II molecules, LAG-3 mediates the immunosuppressive function of Tregs, on which it is also expressed. Indeed, LAG-3 expression increases on activated Tregs, which then exert a potent immunosuppressive effect in a LAG-3-dependent manner. Use of anti-LAG-3 antibodies therefore abrogates the effect of Tregs *in vitro* and *in vivo*, which is demonstrated by an rise in T cell proliferation upon stimulation (211). Early phase clinical trials with IMP321 (a LAG-3 fusion protein that binds MHC-II) have already been reported, showing a tolerable safety profile and, pertinently, an increase in the population of effector memory T cells, in patients with metastatic breast cancer (212) and RCC (213). Trials in pancreatic cancer at a lower dose of IMP321 have not demonstrated the same effect (214).

Co-expression of BTLA, TIM-3 and PD-1 on antigen-specific CTLs from patients with metastatic melanoma defines a population of exhausted, poorly-functioning T cells which produce diminished amounts of IFN-γ, IL-2 and TNF-α compared to T cells expressing only one of these inhibitory molecules (215). The additional findings that treatment with combination therapies (anti-TIM-3/anti-PD-1, anti-BTLA/anti-PD-1/anti-TIM-3 or anti-LAG-3/anti-PD-1) increase the proportion of activated (cytokine-secreting) CTLs would seem to support the rationale for combination therapy in the future (206,215,216). Trials of combination therapy with immune checkpoint inhibitors, including IMP321 and pembrolizumab in melanoma (NCT02676869) and anti-TIM-3 and anti-PD-L1 antibody in advanced solid tumours (NCT03099109) are ongoing.

1.5.3 Radiotherapy

Similar to chemotherapy agents, which induce cell death and promote release of TAAs to prime an anti-tumour immune response, radiotherapy may also exert a similar effect at the targeted site and at distal sites. This phenomenon, known as the 'abscopal effect' is demonstrated clinically when treatment of a particular site with radiotherapy leads to improvement in non-treated areas. Supporting this, *in vivo* models of colon carcinoma have shown that, following radiotherapy, disease response was associated with elevated immune cell infiltration into the tumours, raised IFN-γ production and subsequent enhancement of cytotoxic potential of intratumoural CD8+ T cells from the responding tumours (217). In a phase II trial of radiotherapy plus GM-CSF (to enhance DC maturation) 26.8 % of patients were seen to have abscopal responses in non-irradiated tumour sites (218). The additional increase in OS between patients who did or did not have abscopal responses (20.98 months *vs*. 8.33 months, respectively) supports the hypothesis that having an effective anti-tumour response can be beneficial in the context of radiotherapy.

In *in vivo* models of melanoma, the immune effects of radiotherapy have been shown to be mediated by IFN-γ: production of this cytokine after radiotherapy increased expression of chemokines including CXCL9 (219). Other *in vitro* studies have confirmed that radiation can stimulate an environment which promotes immune cell infiltration: radiotherapy-stimulated breast cancer cells produce the chemokine CXCL16, which attracts T and NK cell populations expressing its receptor CXCR6 (220). In addition to enhancing T cell infiltration into tumour, radiotherapy-induced IFN-γ can stimulate upregulation of MHC-I, which consequently leads to increased CTL-mediated tumour cell cytotoxicity (219). As some of the effects of radiotherapy are mediated by infiltration of antigen-specific T cells within the TME, the effects of radiotherapy on DCs have also been investigated. Indeed, radiotherapy has been shown to enhance expression of co-stimulatory molecules on DCs, such as CD80, which, along with binding of antigen to the TCR, are needed to stimulate T cell proliferation (221).

Although radiotherapy can exert a positive effect on the immune microenvironment, treatment can also initiate the infiltration of other populations into the TME, such as immunosuppressive cytokines, MDSCs and M2 TAMs. Indeed, patients with head and neck squamous cell carcinoma are found to have raised levels of circulating Tregs following treatment with chemoradiotherapy (222). Interestingly, there is also evidence that Tregs are more radio-resistant than conventional CD4+ cells, leading to an apparent increase in

irradiated tissues (222,223). Radiotherapy has also been shown to induce the expression of TGF-β (224); this is one mechanism through which the adaptive anti-tumour responses and the priming of such by tumour cell death is inhibited (225). The increase in TGF-β may be one mechanism by which radiotherapy itself upregulates PD-1 and PD-L1 on immune cell populations (225). Radiotherapy may also promote immunosuppressive effects by encouraging infiltration of M2 TAMs, as shown in *in vivo* models of astrocytoma, glioma and prostate cancer (226). This may be partially attributable to the secretion of chemokines for macrophages, such as stromal cell-derived factor-1 (SDF-1, also known as CXCL-12), which has a role in angiogenesis and is expressed as a result of tissue hypoxia resulting from radiotherapy (227).

Clinical trials of radiotherapy and checkpoint inhibition are underway (NCT02821182, NCT02298946) (228). Combination with anti-PD-1 and anti-PD-L1 inhibitors may be of particular interest given that radiotherapy can stimulate T cell infiltration into the tumour, which is the site of activity of these immunotherapy drugs.

1.5.4 Oncolytic Viruses

Oncolytic viruses (OVs) were initially thought to exert their effect through direct oncolysis of malignant cells, which are preferentially infected due to the presence of mutations or factors which made them more susceptible to OV-mediated killing. As studies into these agents have progressed, it has become clear that the generation of immune responses by OVs may be as important (if not more so) in their direct anti-tumour effects. A variety of OVs are currently being investigated, including RNA and DNA viruses (such as Respiratory and Enteric Orphan virus (reovirus), coxsackie virus and HSV) or genetically-modified viruses such as talimogene laherparepvec (HSV encoding GM-CSF, also known as T-VEC and OncoVEX^{GM-CSF}), discussed in section 5.1.4.3.2.

Initial concerns about the potential neutralisation of OVs before they can exert their effects seem to be unfounded. In a phase I trial of reovirus, the presence of neutralising antibodies (Nab) at baseline did not prevent reovirus from reaching tumour sites (229). In patients with advanced CRC receiving adenovirus (coding for CEA), over half the patients had NAb at baseline yet, despite increasing levels, there was evidence of an antigen-specific immune response to treatment (230).

In vitro treatment of monocyte-derived healthy donor (HD) DCs with reovirus can stimulate secretion of IFN-α, IL-12 and TNF-α. These activated DCs can stimulate NK cells and T cells, leading to increased secretion of IFN-γ by both cell subtypes and more potent cytotoxicity against target tumour cells (231). In samples from HDs and patients, CD69 expression on NK cells was elevated by exposure to reovirus, with enhanced cytotoxicity as shown by greater CD107 expression against relevant tumour cell targets (229,232).

In addition to their effects on innate immunity, OVs can enhance priming of an adaptive immune response (233). Moreover, this effect does not rely on infection of target cells followed by cell lysis and release of TAAs. B16ova melanoma cells, which express low levels of the reovirus receptor junctional adhesion molecule-A are resistant to reovirus infection. When immunocompetent mice were implanted with B16ova tumours and treated with reovirus, isolated splenocytes produced IFN-γ when re-exposed to melanoma antigens. This indicates the activation of a specific anti-tumour response in the absence of direct cytotoxicity (234).

Treatment with OVs can generate an immune milieu capable of eliciting an immunogenic and cytotoxic response. Following infection with reovirus, both human melanoma cell lines and primary melanoma cells produce greater amounts of IL-8 and RANTES which can recruit immune cells into sites of inflammation (235,236). Associated with this, NK cells, DCs and CTLs (primed against melanoma cell lines) all migrate towards tumour-conditioned media (reoTCM) from reovirus-infected melanoma cells in the absence of active virus. Down-regulation of IL-10 is also observed after therapy *in vitro* (235).

Although intravenous (*i.v.)* delivery of OVs may be the most practical route, some are still being administered intratumourally (*i.t.)* in order to circumvent the issue of NAbs completely. The populations of infiltrating cells into the tumour can determine whether a pro- or anti-tumour immune environment prevails and *i.t.* therapy offers an elegant system in which to study the effects of OVs on the TME. Intralesional injection of T-VEC into melanoma metastases has been shown to reduce the populations of immunosuppressive Tregs and MDSCs (237). Conversely, there was an increase in melanoma-antigen specific T cells in the TME (237). Moreover, *i.t.* therapy can result in infiltration of immune cells into non-injected lesions, which may go some way to explain why *i.t.* OVs can affect injected and non-injected tumours (238,239). Given these findings, further studies into the clinical use of OVs may instead focus on intensifying their immune-mediated effects to exploit potential combinations with other immunotherapies such as the checkpoint inhibitors.

Chapter 2 Materials and Methods

2.1 Buffers and Reagents

Table 2.1 Buffers and Reagents

Name	Constituents
Ammonium Chloride Potassium (ACK) Buffer	0.15 M NH_4Cl (Sigma Aldrich, St. Louis, USA) + 0.1 mM ethylenediaminetetraacetic acid (EDTA, Fisher Scientific, Waltham, USA) + 10 mM $KHCO_3$ (Sigma Aldrich) in double-distilled water (ddH_20; pH 7)
ELISA Blocking Solution	Phosphate buffered saline (PBS) + 10 % (v/v) foetal calf serum (FCS; Life Technologies, Carlsbad, USA).
ELISA Coating Buffer	100 mM $NaHCO^3$ (BDH Lab Supplies, Poole, UK) in ddH_2O
Fluorescence-Activated Cell Sorting (FACS) Buffer	PBS + 10 % (v/v) sodium azide (NaN_3) (Sigma Aldrich) + 10 % (v/v) FCS
PBS-T	PBS + 0.05 % (v/v) Tween-20 (Sigma Aldrich)

2.2 Cell Culture

Cell lines were cultured in Sanyo CO_2 incubators at 37 °C in a humidified atmosphere of 5 % CO_2. Cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich) or Roswell Park Memorial Institute (RPMI; Sigma Aldrich) medium and supplemented as shown in Table 2.2. FCS and pooled human AB serum (HS; Gemini Bio-Products, Sacramento, USA) were heat-inactivated at 56 °C for 30 minutes prior to use. Adherent cell lines were passaged when approaching confluence using trypsin (Sigma Aldrich). Cell lines were regularly tested for mycoplasma contamination. All work involving blood and viruses was carried out in either Nuaire or BioMat2 Category II Microbiological Safety Cabinets decontaminated with 2 % (w/v) Virkon (Fisher Scientific) followed by 70 % (v/v) ethanol (Sigma Aldrich). Appropriate personal protective equipment was worn. Waste products were decontaminated in either 2 % (w/v) Virkon or 20 % (v/v) Distel (Tristel Solutions Ltd, Newmarket, UK).

All centrifugations were performed at 400 g for 5 minutes with full acceleration and brake, unless otherwise stated.

Table 2.2 Culture Media

Cell Line	Culture Medium	Name
Mel624 (Melanoma) **Mel888 (Melanoma)** **SW480 (Colorectal)** **SW620 (Colorectal)**	DMEM + 2 mM L-Glutamine + 10 % (v/v) FCS	Complete DMEM
K562 (Chronic Myeloid Leukaemia, CML)	RPMI + 2 mM L-Glutamine + 10 % (v/v) FCS	Complete RPMI
PBMCs	RPMI + 2 mM L-Glutamine + 10 % (v/v) FCS	Complete RPMI

2.3 Reovirus

Reovirus Type 3 Dearing was provided by Oncolytics Biotech® Inc. (Calgary, Canada). Stock titre was determined using a standard plaque assay protocol. Aliquots were stored at 4 °C or - 80 °C for longer term storage.

2.4 Cell Lines

SW480, SW620, K562, Mel624 and Mel888 tumour cell lines were supplied by the American Type Culture Collection (ATCC). K562 cells were selected for use in experiments as the 'gold standard' NK cell target.

2.5 Cell Counts

Cells were observed using Nikon Eclipse TS100 or Olympus CKX31 microscopes and counted using a standard haemocytometer and Trypan Blue (Beckman Coulter, Brea, USA) exclusion method.

2.6 Propidium Iodide Staining for Viability

Where stated, propidium iodide (PI) staining was also used to calculate viability of cells (240). Aliquots of $1x10^6$ PBMCs were washed/centrifuged in PBS and 5µl PI (0.01mg/ml in PBS) was added. Data was acquired immediately using a Becton Dickinson ™ (BD ™, Franklin Lakes, New Jersey, USA) LSRII 4-laser flow cytometer.

2.7 Collection of Blood Samples

Peripheral whole blood was obtained from either HD volunteers or patients undergoing treatment at Leeds Cancer Centre, St James's Hospital, Leeds, UK. Leucocyte cones were obtained from NHS Blood and Transplant at Seacroft Hospital, Leeds. Informed consent was obtained from all patients and donors in accordance with local institutional ethics review and approval.

2.8 Isolation of Blood Components

2.8.1 Plasma

K3-EDTA vacuettes (Greiner Bio-One, Kremsmünster, Austria) containing whole blood samples were were centrifuged at 2000 g for 10 minutes with low acceleration and no brake . Plasma was then harvested using a sterile Pastette, aliquoted into Eppendorf tubes (Scientific Laboratory Supplies, Nottingham, UK) and stored at -80 °C until required.

2.8.2 Whole White Blood Cells

Whole WBCs were isolated from blood collected into K3-EDTA vacuettes: blood tubes were centrifuged at 2000 g for 10 minutes with low acceleration and no brake and the WBC layer harvested. Red cell lysis was performed on ice using ACK buffer (Table 2.1). Cells were placed on ice for a maximum of 5 minutes, with close monitoring. Once lysis was complete, cells were washed in Hank's Buffered Salt Solution (HBSS; Sigma Aldrich) and centrifuged at 400 g for 10 minutes, followed by a further 2 washes and 5 minute spins. If red blood cell contamination persisted, ACK treatment was repeated.

2.8.3 Peripheral Blood Mononuclear Cells

PBMCs were isolated from blood (collected in EDTA vacuettes) or leucocyte cones using density gradient separation. After collection of whole blood samples, the WBC population was harvested as described above, without the need for red lysis. WBC were diluted with HBSS and overlaid onto Lymphoprep© (Axis Shield, Oslo, Norway) at a ratio of 2:1. Tubes were centrifuged at 800 g for 20 minutes with low acceleration and low brake. The PBMC layer was harvested, washed in HBSS and centrifuged at 400 g for 10 minutes. Cells were washed in HBSS and centrifuged twice more for 5 minutes before cell counts performed. PBMCs were

then re-suspended at the required density. Blood from leucocyte cones was directly diluted with HBSS and PBMCs isolated as described above.

2.9 Cryopreservation of Cells

PBMCs and WBCs were cryopreserved at defined densities in appropriate freezing mixture (FM) (Table 2.3). FMs and cryovials (Thermo Scientific, Waltham, USA) were equilibrated to 4 °C prior to use. FM was added in a drop-wise manner, whilst swirling the cells, to minimise cell damage by dimethyl sulphoxide (DMSO; Sigma Aldrich). The procedure was carried out swiftly to preserve cell membrane integrity. Cryovials were transferred into Mr Frosty™ (Thermo Scientific) freezing containers and stored at -80 °C until transfer to liquid nitrogen (LN2), usually within 24 hours.

Table 2.3 Freezing Mixtures

Name	Constituents
'Standard' FM	90 % (v/v) FCS + 10 % (v/v) DMSO
10 % DMSO/RPMI	90 % (v/v) complete RPMI + 10 % (v/v) DMSO
20 % DMSO/RPMI	80 % (v/v) complete RPMI + 20 % (v/v) DMSO
'Clinical' FM	40 % (v/v) RPMI (including 1 mM sodium pyruvate, Sigma Aldrich) + 50 % (v/v) human serum (HS) + 10 % (v/v) DMSO

2.10 Thawing Cells

Cells were transported on dry ice from LN2 to a 37 °C water bath. Vials were closely monitored and removed when a small ice crystal was still visible. Cells were thawed into either PBS or cell culture media, immediately washed with PBS and pelleted by centrifugation.

2.11 Reovirus Treatment of PBMCs

PBMCs were re-suspended at $2x10^6$ cells/ml in complete RPMI and incubated ± reovirus (0 or 1 plaque-forming unit (pfu)/cell) overnight at 37 °C. Next day, PBMCs were harvested and washed free of unbound virus. Reovirus-treated PBMCs were used in functional assays (below) and cell culture supernatants stored at -20 °C.

2.12 Chromium Release Cytotoxicity Assay

Tumour cell lines, which were used as targets, were labelled with 50 µCi of chromium-51 (^{51}Cr; Perkin-Elmer, Waltham, USA) for 1 hour at 37 °C. Excess radiation was removed by 3 large volume washes/centrifugations in PBS. PBMCs were re-suspended at 5x10^6 cells/ml in complete RPMI. Triplicate, halving serial dilutions of effector PBMCs were performed in 96-well U-bottomed plates to ensure decreasing effector:target (E:T) ratios. Control plates for maximum and spontaneous target release were set up using 1% (v/v) Triton-X (Sigma Aldrich) or complete RPMI, respectively. ^{51}Cr-labelled targets were then added at 5x10^3 cells/well. Following a 4 hour incubation at 37 °C, plates were centrifuged, supernatants transferred onto Luma scintillation plates (Perkin-Elmer) and left to dry overnight. A MicroBeta Jet Scintillation counter (Perkin-Elmer) determined ^{51}Cr-release from target cells in counts per minute (cpm). The percentage of lysed target cells was calculated using the formula:

$$\%\, lysis \;=\; 100\; x\, \frac{(sample\; cpm \;-\; spontaneous\; cpm)}{(maximum\; cpm \;-\; spontaneous\; cpm)}$$

2.13 CD107 Degranulation Assay

PBMCs and tumour targets were re-suspended at 5x10^6 cells/ml in complete RPMI and aliquoted into FACS tubes (Sigma Aldrich) at a 1:1 ratio. Following incubation for 1 hour at 37 °C, 10 µg/ml Brefeldin A (Biolegend, San Diego, USA) was added, in addition to anti-CD107a-FITC and anti-CD107b-FITC antibodies (Table 2.4). After a further 4 hours at 37 °C, cells were washed/centrifuged in FACS buffer (Table 2.1). NK cell identification antibodies (anti-CD3-PerCP and anti-CD56-PE, Table 2.4) were added and tubes protected from light at 4 °C for 30 minutes before a final wash in FACS buffer. Cells were fixed in 1% (v/v) paraformaldehyde (PFA; Sigma Aldrich) and protected from light at 4 °C until flow cytometry was performed.

2.14 Preparation of Cryopreserved Cells for Immunophenotyping

PBMCs were thawed as previously described (section 2.10) and subjected to immunophenotyping immediately ('same day') or 'rested' at 37 °C overnight at a density of 2x10^6 cells/ml in complete RPMI before immunophenotyping (section 2.14.1).

2.14.1 Immunophenotyping and Flow Cytometry

Cells were re-suspended in FACS buffer at $5x10^6$ cells/ml and $5x10^5$ cells used per tube for staining with appropriate fluorescently-conjugated antibodies (Table 2.4). Antibody staining was performed in darkness for 30 minutes at 4 °C and unbound antibody washed free with FACS buffer. Cells were fixed in 1% (v/v) PFA and kept protected from light at 4 °C until flow cytometry was performed.

Data acquisition was undertaken using a BD™ LSRII 4-laser flow cytometer and analysed using BD FACSDiva™ software (version 8.0). Expression of cell surface markers was calculated as the percentage positive cells in a given population over the isotype control. Relative median shift (RMS), as a function of median fluorescence intensity (MFI) compared to the isotype control of each fluorophore, was calculated using the formula:

$$Relative\ Median\ Shift = \frac{Observed\ MFI}{Isotype\ MFI}$$

2.14.2 Intracellular Staining of PBMCs for FoxP3

Tregs were stained for intracellular FoxP3 using the FoxP3 Staining Buffer Set (Miltenyi Biotech, Bergisch Gladbach, Germany) following manufacturer's instructions. Following extracellular staining for cell surface markers (section 2.14.1), cells were washed in FACS buffer. Fixation/Permeabilisation solution was added and incubated at room temperature (RT) for 30 minutes. Cells were then washed in 1x Permeabilisation Buffer and anti-FoxP3-APC antibody (Table 2.4) added. Following a further 30 minutes incubation at RT, cells were again washed in 1x Permeabilisation Buffer. Finally, cells were washed in FACS buffer and fixed with 1 % PFA. Data was acquired immediately using a BD™ LSRII 4-laser flow cytometer.

Table 2.4 Fluorescently-labelled Antibodies for Flow Cytometry[1]

Target Molecule	Fluorochrome	Volume used per $5x10^5$ Cells (µl)	Species of Origin	Clone
CCR7	PerCP	10	Mouse	150503
CD107a	FITC	5	Mouse	H4A3
CD107b	FITC	5	Mouse	H4B4
CD127	BV421	5	Mouse	HIL-7R-M21
CD14	FITC	5	Mouse	MϕP9
CD14	V500	5	Mouse	MϕP9
CD14	BV510	10	Mouse	MϕP9
CD14	PerCP	10	Mouse	MϕP9
CD19	BV421	2	Mouse	HIB19
CD19	PE	5	Mouse	HIB19
CD19	APC	5	Mouse	SJ25CI
CD19	PE-Cy7	5	Mouse	HIB19
CD25	PE-Cy7	2	Mouse	M-A251[2]
CD3	PE-Cy7	5	Mouse	SK7
CD3	PerCP	3	Mouse	SK7
CD3	FITC	5	Mouse	SK7
CD3	BV510	5	Mouse	HIT3a
CD4	BV510	10	Mouse	SK3
CD4	PerCP	5	Mouse	SK3
CD4	APC	5	Mouse	SK3
CD56	FITC	10	Mouse	NCAM 16.2
CD56	PE	2	Mouse	AF12-7H3
CD56	BV421	5	Mouse	NCAM 16.2
CD62L	BV605	5	Mouse	DREG-56
CD69	APC	2	Mouse	FN50
CD8	V500	5	Mouse	SK1
CD8	BV421	5	Mouse	RPA-T8
CD8	PerCP	5	Mouse	SK3
CD86	FITC	20	Mouse	BU63[3]
CTLA-4	BV786	10	Mouse	BNI3
FoxP3	APC	20	Mouse	3G3[4]
HLA-DR	PerCP	20	Mouse	L243
PD-1	PE	10	Mouse	EH12.1
PD-1	BV786	10	Mouse	G155-178
PD-L1	FITC	5	Mouse	MIH1
TCRγδ	BV421	10	Mouse	B1

[1] Manufactured by BD Biosciences (San Jose, USA) unless otherwise stated

[2] Biolegend

[3] AbD Serotech (Raleigh, USA)

[4] Miltenyi Biotech (Bergisch Gladbach, Germany)

Table 2.4 continued

Target Molecule	Fluorochrome	Volume used per $5x10^5$ Cells (µl)	Species of Origin	Clone
Isotype IgG1	BV605	5	Mouse	X40
Isotype IgG1	FITC	5	Mouse	G18-145
Isotype IgG1	PE	5	Mouse	MOPC-21
Isotype IgG1	APC	5	Mouse	11711
Isotype IgG1	BV421	5	Mouse	MOPC-21[5]
Isotype IgG1	PE-Cy7	5	Mouse	MOPC-21
Isotype IgG2a	PerCp	5	Mouse	G155-178
Isotype IgG2b	BV510	5	Mouse	27-35
Isotype IgG2a	BV786	5	Mouse	G155-178
Isotype IgG1	BV786	5	Hamster	G235-2356
Isotype IgG1	V500	5	Mouse	X40

Table 2.5 Immune Cell Identification and Activation Markers

Cell Population	Markers + Fluorophores	
	Identification	Activation
CD4+ T cells (CD3+/CD4+)	CD3-PE-Cy7, CD4-BV510	CD69-APC, CCR7-PerCP, PD-1-PE, PD-L1-FITC, CTLA-4-BV786
CD8+ T cells (CD3+/CD8+)	CD3-PE-Cy7, CD8-V500	CD69-APC, CCR7-PerCP, PD-1-PE, PD-L1-FITC, CTLA-4-BV786
Tregs (CD3+/CD4+/$CD25^{high}$ $CD127^{low}$/Fox-P3+)	CD3-BV510, CD4-PerCP, CD25-PE-Cy7, CD127-BV421, FoxP3-APC	CD62L-BV605, PD-1-PE, PD-L1-FITC
NK cells (CD3-/CD56+)	CD3-PE-Cy7, CD56-FITC	CD69-APC, CCR7-PerCP, PD-1-PE, PD-L1-FITC
NKT cells (CD3+/CD56+)	CD3-PE-Cy7, CD56-FITC	CD69-APC, CCR7-PerCP, PD-1-PE, PD-L1-FITC
γδ T cells (CD3+/TCRγδ+)	TCRγδ-BV421	CD69-APC, CCR7-PerCP
Monocytes (CD14+)	CD14-BV510	CD69-APC, HLA-DR-PerCP, PD-1-PE, PD-L1-FITC, CD86-FITC
B cells (CD19+)	CD19-BV421	CD69-APC, HLA-DR-PerCP, PD-1-PE, PD-L1-FITC, CD86-FITC

[5] Biolegend

2.14.3 Immunophenotyping and Flow Cytometry using DuraClone Tubes

Cells were re-suspended in FCS at $5x10^6$ cells/ml. Aliquots of $5x10^5$ cells were added to custom-made DuraClone Dry Reagent Cocktail FACS tubes (Beckman-Coulter, Brea, California, USA, Table 2.6) and incubated in darkness at RT for 15 minutes. Where necessary, anti-CTLA-4-BV786 antibody (Table 2.4) was added to tubes. Cells in tubes A, B, C and D were washed in FACS buffer, fixed in 1 % PFA and kept protected from light until data acquired. Tube E was subjected to Fox-P3 staining as described below.

2.14.4 Intracellular Staining of PBMCs for FoxP3 Using DuraClone Tubes

Intracellular staining of Tregs for FoxP3 was performed using the PerFix-nc kit (Beckman Coulter) following manufacturer's instructions. After the initial 15 minute incubation (section 2.14.3), 5 µl of Buffer R1 (Fixative Reagent) was added to cells and incubated in darkness at RT for a further 15 minutes. 300 µl Buffer R2 (Permeabilising Reagent) was then added and incubated at RT for 1 hour. Finally, cells were washed in PBS and then in 3 ml 1x Buffer R3 before being fixed with 1 % PFA. Data was acquired immediately using a BD™ LSRII 4-laser flow cytometer.

2.15 Immunophenotyping of Fresh Whole Blood using DuraClone Tubes

Following blood sample collection (section 2.7), 100 µl of fresh whole blood was added to DuraClone tubes (Table 2.6) and incubated in darkness for 30 minutes at RT. Cells were incubated for a further 10 minutes at RT following addition of 1x FACS Lysing Solution (BD Biosciences). Cells were washed and fixed in 1% PFA and protected from light at 4 °C until flow cytometry was performed. Intracellular staining of Tregs for FoxP3 was performed as previously described (section 2.14.4).

Table 2.6 DuraClone Dry Reagent Cocktail Panel

		Cell Marker, Fluorophore and Clone								
Tube	**Panel**	**BV421**	**BV510**	**FITC**	**PE**	**PerCP**	**PECy7**	**APC**	**AF700**	**APC-Cy7**
A	**Isotype**	IgG1	IgG1	IgG1	IgG2a	IgG2a	IgG2a	IgG2b	IgG1	IgG1
B	**T cells**	**CD3** (UCHT1)	**CD4** (13B8.2)	**CD8** (B9.11)	**CCR7** (G043H7)	**PD-1** (Polyclonal)	**CD25** (B1.49.9)	**CD69** (TP.55.3)	**PD-L1** (Polyclonal)	
C	**NK/NKT/ γδ T cells**	**CD3** (UCHT1)	**CD16** (3G8)	**TCR γδ** (IMMU510)	**CCR7** (G043H7)	**PD-1** (Polyclonal)	**CD56** (N901)	**CD69** (TP.55.3)	**PD-L1** (Polyclonal)	
D	**Monocytes/ B cells**	**CD19** (J3-119)	**HLADR** (Immu-357)	**CD14** (RMO52)	**CD11c** (BU15)	**PD-1** (Polyclonal)	**CD25** (B1.49.9)	**CD69** (TP.55.3)	**PD-L1** (Polyclonal)	**CD86** (HA5.2B7)
E	**Tregs**	**CD3** (UCHT1)	**CD4** (13B8.2)		**CD62L** (DREG56)	**PD-1** (Polyclonal)	**CD25** (B1.49.9)	**FOXP3** (259D)	**PD-L1** (Polyclonal)	**CD127** (SSDCLY107D2)

2.16 Cytokine/Chemokine Analysis

2.16.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Paired antibodies were used as per manufacturers' instructions or following lab-optimised protocols (Table 2.1 and Table 2.7). Briefly, capture antibody (in coating buffer) was added to Nunc 96-well Maxisorb flat-bottomed plates (Scientific Laboratory Supplies, Nottingham, UK) and stored at 4 °C overnight. Plates were washed 3x in PBS-T and blocking solution was added. After 2 hours, plates were washed 3x in PBS-T and triplicate halving dilutions of appropriate recombinant cytokine protein were performed to generate a standard curve for analysis. Cell culture supernatants were added in triplicate and plates stored overnight at 4 °C. Following 6x washes with PBS-T, biotinylated detection antibodies were added and plates were incubated in darkness at RT for 2 hours. Plates were washed 6x in PBS-T. Streptavidin conjugate (ExtrAvidin®, Sigma Aldrich) was added and plates were incubated for 1 hour at RT. Following final washes of 3x PBS-T and 3x ddH_20, Sigmafast™ p-nitrophenyl phosphate (pNPP) alkaline phosphatase substrate (Sigma Aldrich) was added. Plates were monitored for the development of colour and a Multiskan® EX plate-reader (Thermo Scientific) was used to measure optical density at a wavelength of 405 nm.

Table 2.7 Antibodies for ELISA

Cytokine	Capture (µg/ml)	Detection (µg/ml)	Standard (pg/ml)	Manufacturer
CXCL-10	2	0.05	2000	R&D Systems
TRAIL	2	0.2	1500	R&D Systems
GM-CSF	2	1	2000	Peprotech
IFN-γ	4‡	1‡	10000*	‡BD Bioscience *Peprotech

2.16.2 Luminex Multiplex Assay

Multiplex human chemokine and cytokine analysis was performed using 21-plex (Table 2.8) and 27-plex (Table 2.9) Bio-Plex Pro™ plates (BioRad Laboratories, Hercules, California, USA). Standards, samples and coupled beads were prepared prior to use following manufacturer's guidelines. All incubations were performed on a Unimax 2010 platform shaker (Heidolph Instruments, Schwabach, Germany) at 450 revolutions per minute (rpm). Plate washes were performed on Bio-Plex® handheld magnetic wash plate (BioRad) to prevent loss of beads, using the Bio-Plex® Wash Buffer. Lyophilised standard was reconstituted with Bio-Plex®

Standard Diluent and stored on ice for 30 minutes before use. Serial dilutions were performed on reconstituted standard to generate a standard curve for analysis. Plasma samples were thawed and centrifuged at 10,000 g for 10 minutes to remove any cellular debris and diluted 1:2 with Bio-Plex® Sample Diluent. 50 µl of 1x beads were added to Bio-Plex® 96-well flat-bottom plates and plates were washed twice with Bio-Plex® Wash Buffer. 50 µl of standard and sample were added to each well in duplicate. Plates were sealed to protect from light and incubated at RT for 30 minutes. Plates were then washed 3x as previously described, before 25 µl of 1x detection antibody was added to each well. Plates were incubated for a further 30 minutes at RT. Following 3x washes, 50 µl of 1x Streptavidin-PE was added to each well. The plates were incubated at RT for 10 minutes protected from light. Finally, plates were washed 3x and 125 µl Bio-Plex® Assay Buffer added to each well. Plates were read immediately on a Bio-Plex 100 system and analysed with Bio-Plex Manager™ 4.1 software.

Table 2.8 21-plex Cytokine and Chemokine Panel

IL-1α	Cutaneous T cell-Attracting Chemokine (CTACK)	MIG
IL-α2	CXCL1 (GRO-α)	β-Nerve Growth Factor (NGF)
IL-2rα	Hepatocyte Growth Factor (HGF)	Stem Cell Factor (SCF)
IL-3	Leukaemia Inhibitory Factor (LIF)	Stem Cell Growth Factor (SCGF)-β
IL-12p40	MCP-3	Stromal Cell-Derived Factor (SDF)-1α
IL-16	M-CSF	TRAIL
IL-18	Macrophage Migration Inhibitory Factor (MIF)	TNF-β

Table 2.9 27-plex Cytokine and Chemokine Panel

IL-1β	IL-10	IFN-γ
IL-rα	IL-12p70	IP-10
IL-2	IL-13	MCP-1
IL-4	IL-15	MIP-1α
IL-5	IL-17α	MIP-1β
IL-6	Eotaxin	PDGF-ββ
IL-7	Basic-Fibroblast Growth Factor (FGF)	RANTES
IL-8	G-CSF	TNF-α
IL-9	GM-CSF	VEGF

2.16.3 TGF-β ELISA

TGF-β ELISA was performed using R&D Systems Human TGF-β Duoset kit following manufacturer's instructions. Briefly, TGF-β capture antibody was reconstituted to a working

concentration of 2 µg/ml in PBS. Supplied 96-well microplates were coated with 100 µl diluted capture antibody and incubated overnight at RT. The following day, plates were washed 3x in the supplied wash buffer and 300 µl supplied block buffer was added to each well. Plates were incubated for 60 minutes at RT before being washed 3x with wash buffer. Plasma samples were activated using R&D Systems ELISA Sample Activation Kit. 40 µl thawed plasma (per well) was added to 20 µl 1NHCl and incubated for 10 minutes at RT. Samples were then neutralised by adding a further 20 µl of 1.2 N NaOH/0.5 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). 10 µl of activated plasma was then diluted 1:20 with provided Reagent Diluent, to a final dilution of 1:40. A standard curve was generated using 2-fold serial dilutions of TGF-β in Reagent Diluent with a top standard of 2000 pg/ml. Activated plasma samples and standards (both in triplicate) were added to the prepared microplates, covered and incubated for 2 hours at RT. Following 3x further washes, 100 µl TGF-β detection antibody was added before further incubation for 2 hours at RT. Plates were washed again, as previously described, before the addition of 100 µl of streptavidin-horseradish peroxidase (HRP). After a further 20 minutes protected from light at RT, a final wash was performed. Substrate Solution was added to each well, followed by Stop Solution after 20 minutes. A Multiskan® EX plate-reader (Thermo Scientific) was used to measure optical density at a wavelength of 450 nm.

2.16.4 IL-10 ELISA

BD Biosciences' Human IL-10 ELISA kit was used for measurement of IL-10 in plasma samples, following manufacturer's instructions. For analysis an IL-10 ELISA standard curve was generated using serial dilutions of IL-10 to a top standard of 500 pg/ml. In summary, 100 µl of plasma was added to the 96-well plate, pre-coated in IL-10 antibody, in triplicate. Plates were incubated for 2 hours at RT before being washed 5x with the supplied Wash Concentrate. 100 µl of Working Detector was added to each well and incubated for 1 hour at RT. Plates were washed 7x before adding 100 µl Tetramethylbenzidine (TMB) One-Step Substrate Reagent. Plates were incubated for a further 30 minutes, protected from light, at which time 50 µl stop solution was added to each well. A Multiskan® EX plate-reader (Thermo Scientific) was used to measure optical density at a wavelength of 450 nm.

2.17 ELISpot Assay

Prior to the addition of capture antibody, 96-well polyvinylidene difluoride (PVDF) Multiscreen® ELISpot plates (Merck Millipore, Darmstadt, Germany) were moistened with 100

µl 70 % (v/v) sterile ethanol to allow subsequent penetration by aqueous solutions in the remaining steps. Following 3x washes with PBS, IFNγ capture antibody (Mabtech, Nacka Strand, Sweden) was diluted to a final concentration of 2 µg/ml in PBS and 100 µl added to each well. Plates were incubated for 24 hours at 4 °C and then washed 6x with sterile PBS. Plates were blocked with complete RPMI and incubated for 2 hours at 37 °C before either being used, or stored at -20 °C until required. Cryopreserved plates were thawed at RT for 2 hours before use. On the day of the assay, effector cells were thawed as previously described (section 2.8 and 2.10), re-suspended at the concentration required for the assay and rested at 37 °C for 2-4 hours prior to use. Blocking solution was discarded from the plates and complete RPMI added to each well to prevent the plates drying out. Immune stimuli were prepared as follows and 50 µl of each added to appropriate wells. Cytomegalovirus/Epstein-Barr Virus/Influenza (CEF) peptide pool (Cambridge Bioscience, Cambridge, UK) was used as a pan-immune stimulus and either CEA or MART-1 PepMix™ peptide pools were used as tumour-specific antigens (both JPT Peptide Technologies, Berlin, Germany); all used at 2 µg/ml. Pokeweed mitogen (Sigma Aldrich), was used as a positive control (at 1 µg/ml) and complete RPMI was used as a negative control. Effector cells were added to duplicate wells in variable concentrations to a final volume of 200 µl/well. Plates were incubated at 37 °C for 24 hours. The following day, plates were washed 6x with PBS-T. IFNγ biotinylated detection antibody (Mabtech, Nacka Strand, Sweden) was diluted 1:5000 in PBS to achieve a final concentration of 0.2 µg/ml. 100 µl was added to each well and plates either kept at 4 °C for 24 hours or RT for 4-6 hours, protected from light, before development. Streptavidin alkaline phosphatase (Mabtech, Nacka Strand, Sweden) was diluted 1:1000 in PBS and following a further 6x wash of the plate in PBS, 100 µl added to each well. Following a final incubation at RT for 2 hours, protected from light, 100 µl filtered 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) substrate (Mabtech, Nacka Strand, Sweden) was added in the dark. Once spots became visible (approximately 5-10 minutes), the plate was washed in cold water to halt further development. Plates were air-dried overnight and read using the SD32G2 AID ELISpot reader (AID Diagnostika, Strassberg Germany) and analysed using AID ELISpot software (version 5.0). Each visible spot is representative of one T cell secreting IFN-γ (a spot-forming unit, SFU).

2.18 Sample Collection

2.18.1 CRC Patient Cohort

Informed consent was obtained from patients receiving chemotherapy for metastatic CRC. Samples were collected from patients at three time points corresponding to chemotherapy cycles: baseline (pre-treatment), 6 weeks (following 2 cycles of 3-weekly chemotherapy) and 12 weeks (after 4 cycles of chemotherapy). Patients were followed up until collection of the final blood sample. When analysing the change in immune function over time, only patients who had multiple samples taken were included in the analysis. Furthermore, such patients were stratified into groups based on the change in NLR over the first 6 weeks of therapy, initially denoted as: **'high-high'** (NLR > 5 at baseline which remained elevated at 6 weeks; n=2), **'high-low'** (NLR > 5 at baseline which had fallen to < 5 by 6 weeks; n=10) and **'low-low'** (baseline NLR < 5 which remains so over the first 6 weeks of chemotherapy; n=17). As there were only 2 patients in the 'high-high' group, this is not included in the statistical analysis. For simplicity, therefore, results are annotated as either the **'high'** group (denoting the 10 patients with a high baseline NLR at baseline but low at 6 weeks) or **'low'** group (those 17 patients with an NLR <5 throughout). Following thawing of cryopreserved cells, PBMCs were rested overnight. Functional analysis was performed as allowed by the number of viable PBMCs.

2.18.2 Melanoma Patient Cohort

Informed consent was obtained from patients receiving immunotherapy for metastatic melanoma. Samples were collected from patients at three time points corresponding to immunotherapy cycles: baseline (pre-treatment), 6 weeks (following 2 cycles of 3-weekly ipilimumab or pembrolizumab) and 12 weeks (after 4 cycles of 3-weekly ipilimumab or pembrolizumab). When analysing the change in immune function over time, only patients who had multiple samples taken were included in the analysis. Following thawing of cryopreserved cells, PBMCs were rested overnight. Functional analysis was performed as allowed by the number of viable PBMCs.

2.19 Statistical Analysis

GraphPad Prism® (version 7.02) was used for statistical analysis. For the CRC and melanoma patient groups, Mann-Whitney U tests were used for comparison of baseline samples. For analysis of data over time, Wilcoxon rank test was performed. All graphs show the median and interquartile ranges and $p<0.05$ denotes a significant result (*$p<0.05$, **$p<0.01$ **$p<0.001$). For the validation experiments, paired t-tests were used for comparison of samples. All graphs show the standard error of the mean (SEM) and $p<0.05$ denotes a significant result (*$p<0.05$, **$p<0.01$ ***$p<0.001$).

Chapter 3 Immune Assay Validation

3.1 Introduction

Investigating the immune response to therapy is an essential component of immunotherapy trials, alongside any clinical endpoints. In addition, collecting such data from patients on a range of treatment regimens may reveal novel insights into immunomodulatory effects which can be exploited when developing new therapies. This may also identify potential immune signatures which can be used to monitor biological activity and which may correlate with clinical efficacy or toxicity.

To date, knowledge of patient immune responses following such treatments have been confined to general observations; detailed, structured investigations including functional assessment of patient samples have not been performed in this setting. Analysis of fresh patient blood is the ideal, eliminating the possibility of processing errors and other confounding factors. Pragmatically, within a trial setting this is not usually possible for the majority of assays, particularly in multi-centre studies when samples are collected at numerous time points. In this regard, blood samples could be processed and stored as frozen components for batch analysis or for transportation to a centre where all assays can be performed. If clinical trial samples are cryopreserved in LN2 for long term storage and analysed at a later date, validation of novel assays on these frozen samples is required to be able to understand the effects of cryopreservation on immune cell function. In order to ensure that the results of such analyses are robust, we developed a panel of immune functional assays to interrogate immune responses in detail. Validation of this panel will ensure high quality and reproducible results, striking a balance between maintaining the functional profile of immune cell populations and being able to recover a high enough yield of cells from patient samples to perform a range of analyses.

3.1.1 Immune Functional Assays

In order to fully interrogate the response of a patient's immune system to chemotherapy, immunotherapy or other novel therapies, a panel of immune functional assays should evaluate different facets of the immune response. The assays developed in our panel were used to analyse aspects of the innate immune response, adaptive responses (to both tumour-

specific antigens and non-specific viral peptides) and other parameters including cytokine/chemokine profiles.

3.1.1.1 Immunophenotyping

Immunophenotyping is used to evaluate the expression of molecules on cells which can determine phenotype and activation. Cells are labelled with antibodies bound to a fluorescent molecule which can be detected by specific light emission when passed through (excited by) a laser and quantitatively measured by a flow cytometer. Despite this being a widely used technique, there is still a need for assay validation and standardisation of immunophenotyping protocols: the increased capacity for flow cytometric measurement of multiple cell populations using multiple fluorophores has added to the complexity of being able to ensure accurate compensation strategies. When deciding on an immunophenotyping panel to analyse clinical samples there are many factors to consider, including the emission spectra and brightness of each fluorophore and the level of expression of specific proteins being investigated: proteins present at very low density, for example, could be more reliably detected using brighter fluorophores. Furthermore, comparison of different fluorophores of the same clone but from different manufacturers can yield different results when used to stain matched peripheral blood samples (241), as can using different clones. Isotype controls can be used for each fluorophore to determine the proportion of non-specific antibody staining, above which the true positive population can be quantified (i.e. IgG-PE isotype compared to CD3-PE staining) (242).

Small variations in sample preparation could also lead to differences in the results obtained by flow cytometry. As an example, Kalina *et al.* demonstrated that, if using a red cell lysis step in the processing of peripheral blood samples, both the brand of lysis buffer and the protocol for staining the cells can alter the absolute detected counts (%) and also the MFI of each immune cell population (241). Similarly, the MFI of cell populations decreased over time following staining, suggesting that samples should be analysed within one hour of the cell staining procedure or otherwise be stored at 4 °C in the dark for data collection as soon as possible (241).

Even though some centres have shown that the anti-coagulant used in blood sample collection bottles does not affect yield of immune cells (243), differences in the populations of cells positive for certain cell surface markers have been demonstrated (244). For example, lymphocytes from blood samples anticoagulated with heparin showed increased expression of

HLA-DR in response to stimulation with cytokines such as IFN-γ. This effect was not seen when EDTA was used as an anticoagulant (244).

3.1.1.2 Innate Immune Assays

The innate immune response to stimulus is usually very rapid (within hours of activation *in situ*) and precedes an adaptive response. Degranulating NK cells are identified by surface expression of CD107 which is strongly correlated with cytotoxicity of tumour-cell targets. It can therefore be used as an effective surrogate marker for NK-mediated cytotoxicity. The expression of CD107 and the cytotoxicity of NK cells has also been shown to correlate with cell surface expression of the activation marker CD69 and production of IFN-γ (245).

Chromium-release assays measure the ability of effector immune cells to actively kill tumour targets. Target cells are labelled with ^{51}Cr, which is non-specifically internalised within the cell. Release of ^{51}Cr into the cell culture supernatant following co-culture with PBMCs as a consequence of target cell lysis therefore represents the functional cytolytic activity of effector cells. These assays are utilised in parallel with CD107 degranulation to assess innate responses against tumour targets. Our laboratory has previously shown that NK cells within the whole PBMC population are the effector cells responsible for lysis of target tumour cells in ^{51}Cr assays (229). Hence, it is the NK cell population within PBMCs which has been studied in these assays throughout this thesis.

3.1.1.3 Adaptive Immune Assays

Adaptive immunity involves a T cell response to a previously encountered antigen. An antigen-specific T cell pool responds, after expansion of a small number of memory T cells were primed against the antigen to eradicate the foreign entity. ELISpot assays can detect T cell responses to immune stimuli by the production of cytokines such as IFN-γ (246). They are used to assess tumour-specific T cell responses (in malignancy and infectious disease) by detecting antigen-specific T cells and have a high degree of concordance with other measurements of T cell responses (247). Recall responses to previously encountered antigens (such as previous influenza infection) can be detected by ELISpot assay long after the initial exposure (248). Pre-incubation of PBMCs with the immune stimulus of interest, to allow for processing and presentation of antigen, can be used to increase the measureable T cell response (246). Although there might be a concern that using a 'cultured ELISpot' assay could prime naïve T cells to the antigen of interest, the time period for which the T cells are pre-

cultured is usually less than 14 days (249). Validation of the concentration of protein or peptide of choice is also needed to optimise the result (246). Certain peptides are HLA-restricted and, as such, are specific to individual patients: they can only be presented to T cells on certain HLA molecules. The difficulties inherent in identifying a population of T cells which recognise a specific antigen, given the potential for MHC-restriction between donors, can be abrogated by using pools of overlapping peptides (249,250). Due to the short nature of these individual peptides, minimal processing by APCs is needed (251). Combination of the ELISpot with depletion assays can ascertain whether the responses are mediated by CD4+, CD8+ T cells or NK cells (249), although this may also be dependent on the length of peptides used. Peptides of between 8 and 11 amino acids, for example, are thought optimum for engagement with MHC class I molecules on CD8+ T cells (250,252). Since CD4+ T cells can respond to longer peptides (252,253) - though not reliably those shorter than 10 amino acids - peptide pools of approximately 15 amino acids in length with overlapping sections of 8-11 amino acids can be used to simulate both groups of T cells. The number of PBMCs used in ELISpot assays also has an effect on the number of positive responses yielded, with a higher proportion of reactions detected if $\geq 4x10^5$ PBMCs/well are used (254). Due to the variability in the responses and the heterogeneity of the assay, the Association for Immunotherapy of Cancer Working Group recommends that the assay be performed in triplicate (254).

3.1.1.4 Cytokines

The balance of cytokines and chemokines can directly impact the generation and direction of an immune response. The cytokine/chemokine profile can reveal additional information as to the immune response of a patient to therapy and forms a complementary tool for analysis when used alongside other assays. Multiple methods can be used to monitor the levels of these solutes, including, for example, ELISA and Luminex; the major advantage of Luminex being high-throughput analysis. Another advantage is that the relative amount of sample needed is much smaller than would be required for multiple ELISAs, thereby preserving precious patient samples. However, there are considerable cost implications. Comparisons between ELISA and multiplex bead assays have shown that, for most T_H1 and T_H2 cytokines, there is a high degree of correlation between the two methods of detection (255,256). This stands true for stimulated and non-stimulated patient samples. Although there is a high level of concordance between the two assays, the absolute values of analyte detected can differ, possibly due to differences in the reference standards and the antibodies used in the assay

(256). Multiplex bead assays have been shown to be more sensitive when detecting low levels of cytokine and chemokines (257) which is essential for patient samples.

Once again, processing and storage of samples can alter the outcome of the assays. Levels of IL-8 in plasma from five-day old platelet concentrates, for example, are far higher than concentrates analysed after only two days of storage (258). In contrast, cytokine production from PBMCs stored at room temperature was decreased compared to those that were analysed fresh (259).

3.1.2 Sample Collection and Storage

In order to ensure sample quality and reproducibility of analyses, we validated a number of aspects of sample collection, isolation of immune cells, preparation of cells for analyses and sample storage. We also focussed on the cryopreservation of immune cells to use in batch analysis of immune function.

3.1.2.1 Collection of Whole Blood Samples

There is some concern regarding the viability of cells and the impact of time from blood being drawn to being processed. Storage of samples at room temperature for up to 24 hours does not seem to affect the cell counts of individual immune cell populations, with the exception of NK cells (slight increase by 24 hours) and monocytes (slight decrease by 24 hours) (260). There is some variability in the expression of the activation markers CD86 and HLA-DR on the surface of monocytes if there is a period of greater than four hours between blood sample collection and staining (260).

Following cryopreservation, cell recovery upon thawing is decreased when the time between sample collection and processing/cryopreservation is greater than seven hours and increased when processed less than two hours after collection (261). Similarly, samples taken and left for over eight hours before being processed not only demonstrated decreased cell viability after cryopreservation but T cell responses in IFN-γ ELISpot assays were also diminished (243). It has been suggested that samples anticoagulated with EDTA (rather than heparin) have greater contamination with granulocytes when kept for 24 hours before lymphocyte separation (262), so although yield of PBMCs may be similar, the functional effect of granulocyte contamination in this situation cannot be disregarded. In addition, the storage of

whole blood samples in EDTA tubes (at RT) can affect antigen-specific responses, with a decrease in antigen-specific T cell responses at all time points up to 24 hours (263).

3.1.2.2 Isolation of PBMCs

PBMCs can be isolated from fresh whole blood via gradient density separation (using solutions such as Lymphoprep™ or Ficoll®) or pre-prepared tubes such as Vacutainer Cell Preparation Tube. There is no observed difference between the yield of isolated immune cell sub-populations using either method (243,264). Neither is there a difference in T cell immunological responses (265). However, relatively small variations in the preparation of cells can affect the outcome of immune assays. For example, using different media for density gradient separation can produce discrepancies in the magnitude of T cell response to recall antigens: IFN-γ expression was increased in the population of PBMCs isolated using Ficoll® *vs.* Lymphoprep™ (266).

Although many functional assays focus on the analysis of PBMCs and cytokine/chemokine profiles, granulocytes (the majority of which are neutrophils) form a significant part of the immune defence against pathogens. However, assays measuring neutrophil activity can be difficult and the use of radio-labelled trafficking or chemotaxis assays can be variable and correlate poorly to immune functionality (267). One of the major difficulties with the storage or culture of granulocytes *in vitro* is the cytotoxic and microbiocidal potential of activated neutrophils (98,268). If neutrophils are isolated from human whole blood, subsequent lysis may be due to the release of lysosomes, which initiate cell death. Reduced granulocyte viability is directly proportional to the release of lysosomal enzymes (269). Interestingly, inhibition of immune responses (seen in PBMCs isolated from peripheral blood which have been left at RT for a prolonged period before being processed), may be, in part, due to granulocyte contamination of PBMC isolates (270).

3.1.2.3 Cryopreservation of PBMCs

Cryopreservation of cells must be optimised in order to maximise cell recovery and prevent damage through osmotic processes during freezing. Everything from the FM used to the speed of the freezing process and method of thawing can affect the recovery of immune cells following cryopreservation. Crucially, DMSO, used as a cryopreservative, should be added in a step-wise manner, to protect the cell from sudden osmotic changes and to maintain cell integrity (271).

The FM in which cells are cryopreserved can also vary greatly between laboratories. In some studies, FMs were chilled (ideally below 4 °C) when preparing to cryopreserve cells. This is borne out in studies showing that using warmed solutions decrease T cell responses in immune assays (266,272). In contrast, other studies have found that adding warmed FMs increases the recovery of cryopreserved PBMCs on thawing (273).

The method of cryopreservation is also important and can itself yield differences in the quality and population of cells once recovered. PBMCs must be frozen in a controlled way to avoid formation of intracellular ice crystals, which cause cell membrane disruption and lysis. Common methods include either a controlled-rate freezer (CRF) or a manual method using equipment such as a Mr Frosty™; both function in a similar way by controlling the rate of freezing to 1 °C per minute. When culturing DCs from monocytes, for example, the yield is shown to be greater if PBMCs are cryopreserved using a CRF technique rather than a manual method (274). Despite some benefits of a CRF, use of a Mr Frosty™ is still accepted as good practice in many centres.

There seems to be no consensus on the freezing density of lymphocytes for cryopreservation in the literature to date. Pross and Maroun cryopreserved lymphocytes at densities of between 3 and $5x10^6$ /ml (275), whereas Mata *et al.* (276) and Aziz *et al.* (277) used freezing densities of $1x10^7$ PBMC/ml. Disis *et al.* found there was no difference in viability of thawed PBMCs when cryopreserved in aliquots of $1x10^7$/ml, $2x10^7$/ml or $3x10^7$/ml (278).

3.1.2.4 Storage of Samples

For batch analysis, clinical trial samples are stored long term in LN2 until required. Some studies have shown that storing samples at -70 °C for ≤ 3 weeks does not affect recoverable cell viability (279), while others have shown a decline in thawed PBMC viability when stored at -80 °C compared to those stored in LN2, with a higher proportion of apoptotic cells. Of note, these samples were thawed and analysed after 14 months, which may explain the poorer yield (280). T cell populations within frozen PBMCs also appear to remain constant at time points up to 15 months in LN2 (and when stored at -70 °C for ≤ 3 weeks) (279). There is conflicting data around the preservation of T cell responses over time. Weinberg *et al.* demonstrated that T cell responses to stimulus such as pokeweed mitogen (in lymphocyte proliferation assays) were maintained up to 15 months after storage in LN2 (279). There was, however, variability in responses to different stimuli when PBMCs had been stored at -70 °C. In contrast, other data suggests that long term storage of cryopreserved PBMC (admittedly

from HIV-positive individuals) diminishes both CD4+ and CD8+ T cell responses to a variety of peptide stimulants (measured by % IFN-γ expression on cell population of interest), particularly in cells stored for greater than one year (281). This loss was, as expected, more evident in the CD4+ population. This effect was not seen in the HIV-negative donors (281), emphasising the importance of standardising and validation of assays in the patient population of interest.

In addition, any fluctuations in temperature during storage can decrease cell viability, cell recovery and T cell responses in assays such as the ELISpot (282,283). Even small changes can lead to reduced recovery and jeopardise outcomes from immune assays (282). The source of these variations, such as other samples being removed from the same storage facility, should be considered when planning long-term storage of clinical samples. Providing that different centres adhere to quality assured SOPs the yield and functional immune profile of PBMCs can be maintained if cryopreserved samples can be shipped on dry ice and transferred immediately to LN2 on arrival at a central laboratory.

3.1.3 Preparation of PBMCs for use in Immune Assays

3.1.3.1 Thawing of PBMCs

Variability in the thawing procedure of cryopreserved cells across different laboratories can cause large fluctuations in the viability of PBMCs (254). Despite this, a number of factors do not seem to alter the viability of cryopreserved cells on thawing: duration of centrifugation following thawing, the volume the samples are resuspended in and the density of thawed cells during the washing procedure (278). Interestingly, although there is conflicting data on whether there is benefit to cooling FM before cryopreservation of PBMC, washing PBMCs in cold medium after thawing has a detrimental effect on cell viability (278,284).

3.1.3.2 Resting PBMCs *versus* Immediate Use

There are questions about whether cells which have been cryopreserved can be used in immune assays immediately upon thawing or whether a period of incubation may be beneficial to allow cells to recover from the thawing process. This has to be weighed against the possible loss of cells following a period of resting before use. As an example, PBMCs from a cohort of patients with chronic viral infections (including HBC and HIV) were analysed after being rested overnight and compared to PBMCs used immediately. Although viability of the

cells decreased, it remained above 90% in the rested group (median viability 94.8 % *vs*. 92.5 %) (285). Interestingly, rested PBMCs contained a greater number of dead cells, whereas a higher proportion of apoptotic cells were found in unrested PBMCs. Rested PBMCs, therefore, may have reduced viability due to death of apoptotic cells, leaving a purer population of viable functional immune cells (285,286). Loss of cells due to apoptosis, rather than necrosis, should ensure that the immune milieu of the assay is not skewed by this process. While apoptotic cells do not respond to antigenic stimulation (by IFN-γ production), the presence of apoptotic cells in a viable T cell population can decrease response of viable T cells to viral antigens (287). Resting thawed PBMCs before use increases the number of positive responses to viral antigens by CD8+ T cells and consequently resulted in increased expression of cytokines including IFN-γ and TNF-α in response to antigen (285,288). This effect has also been demonstrated to a lesser extent in CD4+ T cells. This suggests that T cell responses can be enhanced by resting PBMCs after thawing and can increase sensitivity in detecting antigen-specific immune responses without increasing the levels of false positives (as there was no response to tumour associated antigens by HD PBMCs) (288).

Resting PBMCs overnight can enhance pre-existing CD8+ T cell responses to CEF peptides in ELISpot assays but has no effect on patients with inherently low CEF responses, or on CD4+ responses to antigen (289). Enhancement of responses in this context may be useful if the frequency of circulating antigen-specific T cells is low. An enhanced response was not related to changes in T cell populations as determined by immunophenotyping, suggesting that these findings are due to increased functionality of an already stimulated T cell in response to antigen/peptide (289). Resting PBMCs overnight prior to use can also decrease the background (non-specific) readings compared to cells used immediately after thawing (247,249). In these situations, there should be a balance between maintaining functionality of immune cell populations, being able to detect immune responses with a high degree of specificity and doing so while being able to preserve the viability of cell populations within limited clinical samples, given that resting can lead to the loss of up to 50 % of the viable PBMC population (289). We, therefore, specifically investigated the effect of using thawed PBMCs immediately in assays, compared to immune responses in populations of PBMCs rested overnight before being used.

3.1.4 Using Fresh or Cryopreserved Cells in Immune Assays

The cryopreservation of PBMCs is required for the practicalities of batched analysis of patient samples from multiple clinical sites and potentially multiple trials. Bulk analysis also limits the intra- and inter-patient variability, as all samples are analysed at the same time using the same reagents, same machine settings, etc. Quality-assurance measures should therefore include the time from blood collection to processing/freezing and viability/yield of thawed PBMCs (290) in order to ensure that results are reliable and reproducible. We therefore specifically investigated the yield of cryopreserved PBMCs and the practicalities of freezing in order to maintain cell viability and robust immune responses.

3.1.4.1 Immunophenotyping

There is variation in the reproducibility of cellular markers on different immune cell populations following cryopreservation. Jeurink *et al*. demonstrated that cryopreserved immune cells within the whole PBMC population were similar to those in fresh samples (286). While some groups have shown that the relative populations of CD3/CD4+ and CD3/CD8+ T cells within cryopreserved PBMCs correlated with those from paired fresh samples (286,291), others have demonstrated that the frequency of T cells fall post-thawing and did not recover (292). Lemieux *et al.* showed that following cryopreservation of PBMCs from HDs, populations of NK cells and B cells were unchanged but if cells were rested for 24 hours, there was a significant decrease in the frequency of CD3+ T cells and monocytes (292). Previous studies have shown that activation markers on NK cells, monocytes and B cells are comparable whether PBMCs are used fresh, cryopreserved and rested prior to use or cryopreserved and used immediately on thawing (276).

Tregs are a population of particular interest when evaluating the immune response and accurate assessment of Treg activation is essential to determine the immunosuppressive potential of this cell population. Depending on the definition of the phenotype of Tregs, there can be appreciable differences in the proportion of Tregs between fresh and frozen conditions: CD4+CD25+FoxP3+ cells are lower in cryopreserved cells, whereas when defined as CD4+CD25+CD127^{low}, the population is equivalent (293). Other, conflicting data shows that the population of Tregs within PBMCs can be altered by cryopreservation (294). To this end, we investigated the effect of cryopreservation on PBMC populations as assessed by flow cytometry, including activation and identification of specific populations of immune cells.

3.1.4.2 NK Cell Assays

Activation and function of NK cells in fresh and frozen PBMCs have been assessed by a variety of groups. Although populations of NK cells are not diminished following cryopreservation their absolute cytotoxic potential is reduced (286). In contrast, other studies have demonstrated that the relative cytolytic activity of NK cells can be maintained between each condition (fresh *vs.* frozen) (271). In data from HD PBMCs, there is evidence of variation in NK cell function following incubation prior to use in chromium release assays (used immediately on thawing *vs.* being incubated in culture medium for between 3 and 24 hours) (271). Contrary to this, Mata *et al.* compared NK cell activity in fresh cells, frozen/rested cells and frozen/unrested cells. They observed that resting PBMCs after thawing achieved similar results than when using fresh cells in immune assays (276). Specifically, NK-mediated cytotoxicity against target cells, as well as ADCC and CD107 degranulation, were comparable to fresh PBMCs, if cryopreserved cells were thawed and rested overnight prior to use (276). Earlier work by Pross and Maroun supports this finding, showing that cryopreserved NK cells rested at 37 °C for at least five hours before use demonstrate activity comparable to that seen in fresh lymphocytes (275).

3.1.4.3 T Cell Assays

Cryopreserved PBMCs can also be used in the evaluation of adaptive T cell-mediated immune responses, such as ELISpot assays. Following thawing of HD PBMCs, viability of the cells may be decreased but the size of the CD3+ T cell population is not (286). This is dependent, however, on whether the PBMCs have been co-cultured with a stimulator of T cell function, such as phorbol myristate acetate (PMA) (286). While it has been shown that cryopreservation can diminish T cell responses to viral peptides in ELISpot assays (281), other groups have found that responses to recall antigens (including viral peptide pools) are not affected by cryopreservation (273,278). Indeed, Kuerten *et al.* (289) demonstrated that cryopreservation did not significantly affect IFN-γ production in response to CEF viral peptides when compared to fresh PBMCs. Fluctuations in temperature during the long-term storage of PBMCs can also effect T cell responses when cells are thawed for immune assays (283).

In this regard, we specifically investigated the effect that cryopreservation would have on innate and adaptive immune cell analysis, in particular NK cell degranulation/cytotoxicity assays and ELISpot assays.

3.1.4.4 Measurement of Cytokines/Chemokines

There are concerns that cryopreservation may activate immune cells and skew the immune profile of cells used in immune assays. There is conflicting data in this regard, with some data suggesting that levels of IL-10, IL-12, TNF-α, IFN-γ and IL-5 do not vary in cell culture supernatants of thawed PBMCs (286). However, analysis of cytokine/chemokine production from PBMCs collected from a cohort of children with diabetes showed elevated levels of IL-6, IFN-γ, IL-10 and IL-13 following freeze-thawing (295). Exogenous stimulation of PBMCs results in variable production of solutes between the fresh and cryopreserved conditions (greater IFN-γ and TNF-α and lower IL-10 and IL-13) (295). Other studies have shown decreased levels of IL-12 in response to increased IL-10 production from thawed PBMCs (296). The previous study also showed differential expression of IL-10 when thawed PBMCs were stimulated with phytohaemagglutinin (PHA) (296). However, similar evaluation of PBMCs in another cohort of HDs showed no variation in cytokine production (including IL-6, TNF-α and IFN-γ) following PHA stimulation, between fresh and cryopreserved PBMCs (297). The disparities in levels of each cytokine expressed by cryopreserved cells, with or without stimulation, should be evaluated prior to being included in immune panel analysis of clinical trials samples (298).

In conclusion, variability between assays is decreased when performed by the same operator but repeatability can be maintained over different laboratories and different operators if standard operating procedures (SOPs) are set and adhered to.

3.1.5 Rationale for Current Study

We aimed to develop and validate a panel of reproducible immune assays which could be batch analysed and used to evaluate the immune response, in peripheral blood, across a spectrum of patient populations. Specifically the aims of this part of the project were:

1. To optimise viable cell recovery after cryopreservation, including isolation of immune cell populations, freezing densities and storage of samples.
2. To optimise the thawing process of samples in order to be able to use them in immune assays.
3. To explore whether there is a significant change in the response and cell phenotype after cryopreservation.
4. To explore whether there are significant differences in functional assays after cryopreservation.

3.2 Immune Assay Validation: Results

3.2.1 Cryopreservation of Clinical Trial Samples to Optimise Cell Recovery

3.2.1.1 Recovery and viability of PBMCs are maintained if separated from granulocytes prior to cryopreservation

Cryopreservation of immune cells was investigated in order to improve the maximum recovery of viable cells following thawing and better understand differential viability and recovery of different cell types. Fresh peripheral blood was obtained from HDs and either whole WBCs (including granulocytes) or PBMCs (without granulocytes) were isolated, to determine whether these would yield different results.

Cells were cryopreserved at a density of $1x10^7$ PBMCs/ml. On thawing, viable cells were counted using Trypan Blue exclusion and the proportion of recovered cells was calculated. There was a range of viable cell recovery between HDs (Table 3.1).

Table 3.1 Yield of viable PBMCs from HDs using Trypan Blue exclusion

	Cryopreserved Cells	Viable Cell Yield	Recovery of viable cells
HD 1	$21.3x10^6$	$7.52x10^6$	35.3 %
HD 2	$19.5x10^6$	$12.64x10^6$	64.8 %
HD 3	$20.9x10^6$	$9.52x10^6$	45.5 %

Viability can also be assessed by PI staining. In order to ensure that Trypan Blue exclusion was a reliable way to assess the recovery and viability of PBMCs following cryopreservation, PI staining was used to assess the proportion of dead cells within the population of viable, thawed PBMCs from HD 1-3. PI staining of these PBMCs showed that Trypan Blue exclusion had a high degree of fidelity for assessing the viability of PBMCs. Viability of recovered cells in each of the three donors was greater than 90 % (91.6 %, 91.3 % and 91 %, respectively; Figure 3.1). These data indicate that Trypan Blue is a valid method for evaluating the viability of PBMCs isolated from peripheral blood and will therefore be used for the remainder of the project.

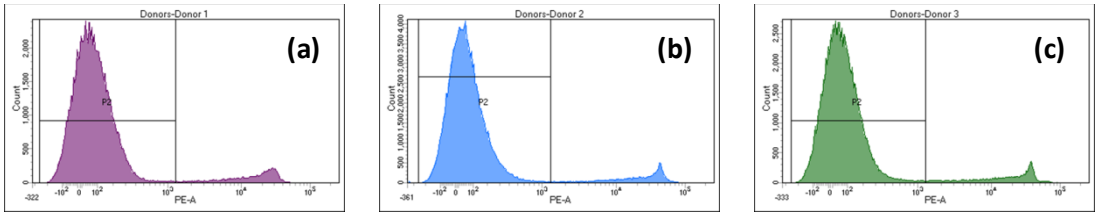


Figure 3.1 Viability of cryopreserved PBMCs verified by PI staining

HD PBMCs were isolated from whole blood samples as already described and cryopreserved in clinical FM at $1x10^7$ PBMCs/ml. Thawed PBMCs were then stained with PI before being analysed by FACS. Levels of dead cells in the three donor samples (HD 1-3; **(a)** **(b)** and **(c)**).

In order to determine the optimum method of sample preparation from patient blood, we sought to compare the isolation and storage of the whole WBC population (including granulocytes) with the PBMC population. Whole WBC populations (from HDs) were cryopreserved at densities of $0.5x10^6$ cells/ml, $1x10^6$ cells/ml and $2x10^6$ cells/ml in different FMs (Table 2.3). Following thawing and counting of viable WBCs, it was clear that the yield of viable WBCs was much lower than anticipated (below 20% in all conditions, Figure 3.2a). However, increasing cell density was associated with greater yield. This was significant when cells had been preserved in standard FM ($0.5x10^6$ WBCs/ml *vs.* $2x10^6$ WBCs/ml, 2.7 % *vs.* 17.3 %, p=0.0066; $1x10^6$ WBCs/ml *vs.* $2x10^6$ WBCs/ml, 5.7 % *vs.* 17.3 %, p=0.0104). At a freezing density of $0.5x10^6$ WBCs/ml there was a significant difference in cell recovery when preserved in standard FM *vs.* 20 % DMSO/RPMI (2.7 % *vs.* 9.3 %, respectively, p=0.0099). At a density of $1x10^6$ WBCs/ml, 20 % DMSO/RPMI was significantly superior to 10 % DMSO/RPMI (12.3 % *vs.* 5.0 %, respectively, p=0.0142). Interestingly, as the freezing density increases, the effect of different FMs seem to be abrogated, with no significant difference seen between media at density of $2x10^6$ WBCs/ml (Figure 3.2a).

As the whole WBC yield was less than expected, the yield of PBMCs (without granulocytes, isolated from HDs) was investigated as an alternative. Higher cell densities of $5x10^6$ PBMCs/ml and $1x10^7$ PBMCs/ml were used, given the results of previous WBC experiments. Overall, recovery of PBMCs at these specific densities was increased compared to WBCs (Figure 3.2b).

There was no significant difference between viable cell recovery from these high freezing densities ($5x10^6$/ml, $1x10^7$/ml) with any FMs. Yield was higher at both cell densities for cells

preserved in standard FM (10 % DMSO/FCS) suggesting that this could be superior in preventing loss of cell viability.

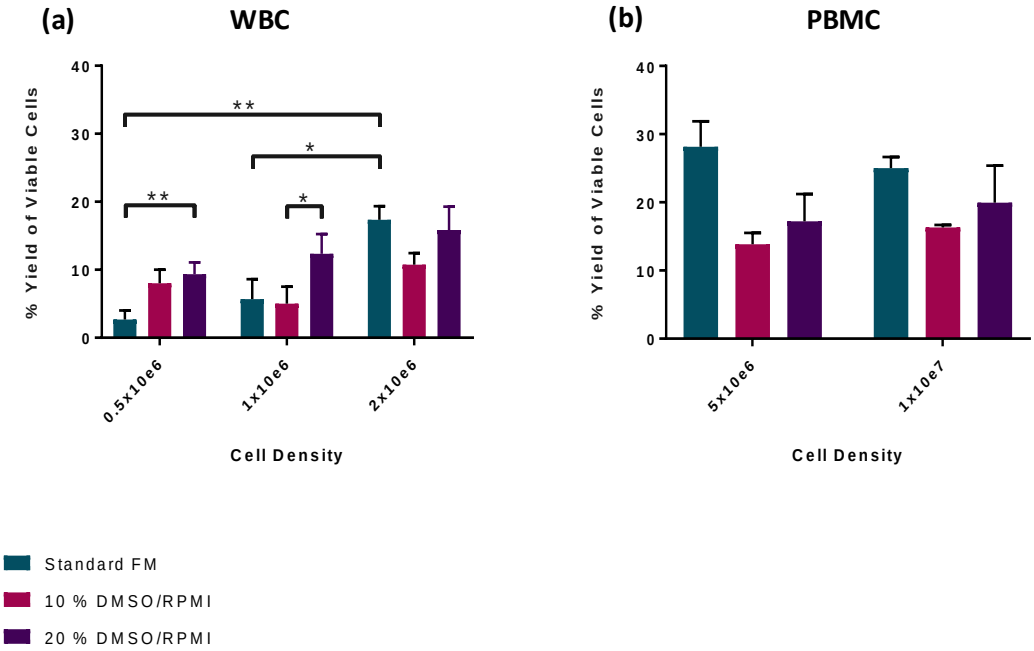


Figure 3.2 Recovery of whole WBC and PBMC populations following cryopreservation

Recovery of viable HD WBCs **(a)** and PBMCs **(b)** following cryopreservation. Cell populations were frozen at stated densities using different FMs. After thawing the yield of viable cells was determined using Trypan Blue exclusion (n=3).

Although some loss of viability is expected when peripheral blood cells are cryopreserved, maximising the cell recovery is essential to be able to perform multiple immune assays. As yields from fresh HD blood did not meet expected levels, a comparison of P BMCs from HD fresh blood and leucocyte cones was performed. Leucocyte cones are a reliable source of high quantities of PBMCs and the usual product of use within the laboratory for research purposes. Once again higher freezing densities ($2x10^7$ PBMC/ml and $4x10^7$ PBMC/ml) were used to investigate whether this would yield a greater number of viable PBMCs on thawing (Figure 3.3). Recovery of PBMCs from fresh blood was higher when cells had been preserved in standard FM compared to 20 % DMSO/RPMI. This was significant at a freezing density of $4x10^7$ PBMCs/ml (standard FM *vs*. 20 % DMSO/RPMI 30.93 % *vs.* 13.33 %, p=0.0156). In comparison, there appeared to be no change in the recovery of cells from cones when comparing both cell density and FM.

Notably, the recovery of viable PBMCs was much lower using fresh peripheral blood compared to cones under all conditions tested. This was significant at a freezing density of $4x10^7$ PBMCs/ml in 20 % DMSO/RPMI (fresh blood *vs.* cones 13.33 % *vs*. 47.20 %, p=0.0151).

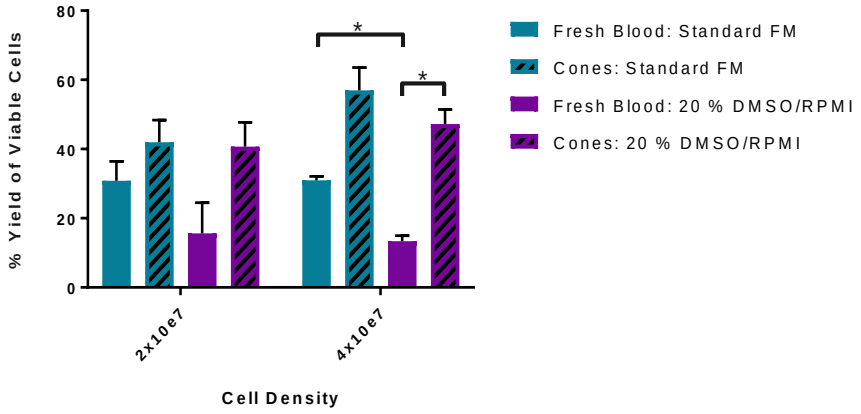


Figure 3.3 Recovery of PBMCs isolated from fresh blood and leucocyte cones.

PBMCs were isolated from HD whole blood and from leucocyte cones. PBMCs were frozen at the specified densities in either standard FM or 20% DMSO/RPMI. Following thawing, the yield of viable cells was determined using Trypan Blue exclusion (n=3).

Overall, these data suggest that there is trend towards better recovery of viable PBMCs after cryopreservation in standard FM. There also appears to be a higher yield when PBMCs are preserved at higher densities. There was still a difference between recovery of HD PBMCs and those from cones, therefore we investigated different FMs to try and improve recovery of HD cells in line with those derived from cones.

3.2.1.2 'Clinical' freezing mixture increased recovery of cryopreserved PBMCs

In order to optimise the cryopreservation process, recovery of HD PBMCs cryopreserved in standard FM (which had been shown to be superior to other mixtures), was compared to that of PBMCs cryopreserved in clinical FM (Table 2.3). The latter has been shown by collaborators to yield an optimum number of viable cells after cryopreservation and may be more appropriate for functional immune analysis at a later stage (personal communication).

Recovery of viable PBMCs was not significantly different when cells were cryopreserved in standard or clinical FM (Figure 3.4). There was also no significant difference in cell recovery between freezing densities.

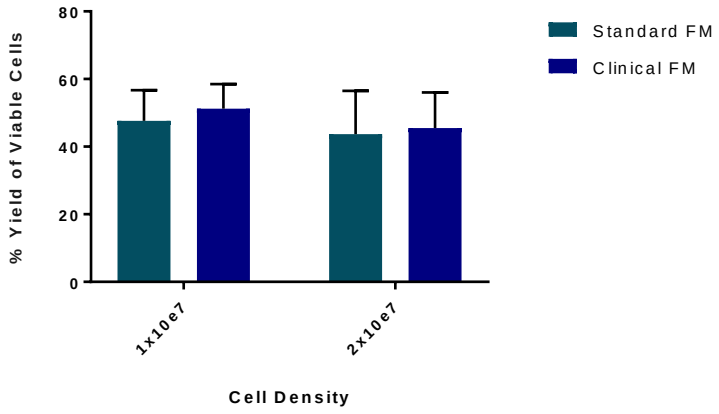


Figure 3.4 Recovery of PBMCs following cryopreservation in standard or clinical FM

Cell populations were frozen at stated densities using different FMs. After thawing, the yield of viable cells was determined using Trypan Blue exclusion (n=3).

This data collectively suggests that to optimise immune cell recovery following cryopreservation, PBMCs should be isolated from granulocytes before being frozen in clinical FM at a density of $1x10^7$ PBMCs/ml. By freezing at this density, the yield of thawed PBMCs will be maximised, while also allowing cells to be frozen in smaller batches so cells are not wasted on thawing.

3.2.2 Functional Analysis of Clinical Trial Samples: Comparing Fresh and Cryopreserved Cells.

Clinical trial samples need to be cryopreserved in LN2 for long-term storage prior to analysis and functional assays require validation for cells which have been cryopreserved.

3.2.2.1 Cytotoxicity of PBMCs against target tumour cells is maintained following cryopreservation

PBMCs were isolated from cones and either used fresh or cryopreserved for a minimum of 8 weeks prior to analysis. Reovirus (1 pfu/cell) was used as an immune stimulant to activate

PBMCs overnight prior to setting up chromium release assays to assess cytotoxicity against three target cell lines (K562, SW620, Mel888). Figure 3.5 shows that both fresh and frozen PBMCs were activated by reovirus, resulting in increased target cell cytotoxicity in comparison to unstimulated PBMCs. This was true for all three target cell lines (e.g. reovirus activation at 1 pfu/cell *vs.* 0 pfu/cell for cryopreserved PBMCs against SW620 cells, 62.3 % *vs.* 23.0 %, respectively, p=0.0146; for fresh PBMCs against SW620 cells at same ratio, 82.3 % *vs.* 44.3 %, p=0.0204; both at 100:1 effector:target ratio). Although the absolute cytotoxicity of cryopreserved cells is moderately diminished compared to fresh PBMCs, this data indicates that previously frozen PBMCs are still functional and able to respond to an immune stimulus following cryopreservation.

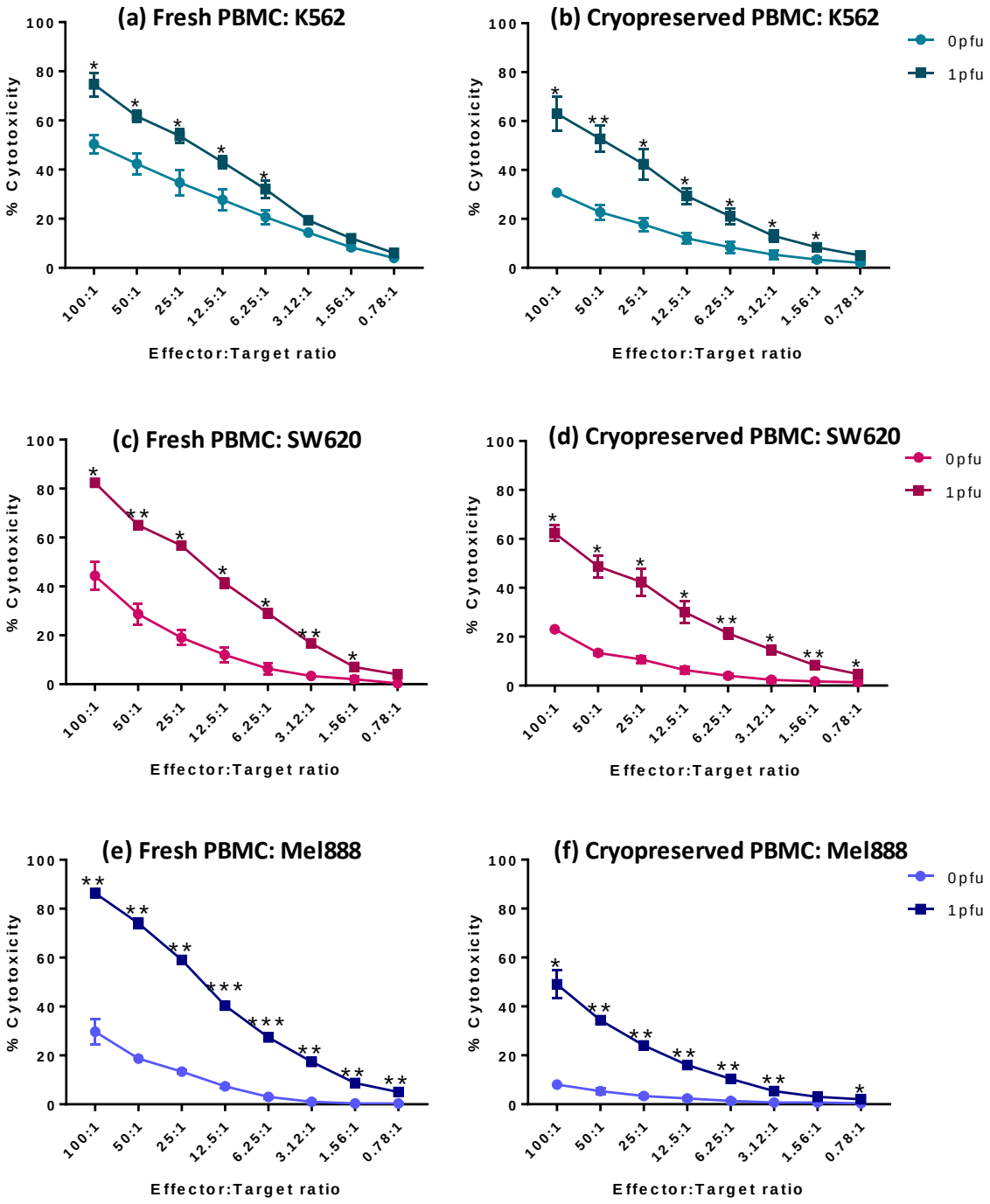


Figure 3.5 Cytotoxicity of fresh and cryopreserved PBMCs

PBMCs from cones were either used fresh **(a, c, e)** or following cryopreservation **(b, d, f)**. Cells were treated with 0 or 1 pfu/cell reovirus overnight at 37 °C before being incubated with ^{51}Cr-labelled target cells (K562 **(a + b)**, SW620 **(c + d)** and Mel888 **(e + f)**). Percentage of dead tumour cells was then determined (n=3). For *p* values see Appendix (Table 8.1).

3.2.2.2 Degranulation of immune cells against target tumour cells is maintained following cryopreservation

CD107 degranulation assays were performed in parallel with chromium release assays to determine the sub-population of immune cells responsible for target cell death. Target cell lines were incubated for 4 hours with PBMCs previously activated ± 1 pfu/cell reovirus overnight. PBMCs were either fresh or had previously been cryopreserved. Cell populations were defined as described in Table 2.5 and degranulating cells were identified using CD107a/b antibodies. NK cells expressed the highest levels of CD107 when compared to NKT and T cells, suggesting that these cells are predominantly responsible for lysis of tumour cell targets via degranulation (Figure 3.6).

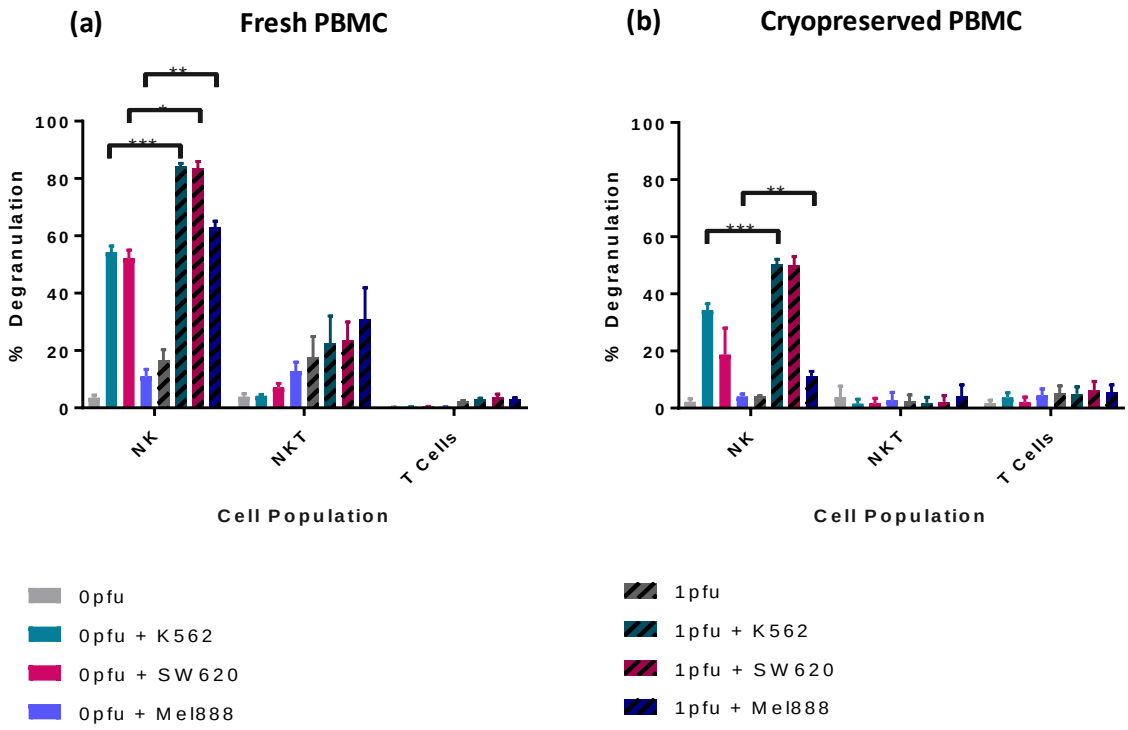


Figure 3.6 Degranulation of fresh and cryopreserved immune cell populations against tumour cell targets

Fresh **(a)** or cryopreserved **(b)** PBMCs were treated ± reovirus at 1 pfu/cell. Following overnight incubation, PBMCs were cultured with target cell lines and anti-CD107 antibodies for 4 hours. Flow cytometry was used to determine the proportion of CD107 expression within immune cell sub-populations (n=3).

Figure 3.7 shows degranulation of NK cells (within the PBMC population) in further detail. Treatment of PBMCs with reovirus increased NK cell degranulation when exposed to tumour targets. This was significant in all target cell lines when using fresh PBMCs (K562: 54.4 % *vs.* 84.1 %, p=0.0007; SW620: 52.2 % *vs.* 83.6 %, p=0.0121; Mel888: 11.0 % *vs.* 63.0 %, p=0.0023). Activation of cryopreserved NK cells with reovirus was also significant against the K562 and Mel888 cell lines (K562: 34.4 % *vs*. 50.3 %, p=0.001; Mel888: 4.0 % *vs*. 11.3 %, p=0.0084). Although the result did not reach significance with SW620 target cells, the trend persisted, with mean CD107 expression 18.7% (0 pfu/cell) *vs*. 50.0% (1 pfu/cell; p=0.0647). When exposed to K562 tumour cells, fresh NK cells expressed significantly more CD107 than cryopreserved NK cells, both with and without stimulation with reovirus (0 pfu/cell: 54.4 % *vs.* 34.4 %, p=0.0399; 1 pfu/cell: 84.07 % *vs.* 50.30 %, p=0.0081). Following reovirus activation, there was also significantly increased CD107 expression on fresh NK cells exposed to both SW620 and Mel888 target tumour cells in comparison to cryopreserved cells (SW620: 83.57 % *vs.* 49.97 %, p=0.0171; Mel888: 63.03 % *vs.* 11.30 %, p=0.0047).

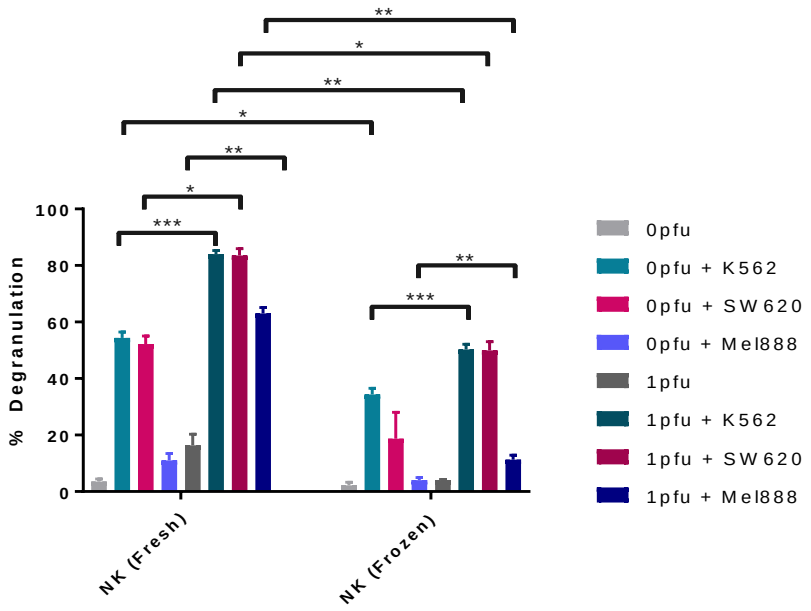


Figure 3.7 Degranulation of fresh and cryopreserved NK cells against tumour cell targets

Fresh or cryopreserved PBMCs isolated from cones were treated ± reovirus at 1 pfu/cell. Following overnight incubation, PBMCs were cultured with target cell lines and anti-CD107 antibodies for 4 hours. Flow cytometry was used to determine the proportion of CD107 expression within immune cell sub-populations (n=3).

Overall, absolute levels of CD107 expression were slightly lower in cryopreserved cells (Figure 3.7), reflecting the results of the chromium release assays but the effect of virus activation was comparable.

3.2.2.3 Cytokine production induced by virus activation is maintained following cryopreservation

PBMCs from cones were isolated and either used fresh or cryopreserved and thawed prior to use. Following an overnight incubation with 0 or 1 pfu/cell of reovirus, cell supernatants were collected and analysed by ELISA for cytokine/chemokine production in response to viral stimulation.

Cell-free supernatants were evaluated for presence of CXCL-10, IFN-α, TRAIL and IFN-γ (Figure 3.8). GM-CSF, TNF-α, IL-28, IL-29 and IL-10 were also evaluated but little or no secretion of these cytokines were detected (data not shown). Analysis showed that there was an increase in the release of inflammatory cytokines in response to treatment with reovirus from both fresh and cryopreserved PBMCs. Levels of IFN-α were significantly different in both conditions (fresh PBMCs: 0 pfu *vs.* 1 pfu, mean ± SEM 0 pg/ml *vs.* 3252 pg/ml ± 575 pg/ml, p=0.011; frozen PBMCs: 0 pg/ml *vs.* 2256 pg/ml ± 638 pg/ml, p=0.0385; Figure 3.8a). Using fresh PBMCs, release of CXCL-10 and TRAIL were significantly increased after virus treatment (CXCL-10: 0 pfu *vs.* 1 pfu, mean ± SEM 99 pg/ml ± 68 pg/ml *vs.* 489 pg/ml ± 109 pg/ml, p=0.0227; TRAIL: 28 pg/ml ± 28 pg/ml *vs.* 167 pg/ml ± 37 pg/ml, p=0.0218; Figure 3.8b and Figure 3.8c). There was a trend for increased cytokine release in response to virus when using cryopreserved PBMCs but this was not significant. Levels of IFN-γ were increased from both fresh and cryopreserved PBMCs in response to stimulation with reovirus, though this did not reach statistical significance (Figure 3.8d).

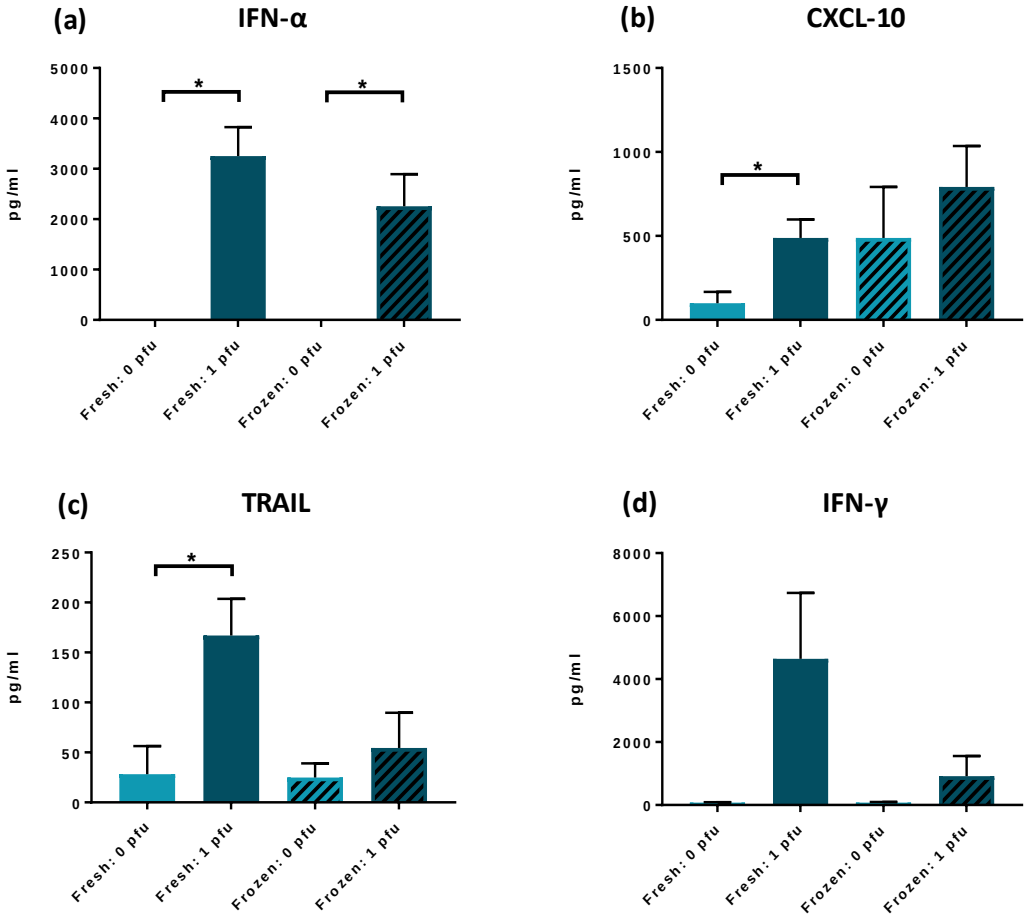


Figure 3.8 Release of cytokines from fresh and cryopreserved PBMCs in response to viral stimulation

PBMCs were isolated from cones and used fresh or following cryopreservation. PBMCs were treated with 0 or 1 pfu/cell reovirus and incubated overnight. Cell–free supernatants were collected and cytokine/chemokine release was analysed using ELISA. Graphs show secretion of IFN-α **(a)**, CXCL-10 **(b)**, TRAIL **(c)** and IFN-γ **(d)** (n=4).

In these conditions, there appears to be an increase in cytokine release from both fresh and cryopreserved PBMCs in response to stimuli; however, the response could potentially be blunted from cryopreserved cells.

3.2.2.4 Immunophenotyping of reovirus-activated PBMCs is maintained following cryopreservation

Our lab has previously validated that the expression of various identification and activation markers are comparable on fresh and cryopreserved PBMCs (personal communication, data not shown). Immune cell populations were identified as shown in Table 2.5 and following

immunophenotyping, flow cytometry was used to determine the expression of activation markers on the cell surface in response to viral activation. These previous investigations were extended to determine whether PBMCs could be used immediately on thawing ('same day') or whether a period of incubation prior to immunophenotyping ('rested') could optimise the expression of activation markers of interest. The frequency of specific cell populations was monitored using identification surface markers. Additionally, cellular activation was measured using other markers (such as CD69). Most immune markers, including CD3, CD4 and CD8 were reproducible in both conditions ('same-day' and 'rested') (Figure 3.9a). This also applied to smaller immune cell populations, such as NK cells and B cells (Figure 3.9b). The only exception to this was in the CD14+ monocyte population. While there was no significant difference in untreated monocytes left resting overnight, stimulation of the PBMC population with reovirus lead to a significant loss of monocytes: 0pfu *vs.* 1pfu 'rested' 64.5 % *vs.* 14.6 %, p=0.0154; 1pfu 'same-day' vs. 1pfu 'rested' 71.8% *vs.* 14.6 % , p=0.0052.

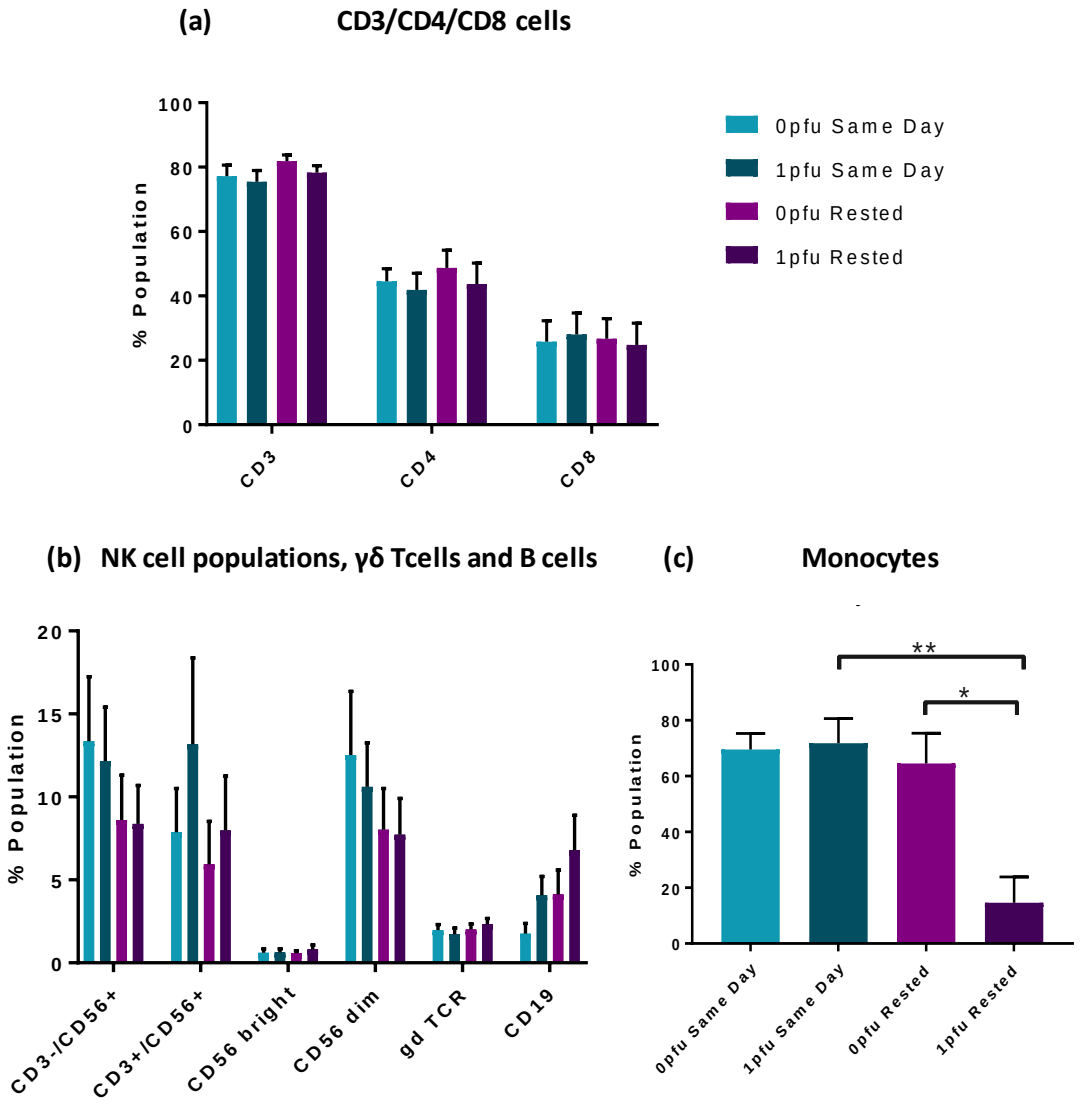


Figure 3.9 Frequency of cell populations in cryopreserved PBMCs used immediately on thawing ('same day') or rested overnight ('rested') before immunophenotyping

PBMCs were isolated from cones, treated with 0 or 1 pfu/cell reovirus and incubated overnight before cryopreservation. Following thawing they were either immunophenotyped immediately ('same-day') or rested overnight and used the following day ('rested'). Expression of cell surface markers CD3, CD4 and CD8 was determined by flow cytometry **(a)**. Similarly, the expression of CD56 (NK cells), CD3/CD56 (NKT cells), CD56 bright and CD56 dim (NK cells), γδ TCR (γδ T cells) and CD19 (B Cells) was determined **(b)**. Expression of CD14 was also determined by flow cytometry **(c)** (n=4).

There was evidence of immune cell activation across all cell types in response to reovirus, as demonstrated by increased CD69 expression. Once again this was reproducible whether PBMCs had been thawed for immediate use in the assay, or rested overnight before immunophenotyping (Figure 3.10).

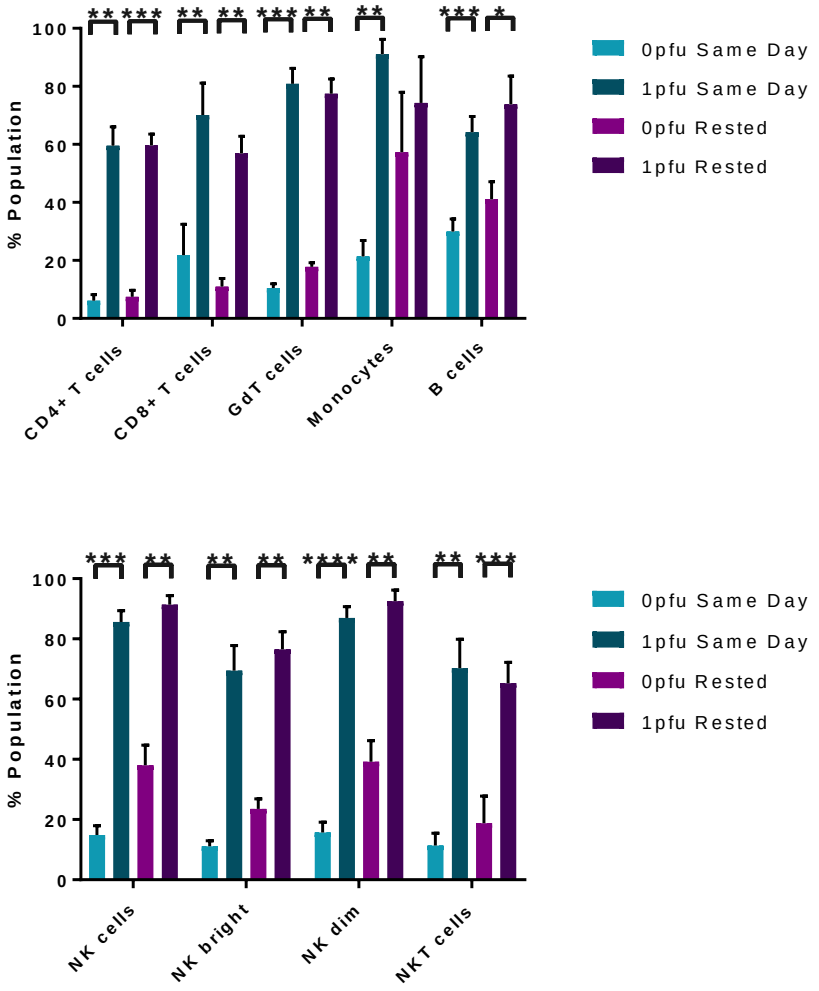


Figure 3.10 Expression of the activation marker CD69 on PBMCs activated with reovirus

PBMCs were isolated from cones, treated with 0 or 1 pfu/cell reovirus and incubated overnight before cryopreservation. Following thawing they were either immunophenotyped immediately ('same-day') or rested overnight and used the following day ('rested'). Expression of CD69 on immune cell sub-populations was determined by flow cytometry. Graphs show the percentage of positive cells (n=4). For *p* values see Appendix Table 8.2)

If the percentage of positive cells within a population was universally high (Figure 3.11a) then RMS was used to determine the magnitude of expression of that particular marker on all cells. Using this method there was, indeed, an increase in response to reovirus (CCR7 on CD3/CD4+

cells, 0 pfu/cell *vs*. 1 pfu/cell, 'same day' 28.9 *vs.* 33.6, p=0.0078; 'rested' 28.5 *vs.* 40.7, p=0.0323, Figure 3.11b).

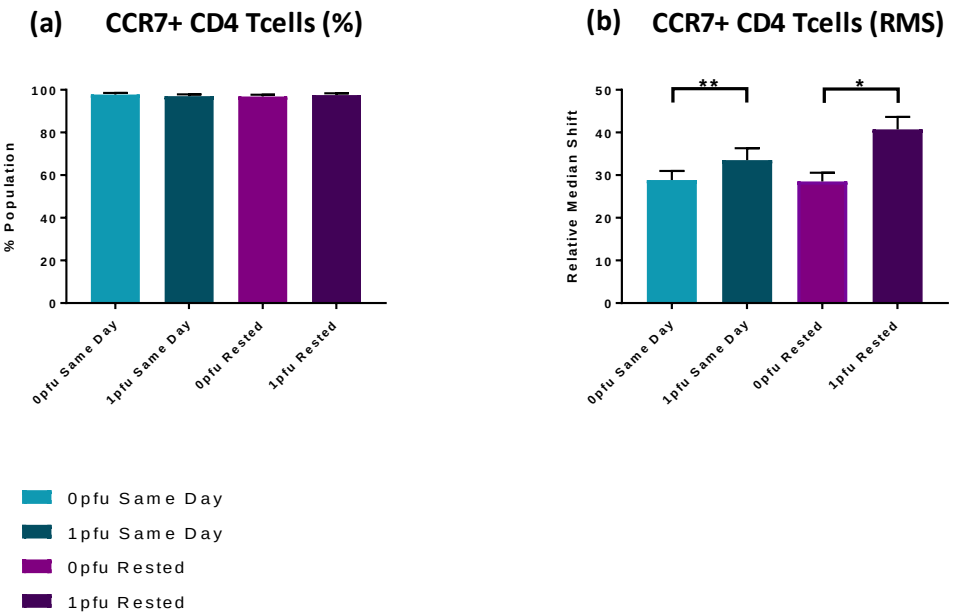


Figure 3.11 Expression of identification and activation cell surface markers on immune cell populations in response to reovirus

PBMCs were isolated from cones, treated with 0 or 1 pfu/cell reovirus and incubated overnight before cryopreservation. Following thawing they were either immunophenotyped immediately ('same-day') or rested overnight and used the following day ('rested'). Expression of CCR7 on CD4+ T cells was determined by flow cytometry and is shown as percentage of positive cells **(a)** and RMS **(b)**; (n=4).

There was no significant difference in the percentage of CCR7 positive CD8+ T cells, NK cells, CD56 bright NK cells, NKT cells and γδT cells investigated in either PBMC condition ('same-day' and 'rested') (data not shown). With the exception of CD4+ T cells (Figure 3.11b), CCR7 expression measured by RMS did not significantly change on any of the other cell populations, in any condition ('same-day' *vs.* 'rested', 0 pfu *vs.* 1pfu, data not shown).

When assessing the percentage positive population, there was no appreciable difference between HLA-DR or CD86 expression on B cells and monocytes treated with reovirus (data not shown). Once again this was reproducible whether PBMCs had been rested or not before treatment with reovirus (data not shown). Similarly, when expression of HLA-DR was assessed by RMS, there was no demonstrable difference between expression of either on B cells or

monocytes in any of the tested conditions (data not shown). The same was true for CD86 expression on B cells. However, there was a significant increase in CD86 expression on monocytes which had been activated with reovirus, in both the 'same-day' and 'rested' conditions (0 pfu *vs*. 1 pfu, 'same day' 4.7 *vs.* 7.9, p=0.0415; 'rested' 6.2 *vs.* 22.5, p=0.0474; Figure 3.12). There was no significant difference in expression of CD86 between cells that were used same day compared to those rested overnight within the reovirus treatment conditions (0pfu and 1pfu).

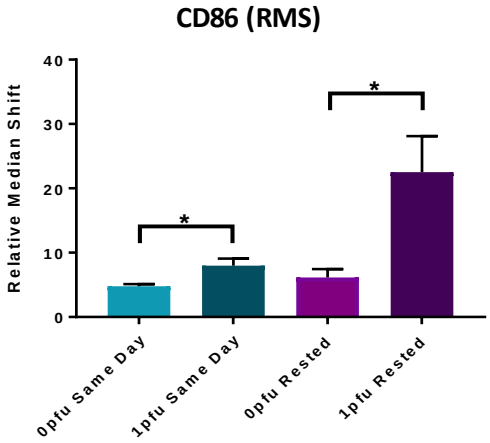


Figure 3.12 Expression of CD86 on monocytes in response to reovirus

PBMCs were isolated from cones, treated with 0 or 1 pfu/cell reovirus and incubated overnight before cryopreservation. Following thawing they were either immunophenotyped immediately ('same-day') or rested overnight and used the following day ('rested'). Expression of CD86 using RMS was determined by flow cytometry (n=4).

In conclusion, these data indicate that immunophenotyping of cryopreserved PBMCs is reliable for the identification and evaluation of activated PBMC sub-populations. Analysis of PBMCs by immunophenotyping was reproducible whether cells were used immediately or rested overnight. Although the monocyte population was diminished by resting overnight, especially when cells were stimulated with reovirus, differences in the expression of activation markers could still be determined.

3.2.3 Functional Analysis of Clinical Trial Samples: Validating ELISpot Assays

Measurement of adaptive immune responses to antigen stimulation can be determined by IFN-γ production by T cells. ELISpot assays are an extremely sensitive assay and IFN-γ responses have been shown to correlate with outcome and survival in a range of cancers. However, due to the multiple steps involved and the sensitivity of the assay, standardisation of the protocol is needed in order to ensure the results are reliable and reproducible.

3.2.3.1 Pooled viral peptides at optimal concentrations can detect recall T cell responses in ELISpot assays

ELISpot assays can be used to determine T cell responses to TAAs (for example CEA or MART-1) or the CEF viral peptide pool, as measured by IFN-γ SFUs. Cryopreserved PBMCs were thawed and rested for 2 hours prior to use in ELISpot assays. Rested PBMCs were incubated overnight on a ELISpot plate with either negative control (complete RPMI, Table 2.2), CEF viral peptide pools or TAA peptide pools. When PBMCs were incubated with increasing concentrations of CEF peptide pool, there was a clear increase in T cell responses in a dose - dependent manner compared to the negative control (Figure 3.13). These data indicated that CEF peptide should be used at a dose of 2 µg/ml in future experiments. It is also apparent that CEF can been used as a reliable positive control in future ELISpot assays.

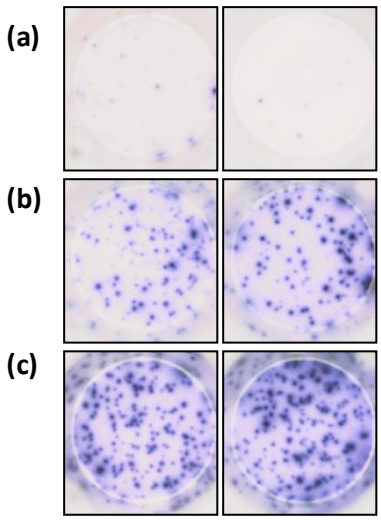


Figure 3.13 IFN-γ T cell responses to viral peptide pool

PBMCs were isolated from cones and cryopreserved. On thawing, they were incubated for 2 hours at 37 °C in complete RPMI. Following this, they were incubated alone (negative control) or with increasing concentrations of CEF peptide at a density of $1x10^5$ cells per well. Images (in duplicate) show examples of the T cell responses to: **(a)** negative control **(b)** CEF peptide at 0.2 µg/ml and **(c)** 2 µg/ml.

3.2.3.2 Incubating PBMCs at high density prior to ELISpot assays does not increase T cell responses to peptide pools

Previous studies have suggested that pre-incubation of cells at high densities for 2 days prior to use in ELISpot assays enhanced the detection of T cell responses to antigen/peptide (299). We tested this within our system and compared PBMCs thawed for immediate use and PBMCs that were cultured at a high density prior to use.

Incubating thawed PBMCs at $1x10^7$/ml for 48 hours prior to use in the ELISpot assay not only resulted in a loss of viable cells (data not shown) but did not enhance the response of T cells to peptide pools (Figure 3.14). In fact, pre-incubation in this manner only served to dramatically reduce the number of responding T cells.

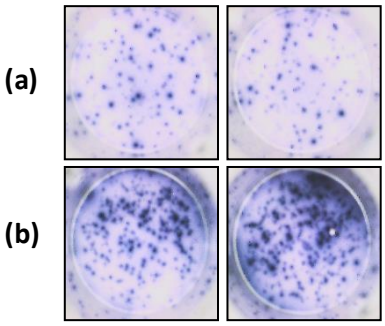


Figure 3.14 IFN-γ T cell responses to CEF viral peptide pool following pre-incubation of PBMCs

PBMCs were isolated and cryopreserved. On thawing, they were incubated at high density ($1x10^7$/ml) for 48 hours at 37 °C in complete RPMI or used immediately. Images (in duplicate) show examples of the T cell responses to 2 µg/ml CEF peptide when cells were: **(a)** pre-incubated for 48 hours or **(b)** used immediately.

To confirm this effect is consistent when using cryopreserved PBMCs from patients (rather than HDs), ELISpot assays were performed using similar conditions of pre-incubation of patient PBMCs. In addition, a comparison of cell densities ($1x10^5$ PBMCs/well and $3x10^5$ PBMCs/well) within the ELISpot wells was performed to determine the optimum cell number required for the assay. As before, pre-incubating PBMCs at high density for 48 hours prior to use in the assay diminished (rather than enhanced) the detection of T cell responses (as shown by a lower number of IFN-γ SFU, Figure 3.15a and Figure 3.15d). Using PBMCs immediately following thawing led to the detection of a far higher number of T cell responses but at increased cell densities the background levels were elevated enough to conceal the individual SFUs (Figure 3.15f). Pre-incubating the cells for 24 hours (*vs.* 48 hours) prior to exposure to peptide pools (at both densities of PBMCs/well) seemed to enhance the detection of T cell responses without reaching excessive levels (Figure 3.15b and Figure 3.15e).

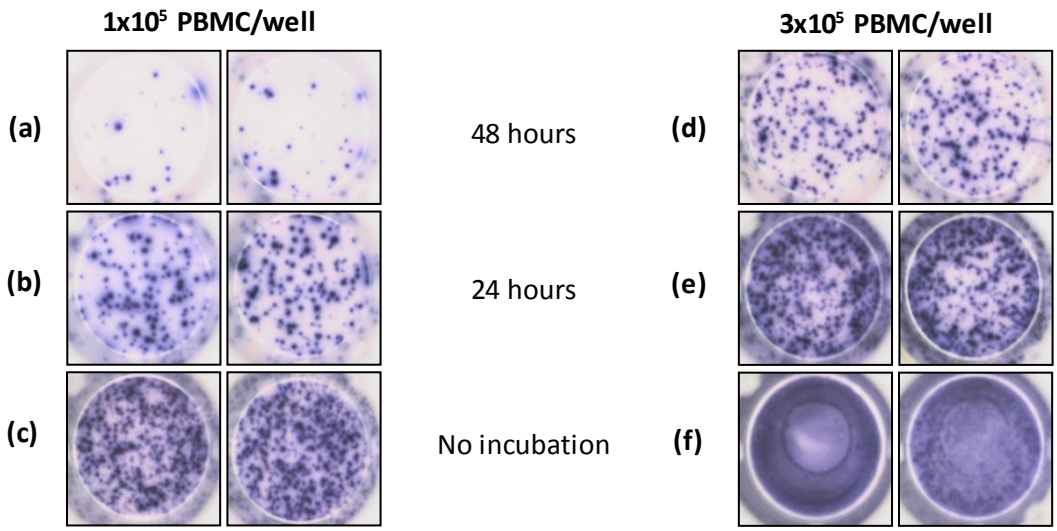


Figure 3.15 Patient IFN-γ T cell responses to viral peptide pool following high-density pre-incubation of PBMCs

PBMCs were isolated from patients with metastatic colon cancer prior to chemotherapy and cryopreserved. On thawing, they were incubated at high density ($1x10^7$/ml) for 24 or 48 hours or used immediately. Images (in duplicate) show examples of T cell response to 2 µg/ml CEF peptide at cell densities of $1x10^5$/well **(a - c)** and $3x10^5$/well **(d - f)**; **(a + d)** pre-incubated for 48 hours, **(b + e)** pre-incubated for 24 hours or **(c + f)** used immediately.

To determine the response to TAAs, PBMCs isolated from the blood of melanoma or CRC patients were used in an ELISpot assay with MART-1 and CEA peptide pools, respectively, as immune stimuli. Cryopreserved PBMCs were thawed and incubated at a density of $1x10^5$ - $4x10^5$ PBMCs/well with the peptide pool overnight. There was no detrimental effect from using higher concentrations of TAA peptide pool (e.g. 1 µg/ml CEA peptide *vs.* 2 µg/ml CEA peptide, Figure 3.16). Increasing cell density beyond $2x10^5$ PBMCs/well did not increase T cell responses (data not shown).

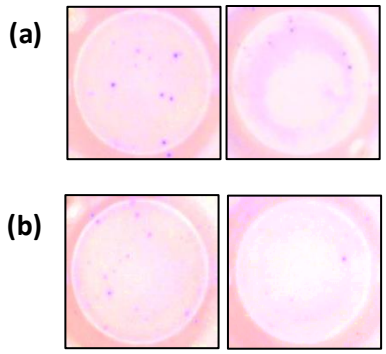


Figure 3.16 Patient IFN-γ T cell responses to CEA

PBMCs were isolated from patients with metastatic CRC and cryopreserved. On thawing, they were incubated at high density ($1x10^7$/ml) for 24 or 48 hours or used immediately. Example images (in duplicate) show T cell responses (at a density of $1x10^5$ PBMC/well when thawed cells were used immediately) to CEA peptide at 1 µg/ml **(a)** and 2 µg/ml **(b)**.

In conclusion, ELISpot assays are a robust method to detect antigen-specific T cells, either in a pan recall response to previously encountered viral peptides or against TAAs when analysing PBMCs from cancer patients. Pre-incubating PBMCs at high density for 48 hours, after being thawed and prior to use in the assay, does not seem to enhance the detection of T cell responses. Pre-incubating cells for 24 hours, however, may mean that differential levels of response to antigen could be detected across a heterogenous group of patients. This method of preparing cells for use would be more in keeping with the other assays and allow batch analysis after 24 hours incubation. Finally, we have shown that peptide pools derived from TAAs can also be used to detect tumour-specific T cell responses in patients.

3.3 Immune Assay Validation: Discussion

In clinical trials of novel anti-cancer agents, including immunotherapy, there is growing interest in translational biological outcomes. In order to gather data about the effect of these therapies on the immune system and the validation of potential biomarkers, large numbers of clinical blood samples need to be collected and analysed. In previous clinical trials in our lab, PBMCs from donor blood samples have been isolated and analysed on the same day. Aside from being very time- and resource-intensive, this can also add variability into sample analysis and is impractical for anything other than immunophenotyping panels, where cells can be stained and fixed to preserve integrity. Batch analysis of these samples, following cryopreservation, in validated immune assays will ensure that information collected is accurate, reproducible and meaningful.

Following cryopreservation and thawing of clinical samples, the recovery (% yield) of viable cells requires assessment. Trypan Blue was routinely used to calculate the proportion of viable immune cells in these validation experiments. Verification of this method with PI staining illustrated that Trypan Blue exclusion was highly accurate and determined that over 90 % were viable cells, a trend seen across different donors (Figure 3.1).

The yield of immune cells isolated from clinical samples must be maximised, especially if patients are undergoing chemotherapy where a drop in cell counts is anticipated. Our data shows that when cryopreserved as a whole population, WBCs have a poor rate of recovery on thawing (Figure 3.2a). This is likely due to the presence of granulocytes within the population, which when thawed, lyse and release products which damage other immune cells, an observation which is supported by other published data (269). Conversely, the recovery of viable cells is increased when the PBMC population is separated from the granulocytes and frozen in isolation (Figure 3.2b). Exposure of the PBMC population to granulocytes, as the result of a prolonged period of time between sample collection and PBMC isolation (within blood sample collection tubes), has been shown to attenuate T cell responses in subsequent immune assays (300). For this reason, blood samples should be processed as soon as possible following venepuncture and cryopreserved as PBMCs, rather than mixed whole WBCs, to optimise both the yield and functionality of viable cells. We therefore aimed to process all clinical samples within 2 hours of venepuncture.

Traditionally, leucocyte cones from HDs have been used to source PBMC populations for use in the lab, due to the high yield of PBMCs from each cone and ease of access. It was notable in

our data that the recovery of cryopreserved PBMCs isolated from HD leucocyte cones was higher than those isolated from fresh donor blood samples (Figure 3.3). The leucocytes cones do not contain granulocytes and, as such, there is no contact with PBMCs *ex vivo*. Hence, the viability of patient clinical samples are likely to have been affected by the presence of granulocytes despite being processed as soon as possible. Consequently, the conditions for the cryopreservation of PBMCs were optimised using fresh blood samples from this point onwards.

We observed that using a standard FM of 10 % DMSO/FCS yielded a higher proportion of viable cells when compared to FM using complete RPMI (Figure 3.2). Other studies investigating the optimum FM to use when cryopreserving PBMCs have found little difference between the use of RPMI or FCS-based FM (243). In addition, our data indicated that increasing the percentage of DMSO in the FM to 20 % did not negatively affect cell recovery, unlike other groups such as Nazarpour *et al.* (Figure 3.2, (272)). This data also showed that the use of FM containing pooled human AB serum (clinical FM, Table 2.3) was similar to standard FM in terms of the percentage of PBMCs recovered following cryopreservation (Figure 3.4). This is in contrast to data published by Disis *et al*, who observed diminished viability of PBMC following cryopreservation in FM containing HS (278). These studies, however, did not include functional analysis of PBMCs cryopreserved in FM containing HS. Personal communication with other groups has supported the use of HS-containing FM in maintaining functional activity of PBMCs. As such, clinical FM was carried forward and used throughout further investigations.

Cell recovery was enhanced by increasing the freezing density of PBMCs (Figure 3.3 and Figure 3.4). There appears to be no consensus on this in published data and other groups have used freezing densities of between $3x10^6$ PBMC/ml and $3x10^7$ PBMC/ml to store samples for analysis (275,276,278). However, the density of PBMCs should be balanced between optimising cell viability and ensuring that all assays can be performed as required, so that an appropriate number of vials can be thawed as needed for analysis without unnecessary waste of precious clinical samples. As such, following these observations, PBMCs collected during subsequent studies were cryopreserved at a density of $1x10^7$ cells/ml in clinical FM: a concentration which has been used successfully in a number of other studies (282,290) and yielded a high percentage of viable cells in our data (Figure 3.4).

Both ^{51}Cr release and degranulation assays were performed using reovirus as an activation stimulus for fresh PBMCs, against tumour targets. Lysis of target cells was significantly

increased by activation of PBMCs with reovirus. This effect was shown to be comparable for PBMCs which had been cryopreserved prior to use (Figure 3.5).

Similarly, reovirus increased baseline CD107 expression compared to untreated controls on T, NK and NKT cells to varying extents (Figure 3.6). NK cells constituted the highest proportion of degranulating cells compared to NKT and T cells, indicating that this population is responsible for lysis of targets observed. CD107 expression and the enhancing effect of reovirus is also comparable between fresh and previously frozen PBMCs (Figure 3.6 and Figure 3.7). Published data has shown similar findings and therefore support our findings that innate immune cell assays can be performed using previously cryopreserved cells (271,276), as they are representative of freshly isolated cells in their response to immune stimulus.

There was evidence of cytokine/chemokine release in response to reovirus in supernatants from both cryopreserved and fresh PBMCs (Figure 3.8). This was variable across all cytokines/chemokines tested. As seen in innate immune assays, the absolute levels of solutes detected when using frozen samples were diminished compared to those found in fresh cell-culture supernatants but the trends were highly significant and representative of the fresh samples. Studies have shown that levels of cytokines produced by thawed PBMCs in culture are variable, depending on the cytokine and whether the cells have been re-stimulated or not (286,297). Activation of PBMCs during thawing, variations in the initial cryopreservation process and death of particular immune cell sub-populations during any point in the freeze-thaw cycle are just some of the reasons why levels of cytokines may vary.

Previous work in our laboratory has determined that activation of immune cell subsets after cryopreservation is comparable to fresh cells where immunophenotyping was performed immediately following collection of the sample (personal communication). This echoes findings in other studies, where the sub-populations of immune cells within PBMCs remain comparable to those present in the same samples following cryopreservation (301). In our investigations, PBMCs stimulated with reovirus before cryopreservation were thawed and either analysed immediately or following overnight incubation ('rested'). In both scenarios, the absolute frequencies of populations such as CD4, CD3 and CD8 were reliable and comparable (Figure 3.9) with the exception of the CD14+ monocyte population (Figure 3.9c).

Reovirus could activate all sub-populations of cells analysed: expression of the activation marker CD69 was significantly increased with reovirus, whether or not PBMCs had been rested after thawing (Figure 3.10). Likewise, CCR7 (Figure 3.11) was up-regulated on some

cells following treatment with reovirus. These data collectively show that cryopreserved cells are comparable to fresh cells when evaluated by immunophenotyping for cell activation by reovirus and that cells can be rested overnight prior to analysis. Although the absolute percentages of marker expression are lower in previously frozen samples, the capacity to become activated after viral stimulation remained. The observed decrease in cytokine production between fresh and cryopreserved PBMCs, although proportional, may explain why absolute levels of immune cell activation (such as CD69 expression or NK cell degranulation/cytotoxicity) is decreased in previously frozen cells (286) as cellular activation is linked to the presence of certain cytokines.

As part of the panel of assays which evaluate immune responses in patients, ELISpot can be used to determine the T cell response to TAAs (anti-tumour response) and viral peptide pools (pan-recall response). Using cryopreserved PBMCs, there was a dose-dependent response to CEF viral peptide pools, with 2 µg/ml being the optimum concentration that was carried forward into the analysis of trial samples (Figure 3.13). There is some suggestion that in order to enhance the detection of T cell responses to antigen stimulation, thawed PBMCs should be incubated at high density prior to being used in ELISpot assays (299). It is suggested that this mimics a tissue-like environment, where T cells are activated through interaction with other cell populations, thereby increasing T cell function and antigen recognition. This process can also lead to a purer population of functional immune cells, due to the loss of dysfunctional cells undergoing apoptosis following thawing (286). In our studies, however, incubating PBMCs at high density for 24-48 hours prior to analysis led to attenuated T cell responses (Figure 3.14 and Figure 3.15). Variability amongst donors to antigen stimulation, both before and after resting, implies that the effect of pre-incubation is not predictable or uniform and other groups have also found this to be true (289). Due to the heterogeneity of the response to viral and tumour antigens between patients, T cell responses from samples incubated for 48 hours before use may be too low to detect in some situations (Figure 3.15). Similarly, if samples are used immediately upon thawing, then responses in some patients may be too numerous to accurately calculate. These data also demonstrate an elevated response when the density of PBMCs seeded into each ELISpot well was increased. Furthermore, when using TAA peptide pools (CEA and MART-1), pre-incubation of PBMCs following thawing prior to the ELISpot assay did not enhance T cell responses (Figure 3.16). Optimising an assay that demonstrates such inter-patient variability requires focus on trying to maximise the 'signal to noise' ratio. As such, the following conditions were shown to be most effective: a density of

$2x10^5$ PBMCs/well, following resting of these PBMCs overnight and stimulation of the T cells with a concentration of peptide pools at 2 µg/ml.

In summary, peripheral blood samples from patients can successfully be cryopreserved for subsequent batch analysis. Although the magnitude in response to immune stimuli is lower in previously cryopreserved cells, this should not affect the overall results when samples are analysed over time and where the response to treatment is compared to a baseline sample from the same patient. The use of optimised and validated protocols will ensure that the viability of cryopreserved samples is maximised and that a high yield of viable cells are recovered on thawing. We have also demonstrated procedures for optimising PBMCs for use in multiple assays: being able to prepare the cells in similar ways reduces the number of cells being wasted and increases the number of experimental conditions possible for each clinical sample, thereby maximising the translational output within a given study. The utilisation of robust SOPs for the investigation and interrogation of immune response will ensure that these assays can be performed on many clinical trial samples, potentially across multiple sites, with a high degree of accuracy and reproducibility.

As part of this project, these assays were used to analyse the immune response to treatment in patients receiving first-line chemotherapy for CRC and patients receiving immunotherapy for metastatic melanoma (Chapter 4 and Chapter 5).

Chapter 4 Neutrophil Lymphocyte Ratio

4.1 Introduction

4.1.1 Colorectal Cancer

CRC is currently the fourth most common in the UK (302), with over 40,000 new cases and 15,000 deaths per year. The Dukes staging system has traditionally been used to stage CRC but the American Joint Committee on Cancer (AJCC) or Tumour Node Metastasis (TNM) staging can also be used to help guide management decisions (303). A screening programme involving the use of faecal occult blood tests was initiated in 2006 for those aged between 60 and 74 (304) and participants with an abnormal test are invited for colonoscopy. Of the 11.6 % of men and 7.8 % of women in whom CRC is detected, over 70 % will have early stage disease (Dukes A or B: LN negative, Table 4.1) (304). The definitive management for potentially resectable disease is surgery, after which long-term outcome is dependent on stage, with a five-year survival of 85 % - 95 % for stage I disease and to 30 % - 60 % for stage III (Dukes C: LN positive disease, Table 4.1). Risk factors for CRC include chronic inflammation (such as inflammatory bowel disease), high fat 'Western' diet, obesity, alcohol excess and smoking, which may explain the increasing incidence in recent years (305). Symptoms may include lower abdominal pain, rectal bleeding, iron deficiency anaemia, change in bowel habit or weight loss. As the majority of tumours are left-sided (in the sigmoid colon or rectum), most are diagnosed on colonoscopy or sigmoidoscopy. For patients who are frail and cannot tolerate a colonoscopy, computerised tomography colonoscopy can be offered as a screening tool but a definitive tissue biopsy may still be sought to assist with diagnosis and management plans (306). Dependent on tumour staging, management with curative intent can also include neoadjuvant or adjuvant chemotherapy in addition to surgery. In the presence of oligometastatic disease, such as liver or lung metastases, surgical resection can still be utilised to improve outcome and prognosis. Local ablative therapies, such as the use of chemoablation, can also be useful in these patients. In the management of metastatic disease, performance status, quality of life and potential toxicities should be considered prior to commencing palliative chemotherapy.

4.1.2 Pathology and Staging of CRC

Staging of CRC is determined using the TNM model, which also corresponds to tumour stage (stage I-IV or Dukes staging A-C, Table 4.1) and can be used to guide management decisions. Although the AJCC staging has recently been updated to the 8th Edition, the 7th edition was used at the time of our study.

Table 4.1 TNM and Dukes staging of colorectal cancer (adapted from 7th American Joint Committee on Cancer, AJCC) (303)

Stage	Dukes Stage	Tumour	Tumour stage
I	**A**	T1	Invading submucosa
		T2	Invading muscularis propria
II	**B**	T3	Invades through muscularis into pericolonic tissues
		T4a	Invades to the surface of the visceral peritoneum
		T4b	Invades into or adherent to other organs or structures
		Nodal Status	**Number of Metastatic Nodes**
III	**C**	N1a	1 regional LNs
		N1b	2-3 regional LN
		N1c	Tumour deposits in mesentery or nonperitoneal tissue without LNs
		N2a	4-6 regional LNs
		N2b	≥ 7 regional LNs
		Metastases	**Site**
IV	-	M1a	Metastases in one organ or site (liver, lung, non-regional LN etc.)
		M1b	Metastases in more than 1 organ/site or peritoneal disease

CRC arises from the bowel mucosa and can be proceeded by a number of mutations which contribute to the development of the disease. The World Health Organisation (WHO) histological classification describes the different pathological subtypes of tumours of the colon and rectum (305). In addition to benign adenomas, neuroendocrine tumours and non-epithelial tumours (including lymphoma), colonic epithelial tumours can be classified as:

- Adenocarcinoma
- Signet-ring cell carcinoma
- Squamous cell carcinoma
- Medullary carcinoma
- Mucinous Adenocarcinoma
- Small cell carcinoma
- Adenosquamous carcinoma
- Undifferentiated carcinoma

In addition, there are a number of genetic mutations which contribute to carcinogenesis and the development of colorectal tumours, including the tumour suppressor genes adenomatous polyposis coli (*APC*), *p53* and *KRAS* (307–309). Mutations in *APC* lead to familial adenomatous polyposis: the usual regulatory function of *APC* in preventing abnormal cellular growth is abrogated and this leads to the abnormal development of polyps which can become malignant. The function of *p53* (also called 'the guardian of the genome') is to detect DNA damage within the cell and either repair it or initiate apoptosis. Loss of *p53* therefore allows the proliferation of genetically abnormal cells with high malignant potential and mutations in *p53* are associated with a range of cancers, including the development of CRC. In contrast, activating mutations in *KRAS* leads to upregulation in intracellular signalling pathways, such as MAPK, initiating abnormal cell proliferation and survival. Sequential mutations in *APC*, *KRAS* and *p53* therefore lead to the development of dysplastic adenomas which consequently transform into carcinomas (308). Other common mutations detected in CRC include *BRAF*, *PIK3CA* (which encodes for the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) protein, part of the PI3K/AKT signalling pathway) and the WNT signalling pathways (310). An important sub-population of CRCs are those tumours which exhibit defects in mismatch repair (MMR) as a result of mutations in genes such as *MLH1, MSH2* and *MSH6.* Mutations in these genes allow the accumulation of small sections of nucleotide repeat sequences (microsatellites) which increase the mutational load of the cell and predispose to the development of microsatellite instable (MSI) CRC. These tumours are also known to have a higher incidence of *BRAF* mutations (311). Analysis of CRC samples from different areas of the bowel reveal a pattern of different mutational profiles relative to location of the tumour. Analysis of mutational signatures of 1443 colonic tumour samples revealed that the incidence of MSI CRC and those with *BRAF* mutations was highest in the ascending colon and reduces along the bowel to the rectum (310). The highest incidence of *KRAS* mutations was found in the caecum (310). More recently, CRC has been further delineated into four consensus molecular subtypes (CMS): CMS 1 (MSI with BRAF mutations and immune infiltrates), CMS 2 ('canonical' subtype and the most prevalent; may have mutations in WNT pathway), CMS 3 ('metabolic' subtype with *KRAS* mutations) and CMS 4 (activation of pathways including TGF-β signalling and VEGF; inflammatory aetiology with poor a prognosis) (312).

Understanding the molecular basis of CRC helps us to appreciate that it is not a singular disease and focuses management. As an example, MSI CRC is the only molecular subtype which responds to immunotherapy. Conversely, tumour with mutations in *KRAS* do not

respond to therapy with EGFR inhibitors. As EGFR is found on the cell surface and is upstream of the MAPK pathway (which includes RAS), activating mutations of the KRAS isoform stimulates this intracellular signalling pathway in the absence of EGRF activation. Blocking EGFR in this situation is therefore ineffective.

4.1.2.1 Immune Response and Microsatellite Instability

MSI is found in up to 15 % of CRC and is due to MMR (313). This is due to either germline mutations in MMR genes or hypermethylation and epigenetic silencing of genes such as *MLH1, MSH2* and *MSH6.* During DNA replication, mismatches between the two strands of DNA can occur (for example due to erroneous insertion of nucleotides in the daughter DNA replication strand causing frameshift mutations) (314). MMR proteins recognise the mismatch (such as pairing guanine with thymine instead of cytosine) and form a complex (usually by binding to other MMR proteins) which excises, resynthesises and replaces the abnormal section of DNA (314). Abnormalities in the MMR gene lead to an accumulation of microsatellites: small sections of nucleotide repeats which are usually present throughout the genome but in the case of tumours deficient in MMR (dMMR), lead to increased mutational load and are associated with a number of cancers. Germline mutations in MMR genes are specifically associated with familial CRC such as hereditary nonpolyposis CRC (also known as Lynch Syndrome) (315). CRCs with dMMR/MSI have a number of molecular and pathological features: i) a tendency for proximal tumours diagnosed at a younger age and earlier stage; ii) an increased association with mutations in the *BRAF* tumour suppressor gene (particularly in sporadic dMMR cancers) and iii) higher grade at presentation and increased lymphocytic infiltration of tumours when compared to microsatellite stable (MSS) CRCs proficient in MMR (pMMR) (311). Although some studies have shown that dMMR tumours have a poorer prognosis than pMMR tumours (316), this conclusion may have been due to co-existing *BRAF* mutations within the dMMR group. In the combined analysis of 3063 patients with metastatic CRC (mCRC) from four large clinical trials, patients with dMMR demonstrated worse progression-free survival (PFS) and OS compared to those with pMMR (hazard ratio (HR) 1.33 and 1.35, respectively) (316). However, within this large patient cohort, nearly 35 % of patients with dMMR also had sporadic mutations in *BRAF*. As such, there was no significant difference in OS for patients with *BRAF* mutations and either pMMR or dMMR (OS 11.3 months vs. 11.7 months, respectively) (316). In a systematic review of 32 studies reporting outcomes in 7,642 patients with stage I-IV disease, MSI CRC was been shown to have a better prognosis than MSS CRC, with a HR for OS of 0.65 (95 % confidence interval (CI) 0.59 to 0.71)

(317). When considering these data, it is also worth noting that there is no standardised method to determine dMMR/MSI status.

There is also evidence that dMMR/MSI can predict response to chemotherapy, particularly in the adjuvant setting. If CRC with dMMR has a better prognosis (as suggested by data from a systematic review which included early stage patients (317)), the benefits of adjuvant chemotherapy may be diminished, particularly in light of evidence that MSI CRC may be resistant to 5-FU (318,319). Although tumours with dMMR can have a better outcome than those with pMMR, the current standard-of-care does not differentiate when decisions are made regarding adjuvant chemotherapy. While there is also some evidence for a benefit in giving adjuvant 5-FU-based chemotherapy to patients with dMMR, this may be limited to patients with germline tumours (Lynch Syndrome), rather than sporadic mutations in MMR genes (320).

Although CRC tumours express inhibitory checkpoint ligands, early phase clinical trials of both anti-PD-1 and anti-PD-L1 antibodies have so far proved disappointing in unselected patients with mCRC (321,322). These results have been echoed in trials of anti-CTLA-4 antibody in mCRC patients (323). However, there is emerging evidence that checkpoint antibody therapy may have efficacy in patients with MSI tumours, including the mCRC cohort (324). Although tumour cell PD-L1 positivity has been shown to predict response to anti-PD-1 therapy, lower levels of expression in unselected patients may explain why these therapies are not effective in all cases of CRC (325). However, as tumours with dMMR/MSI express higher levels of PD-L1, this may be exploited in the use of immunotherapy in these CRCs. Given that the immune milieu may have already primed an anti-tumour immune response in these cancers, it seems rational that checkpoint inhibitors may further enhance this effect.

4.1.3 Management of Localised Disease

The primary modality for treatment of localised disease remains surgical resection. For patients with borderline resectable disease (high TNM tumour status, close resection margins), the National Institute for Health and Care Excellence (NICE) guidelines recommend consideration of neo-adjuvant chemotherapy (306). Evidence regarding this is conflicting: some published data supports the use of the anti-EGFR-antibody cetuximab with 5-FU and irinotecan chemotherapy (FOLFIRI) in patients with KRAS wild-type (WT) tumours (326). Although OS was not increased in this randomised study, response rates (RRs) were increased in the group of KRAS-WT patients who received cetuximab. In contrast, more recent data has

suggested that the addition of cetuximab to doublet chemotherapy (either FOLFIRI or 5-FU/capecitabine with oxaliplatin) in the neoadjuvant setting decreases PFS (14.1 months in cetuximab group *vs*. 20.5 months in chemotherapy alone group) (327).

Following surgery, patients with Dukes C disease or Dukes B with high risk features such as vascular invasion or presentation with perforated tumour, are considered for adjuvant chemotherapy (306). In patients with resected stage II disease, or those who are over 70 years of age, the addition of oxaliplatin to 5-FU (or the oral pro-drug capecitabine) offers no increased benefit in terms of DFS or OS (328–330). In patients with LN-positive disease, the addition of oxaliplatin to 5-FU in the adjuvant setting prolongs DFS (331–334).

4.1.4 Management of Advanced Disease

4.1.4.1 Chemotherapy

The management of mCRC is based around combinations of the cytotoxic agents oxaliplatin, 5-FU and irinotecan (335). Doublet 5-FU-based treatment has been shown to be superior to single-agent chemotherapy (336,337) but initial choice of regime (irinotecan/5-FU, FOLFIRI or oxaliplatin/5-FU, FOLFOX) may depend on a number of factors including potential toxicities, patient co-morbidities and end-organ function. There is also evidence that patients receiving FOLFOX with disease control and who have a normal platelet count (higher platelet counts being associated with poorer prognosis (338)) can receive intermittent chemotherapy with a treatment break at 12 weeks (339). In a trial of nearly 800 patients, first-line chemotherapy with FOLFOX was shown to be superior to FOLFIRI in terms of time to progression (TTP), RR and median OS (340). Conversely, an (albeit smaller) phase III trial of patients randomised to FOLFOX followed on progression by FOLFIRI and vice versa, reported similar PFS in both treatment arms (341). In the group receiving first-line FOLFIRI, median PFS was 21.5 months *vs.* 20.6 months in the FOLFOX group. Similarly on second-line therapy, those receiving FOLFOX had a median PFS of 14.2 months compared to 10.9 months for those patients receiving FOLFIRI (341). Although this may suggest a preference for FOLFIRI upfront, neither difference in PFS was significant and current NICE guidelines suggest that either regime may be used first-line (306). Additional evidence from clinical trials following progression on first-line chemotherapy supports the use of doublet therapy over single agent 5-FU in patients with a satisfactory performance status and adequate organ function (342). In patients who had progressed on 5-FU-based combination therapy, single agent irinotecan increased OS

compared to best supportive care but, surprisingly, was also shown to improve quality of life and most disease-related symptoms (343).

4.1.4.2 Biological Therapy

The anti-EGFR-antibodies cetuximab or panitumumab can be used in *KRAS*-WT patients in the metastatic setting in combination with either FOLFOX or FOLFIRI (306). The addition of cetuximab significantly increases RR in patients with *KRAS*-WT tumours and reduces risk of disease progression (HR 0.57; p=0.00163) (344). Similarly, combination therapy with cetuximab plus FOLFIRI increases PFS, RR and OS (median OS 23.5 *vs.* 20.0 months, HR 0.796, p=0.0093) compared to FOLFIRI alone in patients without *KRAS* mutations as first line therapy for mCRC (345). Similar results have been demonstrated in second-line therapy: patients treated with cetuximab and irinotecan following progression on oxaliplatin and 5-FU demonstrated an increased PFS and RR compared to irinotecan alone (median PFS 4.0 *vs.* 2.6 months; HR 0.692) (346). The addition of panitumumab to irinotecan as second-line therapy has also been shown to improve PFS but without an increase in OS (347). Interestingly, there is also some evidence that adding cetuximab to irinotecan can re-sensitise patients who have previously progressed on irinotecan-based treatment regimes, with greater TTP and RR observed. Despite this, there was only a modest gain in median survival of just over 1.5 months (348).

Although there is some evidence for the addition of the anti-VEGF anti-angiogenic antibody bevacizumab in the management of mCRC, results from clinical trials have been relatively modest and there is currently no predictive biomarker to select patients likely to respond to anti-VEGF therapy. The addition of bevacizumab to first-line FOLFOX has been shown to increase both PFS and OS by 1.4 months (349). Combining bevacizumab with FOLFIRI demonstrated slightly more promising results, with a 4.4 month improvement in PFS and an increase in survival of 4.4 months (350). Similarly, the use of bevacizumab second-line following progression on FOLFIRI was shown to extend survival by just over two months (12.9 months for patients receiving FOLFOX plus bevacizumab vs. 10.8 months for FOLFOX alone) (351). There is also some evidence that older patients who may not tolerate further triplet chemotherapy can benefit from bevacizumab plus capecitabine (352).

Direct comparisons between these biological therapies have generally favoured the addition of anti-EGFR-antibodies, with combinations of FOLFIRI and cetuximab and FOLFOX plus panitumumab conferring an increase in OS compared to the same chemotherapy backbone

with bevacizumab (353,354). This is likely due to the improved outcomes in *KRAS*-WT patients who were selected in these clinical trials to receive anti-EGFR therapy. The combination of both cetuximab and bevacizumab to oxaliplatin and capecitabine (CAPOX) chemotherapy leads to shortened PFS compared to CAPOX and bevacizumab alone (355). Bevacizumab containing chemotherapy is not currently recommended by NICE (306).

4.1.4.3 Targeted Therapy

The use of targeted, oral small molecules in mCRC is still being investigated. Although initially promising, clinical trials of the oral anti-VEGF TKI cediranib did not meet the criteria for non-inferiority compared to bevacizumab when combined with FOLFOX chemotherapy (356). Regorafenib is another TKI with multiple targets including VEGF. Monotherapy with regorafenib has been shown to increase OS by 1.4 months when compared to placebo in a pre-treated population of patients with mCRC (357). Despite the high incidence of adverse events (AEs) in patients receiving regorafenib (93%), 61 % of patients receiving placebo also described AEs, a proportion of which may have been attributable to disease burden in this patient population.

4.1.4.4 Immunotherapy

Although there is evidence regarding the role of both inflammation and immune responses in the pathogenesis of CRC, responses of unselected patients in this group have been disappointing. Conversely, the use of checkpoint inhibitors in patients with dMMR colonic tumours have shown more promise, with an objective RR of 40 % compared to 0 % in patients with pMMR tumours (324). The median OS in the pMMR group was five months but was not met in the dMMR group. Although currently not used in routine practice, clinical trials to confirm the utility of checkpoint inhibitors in dMMR mCRC are ongoing (NCT02060188, NCT02563002). Early results are promising (358,359) and evaluation into the use of anti-PD-1 antibodies in CRC with dMMR by NICE is also underway. The activity of anti-PD-1 inhibitors in this subset of CRC is likely attributable to a number of factors including the increased infiltration of TILs into the TME of these tumours (360,361). The expression of inhibitory molecules such as PD-L1 have also been shown be higher in tumours with dMMR in some studies (360,361), although other analyses of CRC tumours have found an inverse relationship between the dMMR and PD-L1 expression (362).

Although there have been many advances in the treatment of mCRC, current treatment strategies still incorporate traditional cytotoxic chemotherapy with either FOLFIRI or FOLFOX, sequentially first- and second-line, potentially with the addition of anti-EGFR therapy in patients with *KRAS*-WT tumours. Other therapies, including targeted agents and immunotherapy are currently not used in routine clinical practice, meaning that the age-standardised one year survival for stage IV CRC is still only 44 % for men and 35 % for women (302). The success of drugs such as cetuximab and panitumumab being incorporated into treatment paradigms is likely due to the availability of a biomarker (*KRAS* status) to select patients who can clearly benefit from therapy. The current project aims to further evaluate the effect of standard of care chemotherapy on the immune response in CRC, in the hope that this will reveal the potential for further exploration into novel immunotherapeutic strategies.

4.1.5 Immune Response in CRC

4.1.5.1 Inflammation in CRC

There is a strong association between colonic inflammation and the development of CRC. Indeed, IL-10 knockout mice, who develop intestinal inflammation and colitis, have a higher mutational burden within the colon and are therefore predisposed to the development of colonic tumours (363). The role of inflammation in the pathogenesis of CRC is illustrated by the results of multiple clinical studies of anti-inflammatory drugs such as aspirin. Data from 5 randomised trials with a prolonged follow-up of 20 years demonstrated that prophylactic aspirin leads to a reduced risk of developing CRC (HR 0.76, (95 % CI 0.6-0.96, p=0.02). There was also a confirmed decrease in CRC mortality (HR 0·65, 95 % CI 0·48-0·88, p=0·005) (364). Cyclooxygenase-2 (COX-2) is an enzyme that is involved in the production of prostaglandins as part of the inflammatory process and it is through the inhibition of COX-2 that aspirin may exert it's anti-inflammatory effect. COX-2 is upregulated in adenocarcinomas of the colon and has also been linked to tumour angiogenesis (365).

Understanding the immune landscape in CRC, the immunogenic features of inflammation and the potential prognostic markers in CRC, including the NLR, will help us to understand how the modulation of immune function in response to chemotherapy could potentially be exploited in the development of novel therapies.

4.1.5.2 Immune Infiltrates in the Tumour

The balance between pro-immune and immunosuppressive infiltrates within the TME can have a striking effect on prognosis in CRC. Histopathological staining and co-localisation of immune cells can provide information regarding outcome and survival in CRCs (42). Specifically, primary tumours from CRC patients without disease recurrence are shown to have greater densities of CD3/CD8+ T cells compared to those who subsequently relapsed. Granzyme B positivity is also associated with increased survival (42,366). Similarly, immune infiltration of CRC tumours (367), increased CTL infiltration and infiltration of CD8+ and memory T cells (368) are associated with improvements in DFS and OS. This raises the possibility that immune infiltration in the tumour could have potential as a prognostic biomarker, perhaps in patients who have early-stage disease being considered for adjuvant chemotherapy. Although a T_H1 gene expression profile in CRC tumours is predictive of improved outcome, no such relationship is seen with a T_H2 gene profile, which could be postulated to confer a worse outlook (33).

Immunoscore® is a system for analysing and categorising immune infiltrates in the core (CT) and invasive margin (IM) of tumours, through immunohistochemistry of formalin-fixed, paraffin-embedded (FFPE) tumour blocks. The density and distribution of CD3+ and CD8+ cells within the TME are then analysed and an Immunoscore® generated of between zero and four (with zero being the lowest level of immune cell infiltration and cell density and four the highest) (369). MSI tumours, which by definition have a higher mutational load and potentially higher numbers of tumour neo-antigens to generate an immune response, generally have a higher Immunoscore®, increased infiltration of T_H1 cells and, pertinently, higher levels of cells expressing of PD-1 and PD-L1 (370,371). In keeping with this, MSI tumours also have increased expression of immune genes such as those involved in antigen presentation, cytokine signalling and IFN-γ pathways, amongst others. Although patients with MSI CRC have an improved prognosis, there is data to suggest that this may be a reflection of the immune response in these patients: MSI tumours generally have a higher Immunoscore® but an improved disease-specific survival was also seen in patients with a score of four compared to zero, which included those with MSS tumours (370). Increased PD-1/PD-L1 expression was also seen in MSS tumours with a higher Immunoscore®.

4.1.5.3 Innate Immunity in CRC

The presence of intra-tumoural NK cells is associated with an improved prognosis in patients with CRC (372–374). In further support of this, NK cell infiltration into CRC tumours has been shown to decrease with advancing stage of disease and the presence of metastases (375). Patients with CRC also appear to have diminished NK cell function compared to healthy controls (376). NKG2D is a stimulatory NK cell receptor whose ligands may be upregulated in response to cell stress or malignant transformation (377,378). These cells can then be marked for destruction through NK cell-mediated mechanisms. As such, mice who are deficient in NKG2D are more susceptible to the development of aggressive tumours, suggesting a strong role for NK cell receptors in cancer surveillance (377). There is also evidence, however, that this and other NK cell-activating receptors can be down-regulated in CRC, potentially blunting the process of immune surveillance (379). In addition, persistent exposure to over-expressed NK cell ligands can actually lead to a desensitisation of NK cells to this stimulus, which can further disrupt the normal mechanisms of NK cell-mediated tumour cell cytotoxicity (380). NK cells from patients with CRC have been found to under-express activating receptors (including NKG2D, NKp46 and NKp30) and over-express inhibitory receptors such as NKG2A (381). Indeed, the presence of NKp46 has been shown to correlate with increased RFS (381). Interestingly, it would appear that the inhibition of NK cell-mediated anti-tumour activity can be reversed with a combination of IL-2 and cetuximab, which is used clinically in *KRAS*-WT CRC and can initiate ADCC as an additional anti-tumour response (381).

4.1.5.4 Adaptive Immunity in CRC

Pre-treatment immune function has been shown to be prognostic in CRC, with high levels of MDSCs and low levels of CD4+ or CD8+ T cells being associated with poorer outcomes and decreased PFS (382). Patients with CRC also have a higher frequency of peripheral Tregs than either matched healthy controls or patients with non-malignant bowel disease (56). Depletion of this population of immunosuppressive cells led to enhancement of existing T cell responses (as measured by IFN-γ ELISpot) and also unmasked antigen responses in patients who had previously had none (56). This suggests that peripheral Tregs in CRC may suppress anti-tumour responses.

FoxP3+ Tregs are higher in malignant colorectal tumours compared to matched PBMCs from the same patient group or healthy controls (383). In this study, evaluation of PBMCs following

excision of a primary tumour revealed a reduction in the expression of FoxP3 on peripheral Tregs, which corresponded to an increase in IFN-γ production to the TAA 5T4 (383). Suppression of these antigen-specific T cell responses by Tregs have been linked to tumour recurrence, with all patients in this data set with a 'suppressive' phenotype having detectable recurrent disease 12 months after surgery (although in a very small group of patients; n=4 for 5T4 responses and n=7 for CEA responses) (383). Conversely, in a similar (larger) group of pre-operative patients, baseline IFN-γ response to CEA (either before or after the depletion of Tregs) correlated with an increased risk of tumour recurrence (384).

4.1.5.5 Cytokine Production

In a group of 148 patients with CRC, serum cytokine profiling showed differences between the patients and matched healthy controls (385). T_H1 cytokines such as IFN-γ were higher in CRC patients and there was demonstrable variation in cytokine profile across varying clinicopathological features. Patients with metastatic disease (stage IV) had higher serum levels of the anti-inflammatory IL-1 receptor agonist (IL-Ra), the T_H2 cytokine IL-4, pro-inflammatory IL-6 and the chemokine IL-8. There was also a suggestion of a more T_H2 environment in patients with metastatic tumours.

Similarly, in another cohort of patients with CRC, analysis of plasma cytokines and chemokines showed an increase in IL-6, IL-8, HGF, M-CSF and VEGF (386). Moreover, specific patterns of cytokine release (associated with inflammation and angiogenesis) were found in patients with a high NLR (defined in this paper as > 5). These data suggest that the cytokine/chemokine profile of CRC patients can vary between those at different stages of disease and with different inflammatory milieu, potentially reflecting the pathophysiology of the disease.

4.1.6 The Neutrophil Lymphocyte Ratio

As already illustrated, the relationship between inflammation, immune response and malignancy is complex. Measurement of the NLR is both simple and reproducible and as such it has the potential to be used as a pragmatic surrogate marker for inflammation and immune response in cancer. There is evidence that poorer cancer survival can be associated with either low lymphocyte counts or high neutrophil counts in the peripheral blood of unselected cancer patients (387). The NLR has the potential to become an easily measureable biomarker, as it includes parameters which are routinely recorded for patients undergoing chemotherapy.

For the purposes of this work, NLR was calculated using the formula:

$$NLR = \frac{Peripheral\ Blood\ Neutrophil\ Count}{Peripheral\ Blood\ Lymphocyte\ Count}$$

If patients only have total WBC and neutrophil count recorded, the derived NLR (dNLR) can also be used. The dNLR works on the assumption that the total WBC count is comprised predominantly of neutrophils and lymphocytes. Hence, dNLR is calculated using the formula:

$$dNLR = \frac{Peripheral\ Blood\ Neutrophil\ Count}{Peripheral\ WBC\ Count\ -\ Peripheral\ Blood\ Neutrophil\ Count}$$

There is some recognition that NLR may be more accurate than dNLR; decreasing lymphocyte populations in cancer patients may mean that other cells populations, which are not neutrophils, can make up the proportion of WBC (388–390).

Both NLR and dNLR have been found to have prognostic value for patients with a diagnosis of cancer, including CRC patients undergoing surgical resection (391) and those with advanced CRC, with NLR < 4 and dNLR < 2 being associated with improved OS (388). While prognostic biomarkers give information about the course and outcome of a disease independent of treatment, predictive biomarkers can be used to stratify patients into groups more likely to respond to treatment, such as *KRAS* status and anti-EGFR therapy in CRC.

Although the NLR has been shown to be a robust prognostic biomarker in a number of different tumour types, it remains unclear whether a high NLR (usually conferring a poorer outcome) is due to neutrophilia, lymphopenia, or a combination of the two. As a biomarker it is pragmatic, easily measurable and has been validated in many large data sets (392–397) but without actually understanding what it means and what it may represent. Although it may seem a blunt instrument with which to dissect the complex relationship between cancer, chemotherapy and immunity, this project aims to clarify whether the NLR could potentially be a surrogate marker for immune responses and how this is modulated in the context of anti-cancer therapy.

4.1.6.1 NLR and Prognosis in CRC

Many scoring systems have been developed to stratify patients into high or low risk prognostic groups depending on parameters such as CRP, lactate dehydrogenase (LDH), serum albumin, platelet counts and peripheral WBC count (398–401). The NLR has been shown to be

an independent prognostic factor in many different malignancies but the exact level at which it becomes significant can vary. A meta-analysis of over 40,000 patients showed that an NLR > 4 was a prognostic factor in a variety of solid tumours, although this varied between the groups (HR for OS was 2.35 in mesothelioma compared to 1.43 in HCC and 1.91 for CRC). Different values of NLR have also been demonstrated to be significantly associated with PFS (cut-off NLR > 3) and DFS (NLR > 5) (395). In metastatic disease, high NLR (defined as > 5) corresponded with a poorer OS in patients with untreated mCRC and patients entering phase I trials refractory to standard therapies (386). Another systematic review highlighted the differences in threshold NLR between different studies of unselected cancer patients, ranging from > 3.33 to > 5 (397). In reviewing studies of operable cancer, the most commonly used NLR was > 5. NLR was also able to predict survival and recurrence after neoadjuvant therapy (397).

NLR has been associated with factors such as increased incidence of tumour obstruction or perforation, later stage tumours (T4b) and raised CEA (394,402). High NLR (in this case > 5) is also associated with a more aggressive tumour type, with statistically significant correlations with a higher TNM tumour stage, increased nodal involvement and vascular invasion. This translated to a decrease in PFS and OS (393).

The Continuous or Intermittent trial of chemotherapy in mCRC showed that patients who had a higher platelet count (potentially an indirect marker of inflammation) benefitted from continuous chemotherapy, without a treatment break. Further post-hoc analysis also showed a correlation between dNLR and OS (median survival of 18.6 months in patients with dNLR < 2.2 vs. 12.5 months if dNLR ≥ 2.2) (403). An NLR of greater than 2.2 was also seen to be predicative of a poorer survival in a group of unselected patients with CRC prior to treatment (404).

NLR and platelet-lymphocyte ratio (PLR) also appear to have prognostic significance in patients with resectable CRC with synchronous liver metastases (338). Elevated NLR and PLR were associated with a worse OS and with poorer response to chemotherapy. Interestingly, normalisation of these parameters following the first cycle of chemotherapy was associated with improved disease control (either stable disease, partial response (PR) or complete response (CR)). In early stage CRC, in which surgery alone can be curative, an NLR of greater than 2.6 was similarly associated with a poorer OS and PFS (405). These findings were mirrored in another study which chose a higher level of NLR (> 5) to stratify risk (394). An NLR of > 5 is prognostic in CRC patients undergoing first line palliative chemotherapy (406),

localised CRC and CRC with liver metastases in a systematic review of over 10,000 patients (407).

While there has been a paucity of data comparing NLR in cancer patients to a healthy population, Pedrazzani *et al.* compared NLR from 603 patients with resected CRC to 5270 healthy controls (402). Surprisingly, although these patients did not have metastatic disease, there was a significant difference between the absolute NLR values for those with resected CRC compared to healthy controls (3.1 ± 1.8 *vs.* 1.8 ± 1, respectively; $p<0.001$) (402). Moreover, there was no correlation between age and NLR in either group.

4.1.6.2 Neutrophils in CRC

In patients with previously untreated mCRC, a neutrophil count above the upper limit of normal (ULN) was associated with a poorer OS. There was a trend to a similar effect with decreased lymphocyte count (below lower limit of normal, LLN) but it was not statistically significant (386). This implies that the neutrophil count, rather the lymphocyte count, could be the prognostic element in the calculation of NLR. Indeed, in a large study of pre-operative patients undergoing elective resection for CRC, neutrophil count alone was found to be an independent factor for cancer-specific survival (CSS) (408). Similarly, in HCC, it was an elevated neutrophil count that correlated with increased risk of HCC recurrence, rather than the lymphocyte count (409).

There is evidence that neutrophils may exert an inhibitory effect on the TME. The activity of CTL against tumour targets *in vitro* can be inhibited by the addition of granulocytes (410). Not only is this effect dose-dependent but is independent of normal granulocyte function, with a similar phenomenon seen when the cells were heat-inactivated. Similarly, the removal of granulocytes in an *in vivo* model of ultraviolet (UV)-induced tumours lead to inhibition of tumour growth, indicating that the tumour may be dependent on granulocyte function (411). Interestingly, this was in a sub-group of tumours that had been able to subvert a T cell-mediated anti-tumour immune response.

Neutrophils are known to produce a wide range of cytokines and chemokines which modulate all aspects of the immune response. These may include both regulatory cytokines such as IL-4 and TGF- β and pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 (268). The blockade of TGF-β can also lead to an increase in TANs which are more cytotoxic to tumour cells and exert a more anti-tumour phenotype (so called 'N1' versus the pro-tumourgenic 'N2') (100).

Neutrophil-derived ectosomes (extracellular vesicles created by 'budding off' of the cell membrane) have been shown to have immunosuppressive properties, increasing expression of TFG-β when cultured with macrophages (412). These ectosomes were also able to block the release of TNF-α by macrophages. Although this effect may be utilised successfully in the resolution of inflammatory processes, it may exert a harmful pro-tumourgenic effect in the TME.

4.1.6.3 Lymphocytes in CRC

Pre-operative lymphopenia has been shown to be a poor prognostic factor in terms of OS in patients with resected CRC (413). In the palliative setting, lymphopenia can be associated with decreased OS and PFS in CRC patients receiving first-line chemotherapy (414). Additionally, the risk of developing a low lymphocyte count due to chemotherapy is related to increased age (greater than 60 years) and raised CEA (415). Although this data suggests a strong association between cancer and lymphopenia, the underlying mechanisms remain unclear.

Fas to Fas-L on T and NK cells initiates a pro-apoptotic pathway (416). Despite most tumour cells expressing Fas, they can be resistant to Fas-L-induced cytotoxicity. Conversely, tumour cells may also express CD95L, which has been postulated as a potential immune-evasive mechanism (416). In fact, high levels of Fas-L on colorectal tumour cells have been shown to be associated with increased tumour stage and liver metastases (417,418) and high serum levels of Fas-L with poorer outcome and survival (419). Lymphopenia, therefore, may be related to higher levels of Fas-L-expressing tumour cells, initiating apoptosis of T cells. This hypothesis has been supported in HCC (420) and in CRC (418), where apoptosis of T cells was increased when co-cultured with Fas-L-expressing tumour cells.

Although the mechanisms determining the presence of lymphopenia in cancer patients are multiple, complex and poorly understood, we need to consider whether this is a cause or effect phenomenon: is the lymphocyte count diminished because of the tumour or has the tumour developed because lymphopenia is indicative of a blunted anti-tumour immune response? Analysis of immune responses in patients with changing NLR may help to understand this process further.

4.1.7 Rationale for Current Study

It is impossible to fully describe the network of complex relationships between the development and pathogenesis of CRC and the different immune responses to both disease and therapy as outlined above. However, the differences in immunogenicity and outcome in patients with dMMR/MSI colorectal tumours illustrates that modulation of the immune response in CRC patients may offer new and more effective therapeutic strategies. The challenge, therefore, is to determine whether there are any other CRC patients who may benefit from these novel therapies. The aims for this work were specifically:

1. To determine whether the NLR, which has proven to be a robust prognostic biomarker in CRC, actually reflects modulation of the immune milieu in CRC patients by comparing functional immune responses in patients with a high NLR and low NLR.
2. To determine the effect that standard-of-care chemotherapy had on immune responses and whether this was reflected in changes in the NLR over treatment.
3. Investigate whether chemotherapy could modulate the immune response in these patients and, if so, could this information reveal the potential for immunotherapeutic strategies in patients who previously would not have benefited from such treatment.

4.2 NLR Results

4.2.1 Patient recruitment and demographics

Thirty patients with CRC were consented to the study between November 2014 and November 2016. Samples were initially collected from patients at three time points corresponding to chemotherapy cycles: baseline (pre-treatment), 6 weeks (following 3 cycles of 2-weekly chemotherapy) and 12 weeks (after 6 cycles of chemotherapy). One patient (NLR-14) withdrew prior to receiving any chemotherapy or having any translational blood samples taken and so was excluded from the analysis. Survival data was also collected. Patient recruitment and sample collection is illustrated in Figure 4.1.

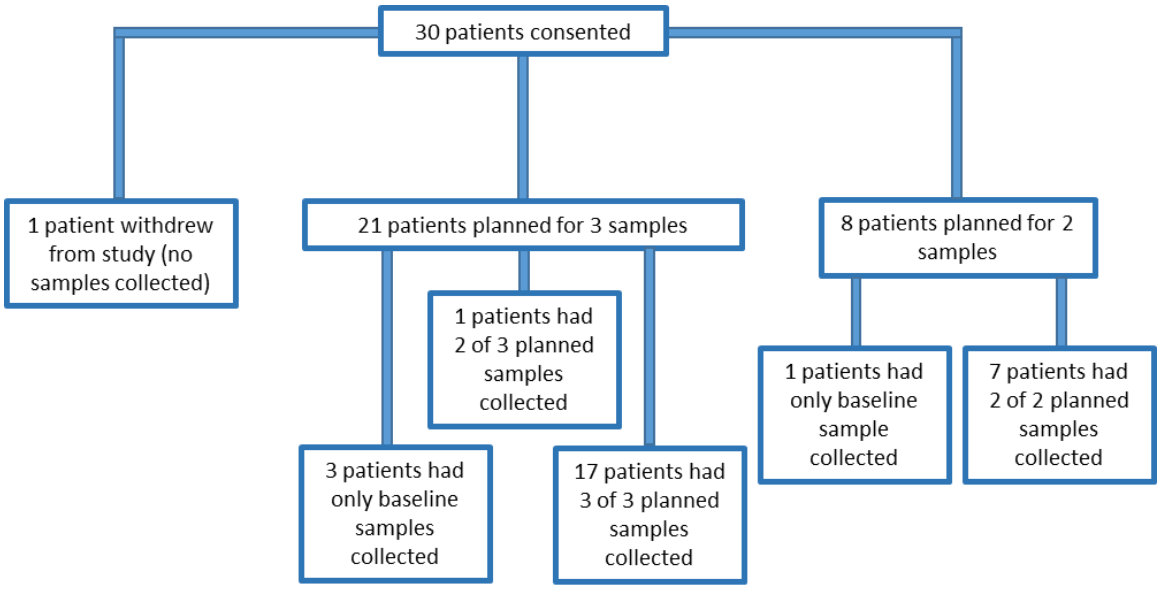


Figure 4.1 Patient recruitment

Following a preliminary analysis of data (after 22 patients had been consented), there was considered to be more interesting findings seen in the first 6 weeks of treatment, as well as potentially too many confounding factors to gain reliable data from samples taken at 12 weeks. These may include dose reductions, dose delays, toxicities from chemotherapy (particularly neutropenic infection) and patient drop-out due to illness or PD. Consequently, from patient NLR-23 only baseline and 6 week samples were collected. For this reason, most of the data presented covers this period, unless there was a strong signal from data at 12

weeks. Interestingly, there was enough data by 6 weeks to suggest changes in the immune response at this time point, which is earlier than the traditional 12 weeks at which patients on chemotherapy would be radiologically re-assessed. Four of twenty-nine patients (14%) only had a single baseline sample collected. From patient 23 onwards, samples were collected only at baseline and 6 weeks (after discontinuation of the 12 week sample due to modifications to the study protocol). Seven out of these eight patients (88%) had both planned samples collected. A further two patients with mCRC who consented to sample collection but who were not planned to receive chemotherapy were included in some of the baseline analysis to increase the proportion of patients in the high NLR group. Sample collection for all patients is shown in Appendix Table 8.3. For the purpose of the analysis, high NLR was defined as ≥ 5 unless otherwise stated. Demographics for the 29 patients who had at least one sample collected are shown in Table 4.2. There was a similar proportion of male and female patients, with a median age of 70.5 years in males and 63 years in females. All patients received 5-FU or capecitabine as part of their chemotherapy regime; 93% had combination chemotherapy and only 2 of the 29 evaluable patients received an additional biological therapy. Most patients received a combination of irinotecan/5-FU, reflecting local practice. As expected, most tumours arose in the distal colon, sigmoid or rectum. Most patients (66 %) had liver metastases at baseline and 17 % of all patients (28 % of those in whom mutational status was known) had mutations in *KRAS*. The proportion of patients with ≥ 2 metastatic sites was greater in the high NLR group (83 % *vs.* 47 %), as was the proportion of those with liver metastases (75% of those with a high baseline NLR vs. 53 % with low baseline NLR).

Table 4.2 Patient Demographics (n=29)

Patient Demographics (n/%)					
Sex		**Median Age and Range**		**ECOG Performance Status at Baseline**	
Male	14 (48 %)	Male	70.5 years (31 – 86 years)	0	10 (34 %)
Female	15 (52 %)	Female	63 years (52 – 76 years)	1-2	19 (66 %)
		Both	65 years (31 – 86 years)	3-4	0
Primary Tumour Site				**First-Line Chemotherapy**	
Right sided		7 (24 %)		Irinotecan/5-FU	19 (66 %)
Transverse colon		1 (3.5 %)		Oxaliplatin/5-FU	6 (21 %)
Left sided		20 (69 %)		Capecitabine only	2 (7 %)
Unknown primary site		1 (3.5 %)		Irinotecan/5-FU + Monoclonal antibody[1]	2 (6 %)
Presence of Liver Metastases		**Number of Metastatic Sites**		**Mutational Status**	
Yes	19 (66 %)	0	0	KRAS	5 (17 %)
No	10 (34 %)	1	10 (34 %)	BRAF	3 (10 %)
		2	13 (45 %)	PI3KCA	1 (3.5 %)
		≥3	6 (21 %)	Failed testing	1 (3.5 %)
				No mutations detected[2]	8 (28 %)
				Not tested	11 (38 %)

[1] One patient received bevacizumab and one received cetuximab in addition to irinotecan/5-FU

[2] Mutational analysis was performed for patients also enrolled onto the FOCUS4 clinical trial and included MSI/dMMR as well as mutations in *KRAS*, *BRAF*, *PI3KCA*, *PTEN* and *NRAS*.

4.2.1.1 Baseline NLR values

The median NLR was 3.63 (range 1.68 - 25.67). Although there were a greater number of patients (59 %) with a low NLR, 41 % of patients had a baseline NLR ≥ 5. The spread of values for the baseline (pre-treatment) NLR are shown in Figure 4.2. Of note, the greatest proportion of patients had an NLR of between 2 and 4 (48 %).

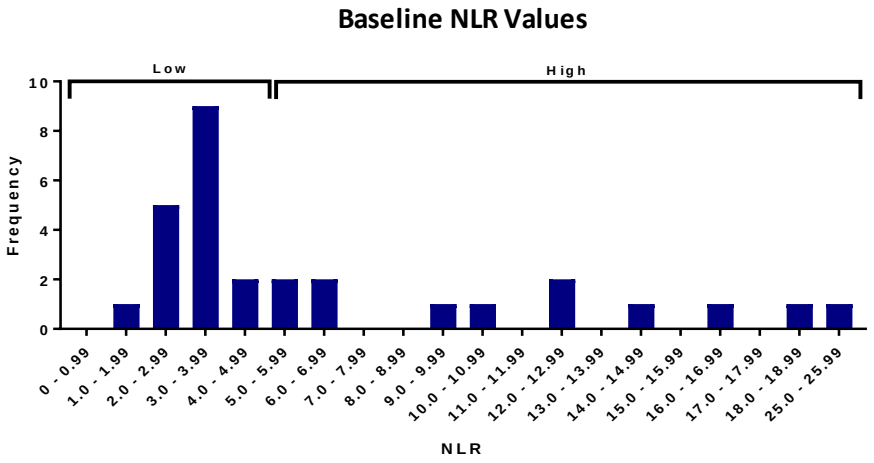


Figure 4.2 Baseline NLR values

Range and frequency of the baseline NLR values of all 29 evaluable patients. A high NLR is defined as ≥ 5 and low NLR < 5.

4.2.1.2 Baseline peripheral blood immunological parameters

The immunological blood parameters of patients were ascertained by routine hospital blood sampling and are summarised in Table 4.3.

Table 4.3 Peripheral blood parameters (n=29)

Peripheral Blood Parameters (n/%)			
Baseline NLR		**Baseline Platelet Count**	
≥5	12 (41 %)	≥ULN	7 (24 %)
<5	17 (59 %)	<ULN	22 (76 %)
Baseline Neutrophil Count		**Baseline Lymphocyte Count**	
≥ULN	11 (38 %)	≤LLN	8 (28 %)
<ULN	18 (62 %)	>LLN	21/29 (72 %)
Serum CEA (n=25)			
<5	6 (24 %)		
≥5	19 (76 %)		

Approximately one third of patients had serum parameters that have been previously demonstrated to be associated with a poorer prognosis (339,389,408), with raised neutrophil count and/or platelet count seen in 38 % and 24 % of patients, respectively and lymphopenia in 28 %. Of note, 76 % of patients had a raised serum CEA, which is slightly higher than has been seen in previous trials of chemotherapy in the metastatic setting (403).

4.2.1.3 Survival outcomes

No patients were lost to follow-up for survival data. Data was censored on 23rd July 2017. Median survival for the whole group was 274 days. Median OS for patients with a high baseline NLR was significantly decreased compared to those with a low baseline NLR (6.6 months *vs*. 18.8 months respectively, p=0.0024, HR 3.6, 95 % CI 1.25 – 10.35, Figure 4.3). Although most patients (10/12) in the high NLR group demonstrated a normalisation in NLR over the first 6 weeks of chemotherapy, this did not change survival outcomes (data not shown).

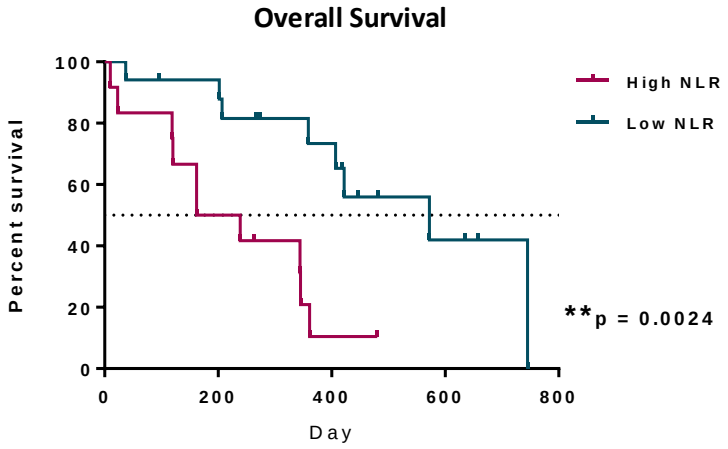


Figure 4.3 OS curves for patients stratified by baseline NLR

Patients were grouped according to baseline NLR (high ≥ 5, low < 5, n=29). Survival was calculated in days from the first dose of chemotherapy. All patients were evaluable in the survival analysis.

4.2.2 Evaluation of Patient Immune Response to Chemotherapy Treatment and the Association with NLR: Immunophenotyping

4.2.2.1 Patient immune system activation is not altered during treatment, irrespective of NLR

The activation potential of patient immune systems were assessed by analysis of cell surface markers by flow cytometry. There was no significant difference in baseline levels of activation markers in either NLR group (low *vs*. high), including CD69 (Figure 4.4), HLA-DR, CCR7 and PD-1/PD-L1 on CD4+ and CD8+ T cells, NK/NKT cells, monocytes or B cells (data not shown, Appendix Table 8.4).

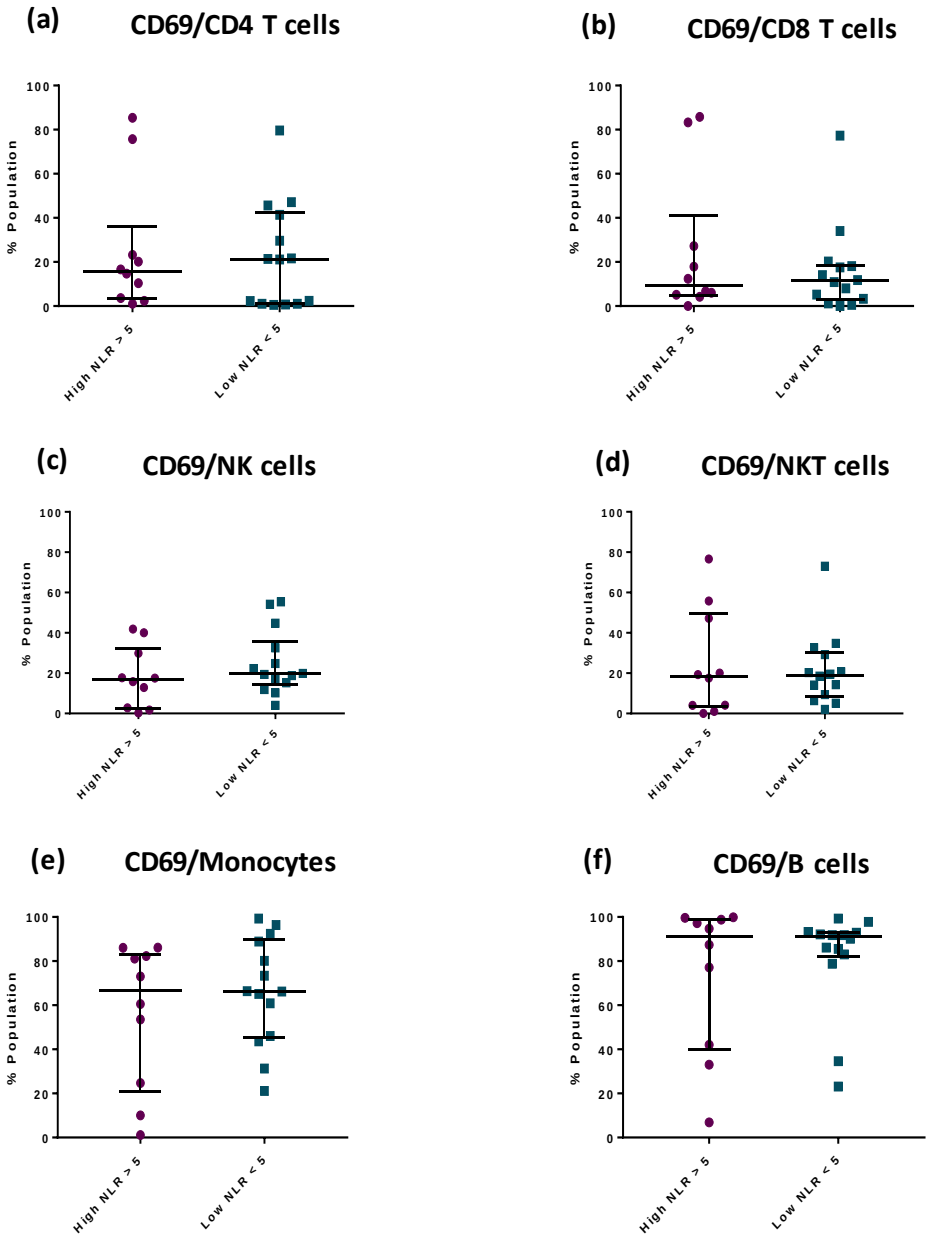


Figure 4.4 Baseline CD69 expression on immune cell subsets (in high *vs.* low NLR patients).
Cryopreserved PBMCs were immunophenotyped and CD69 expression on immune cell sub-populations determined by flow cytometry. The percentage CD69 expression is shown on CD4+ T cells **(a)** CD8+ T cells **(b)**, NK cells **(c)**, NKT cells **(d)**, monocytes **(e)** and B cells **(f)** (all n=24).

Similarly, when looking at the patient cohort as a whole, there was no observed change in expression of any of the aforementioned markers on any of the specific cell populations after 6 weeks of treatment, including CD69 (Figure 4.5 and Appendix Table 8.5). This is true of both NLR groups i.e. NLR ≥ 5 at baseline which had fallen to < 5 by 6 weeks ('high-low') and those with a baseline NLR < 5, which remained so over the first 6 weeks of chemotherapy ('low-low')

(data not shown). This indicates that at a cellular level, patient immune responses, determined by Immunophenotyping, seemingly remain unchanged.

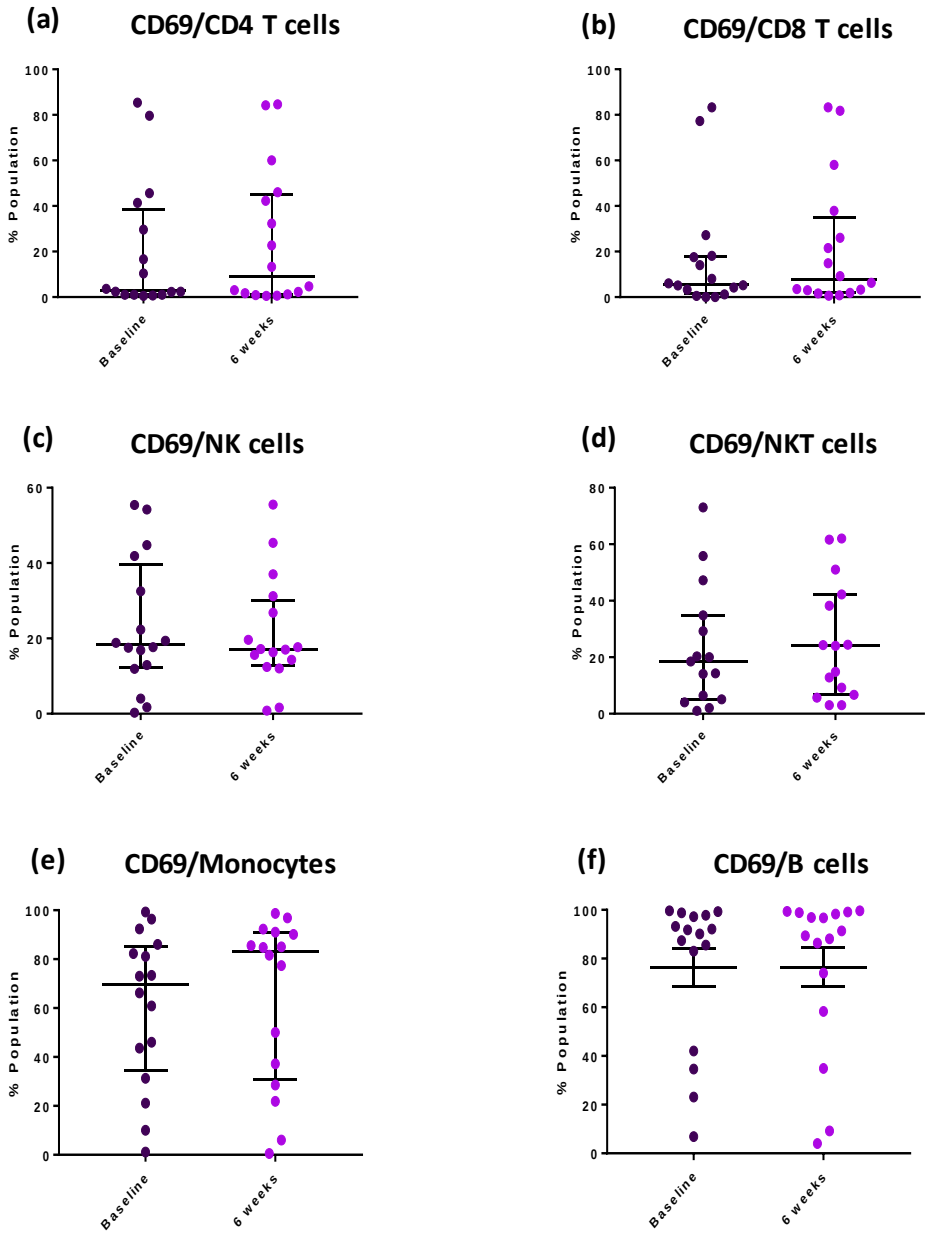


Figure 4.5 CD69 expression on immune cell sub-populations over the first 6 weeks of chemotherapy (whole patient cohort)

Cryopreserved PBMCs were immunophenotyped and CD69 expression on immune cell sub-populations determined by flow cytometry. The percentage expression of CD69 after the first 6 weeks of chemotherapy is shown on CD4+ T cells **(a),** CD8+ T cells **(b)**, NK cells **(c)**, NKT cells **(d)**, monocytes **(e)** and B cells **(f)** (n=15 for NKT cells, all other graphs show n=16).

4.2.2.2 Immune checkpoint molecules increase in response to chemotherapy on select immune cell populations.

PBMCs were evaluated for the immune checkpoint markers PD-1 and PD-L1. There was no significant difference in the expression of PD-1 between the high and low NLR groups on any of the immune cell subsets evaluated at baseline (Figure 4.6). Similarly, PD-L1 expression did not vary (data not shown).

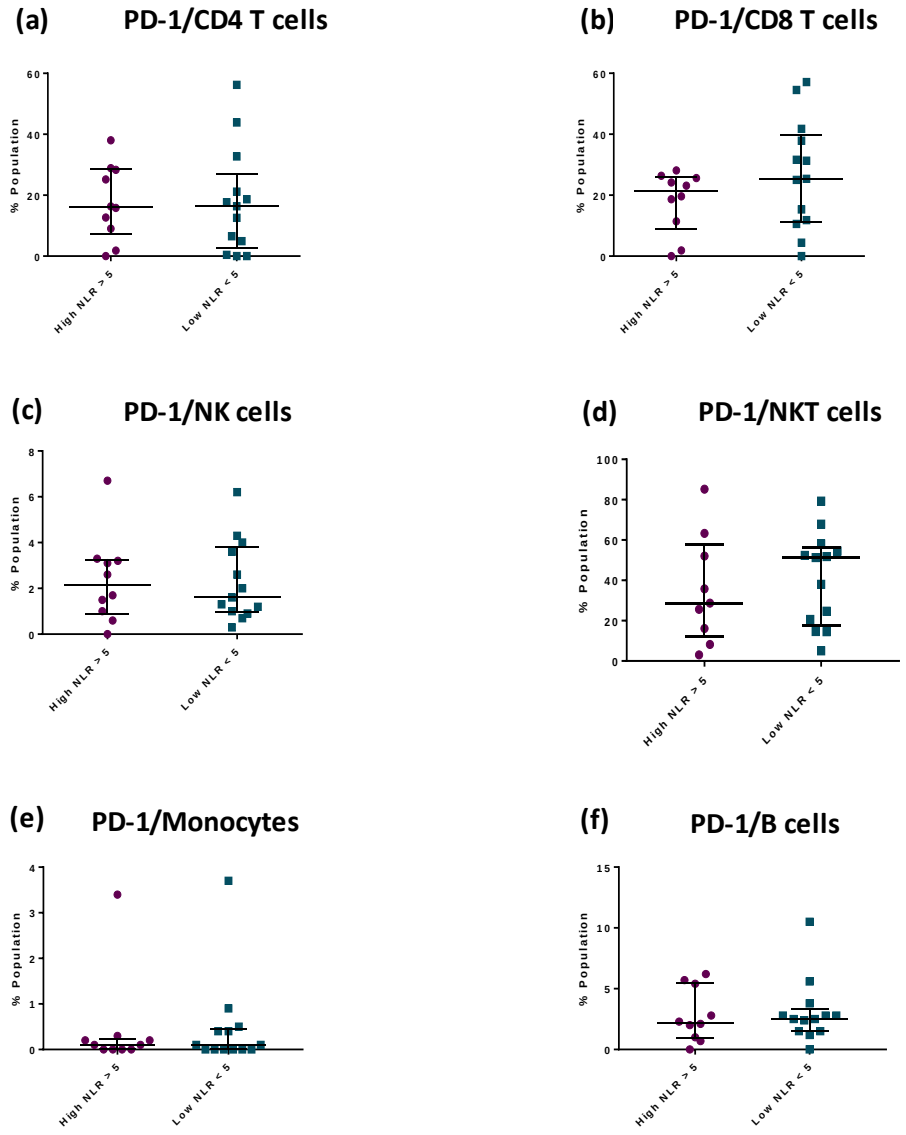


Figure 4.6 PD-1 expression on immune cell sub-populations at baseline (in high *vs.* low NLR patients)

Cryopreserved PBMCs were immunophenotyped and PD-1 expression on immune cell sub-populations was determined by flow cytometry. The percentage expression of PD-1 is shown on CD4+ T cells **(a)**, CD8+ T cells **(b)**, NK cells **(c)**, NKT cells **(d)**, monocytes **(e)** and B cells **(f)** (n=24).

Furthermore, when looking at the whole patient cohort, there was no significant change in the expression of PD-1 on CD4+ or CD8+ T cells or NKT cells after the first 6 weeks of chemotherapy (data not shown, Appendix Table 8.5). However, PD-1 expression increased on NK cells after the first 6 weeks of chemotherapy (p=0.0143, Figure 4.7a). Upon stratification by NLR, this elevation in NK PD-1 expression seemed to be attributable to the patients who had a low NLR at baseline (p=0.0488, Figure 4.7b, c).

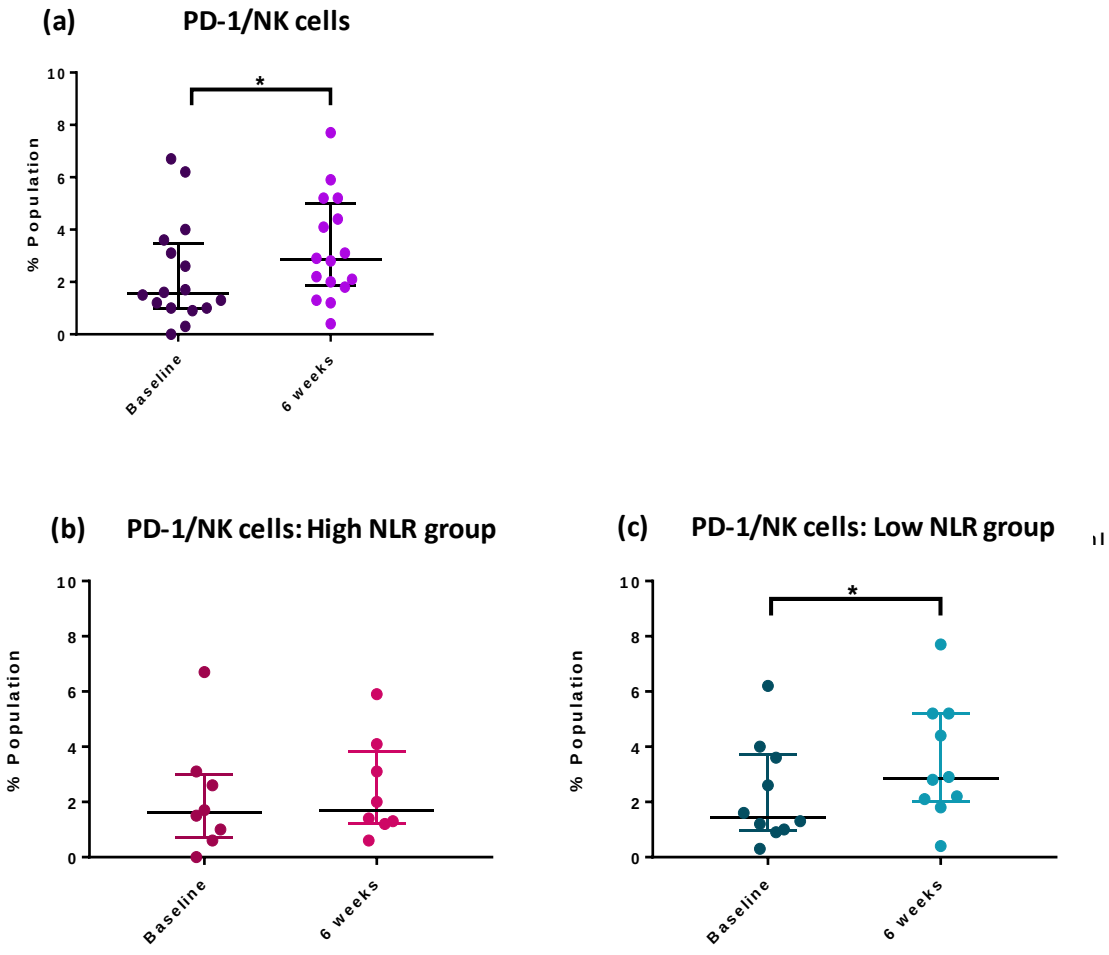


Figure 4.7 PD-1 expression on NK cells over time

Cryopreserved PBMCs were immunophenotyped and PD-1 expression on NK cells was determined by flow cytometry. The percentage of positive cells is shown for **(a)** PD-1 expression on NK cells after the first 6 weeks of chemotherapy (whole patient cohort), **(b)** PD-1 expression on NK cells of patients with high baseline NLR and **(c)** low baseline NLR (n=16).

PD-1 expression significantly increased after the first 6 weeks of chemotherapy on both B cells and monocytes (p=0.0417 and p=0.0156, respectively, Figure 4.8), although there was no appreciable difference between the high and low NLR groups within these cell types (data not shown).

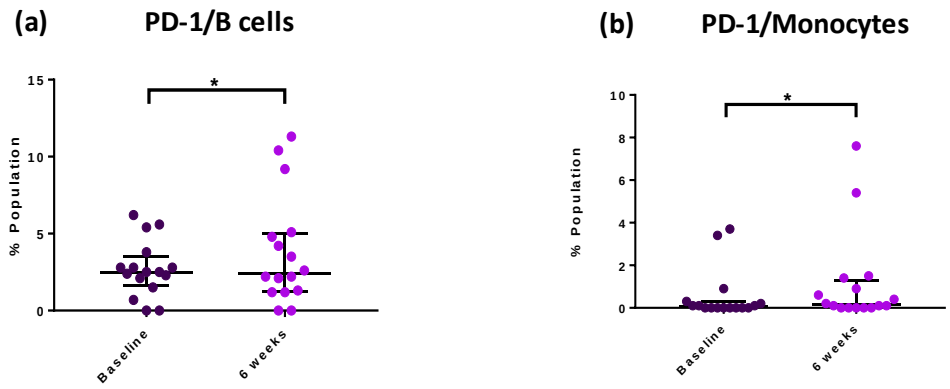


Figure 4.8 PD-1 expression on B cells and monocytes over the first 6 weeks of chemotherapy (whole patient cohort)

Cryopreserved PBMCs were immunophenotyped and PD-1 expression was determined by flow cytometry. The percentage of positive cells is shown on **(a)** B cells and **(b)** monocytes at baseline and following 6 weeks of chemotherapy (n=16).

4.2.2.3 Relationship between NLR status and frequency of peripheral Tregs: effect of chemotherapy

Patients with a high NLR at baseline had significantly fewer peripheral FoxP3+ Tregs than those with a low NLR (p=0.0288, Figure 4.9a). High NLR patients also had significantly reduced expression of CD62L on Tregs (p=0.0165, Figure 4.9b). CD62L (L-selectin) is a homing receptor, which, when expressed on the surface of Tregs initiates homing to LNs.

There was no difference noted in expression of either PD-1 or PD-L1 on Tregs between the high and low NLR groups at baseline (Figure 4.9c, d). Similarly, there was no appreciable change, following the first 6 weeks of chemotherapy, in either PD-1 or PD-L1 expression on Tregs, either in the patient cohort as a whole or when stratified by baseline NLR (data not shown).

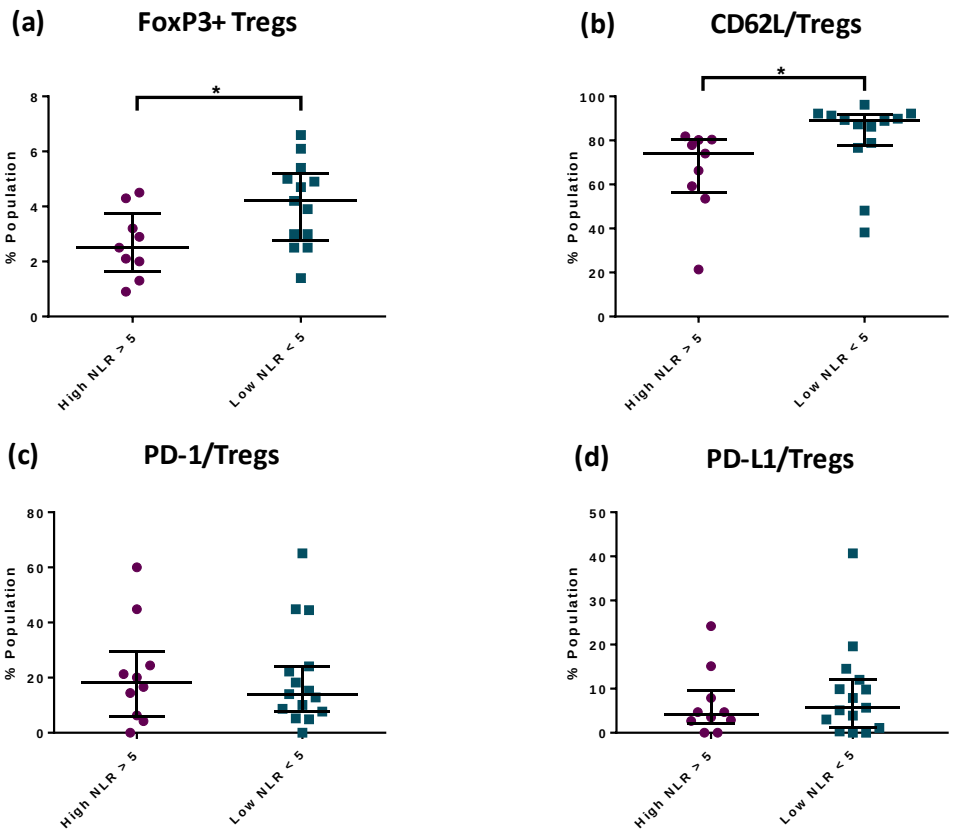


Figure 4.9 Characteristics of FoxP3+ Tregs in patients with high or low baseline NLR

Cryopreserved PBMCs were immunophenotyped and percentage positive cells determined by flow cytometry. Total percentage FoxP3+ Tregs as a proportion of CD4+ T cells are shown **(a)** as well as CD62L expression **(b)** (n=22). Also displayed is cell surface expression of PD-1 **(c)** and PD-L1 **(d)** (n=24).

When looking at the patient cohort as a whole, there was a trend to a reduction in FoxP3+ Tregs following the first 12 weeks of chemotherapy, which approached statistical significance between baseline and 6 weeks (p=0.0597) and baseline and 12 weeks (p=0.0625, Figure 4.10a). In addition, Tregs decreased over time in both the high and low baseline NLR groups, although due to lower patient numbers within each group this was not significant (Figure 4.10b, c, d). The reduction in Tregs was independent of the CD4+ population, as shown by the consistent proportion of CD4+ cells over these 12 weeks alongside a decrease in the Treg/CD4 ratio (Figure 4.10e, f)

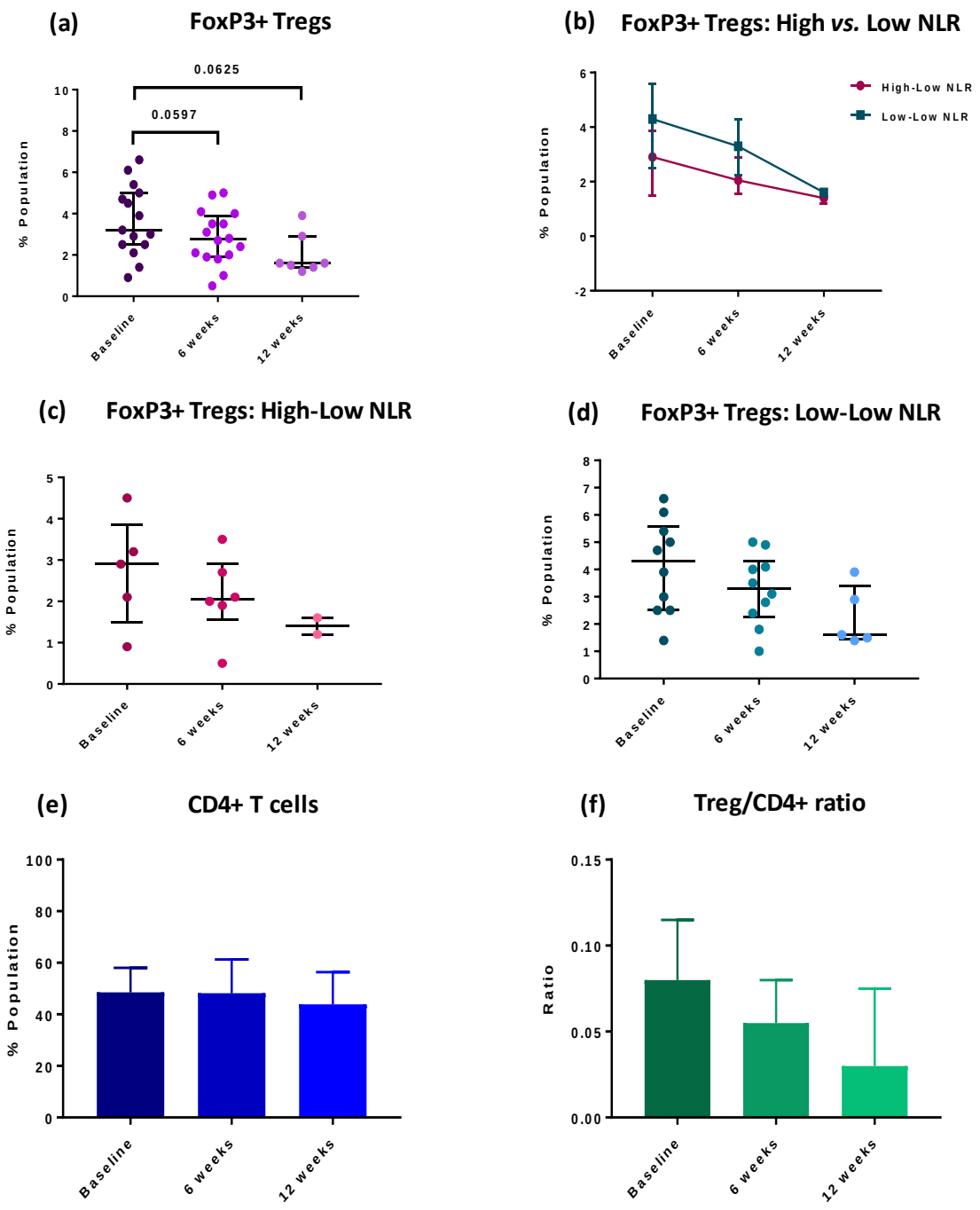


Figure 4.10 Changes in the Treg population over the course of chemotherapy

Cryopreserved cells were immunophenotyped and percentage positive cells in the CD4+ T cell population determined by flow cytometry at baseline, 6 weeks and 12 weeks. This is shown for all patients **(a)** (n=16) and stratified into high or low NLR (**(b)**, both groups, n=16). This data is shown for individual patients in **(c)** (n=6) and **(d)** (n=10). The total CD4+ population for all patients is shown **(e)** as is the Treg/CD4+ ratio **(f)**.

4.2.2.3.1 Tregs and survival

When analysing data from all NLR patients, there was a significantly prolonged OS in those who had a higher frequency of peripheral FoxP3+ Tregs than the median (median OS not reached *vs.* 6.6 months, p=0.0054, HR 6.424, 95 % CI 1.796 to 22.98, Figure 4.11).

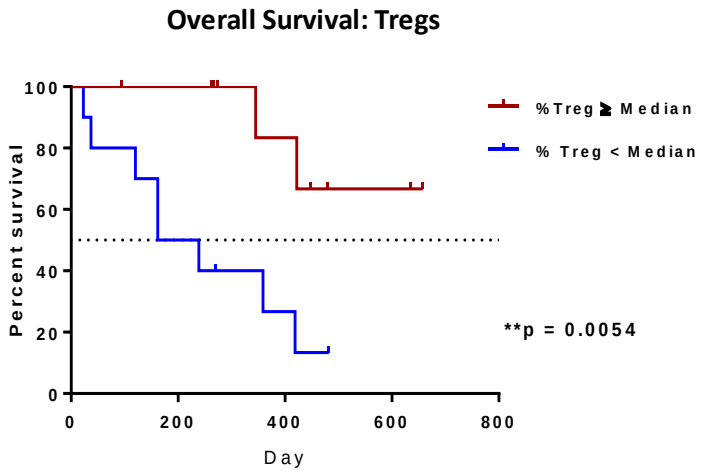


Figure 4.11 Overall survival for all NLR patients stratified by frequency of peripheral blood Tregs

Patients were divided into those with a higher or lower than median frequency of Tregs in the CD4+ T cell population. Survival was calculated as days from receiving the first dose of chemotherapy. All patients with both measurable baseline Tregs and survival data were evaluable in the analysis (n=20).

4.2.3 Evaluation of Patient Immune Responses to Chemotherapy Treatment and Association with NLR: Cytokine/Chemokine Profile

4.2.3.1 NLR is associated with a distinct cytokine/chemokine profile at baseline

Patient plasma was evaluated for a total of 49 cytokines and chemokines by Luminex or ELISA: of these, those which were below the lower detection limits of the assay or where the majority of samples tested fell below the lower limit of the standard curve are shown in Appendix Table 8.6.

As Figure 4.12 and Table 4.4 demonstrate, of the chemokines and cytokines analysed, 4 (HGF, IL-8, IL-10 and TGF-β) were significantly higher at baseline in the patients with a high NLR in

comparison to those with a low NLR (p=0.0287, p=0.0183, p=0.0014 and p=0.0005, respectively). Comparatively, 8 cytokines and chemokines were significantly higher at baseline in those patients with a low NLR: IL-16 (p=0.0033), CTACK (p=0.0009), Eotaxin (p=0.0208), IP-10 (p=0.0141), MIG (p=0.0035), RANTES (p=0.0183), SCF (p=0.0029) and TRAIL (p=0.0006) (Figure 4.13). There was no significant difference in baseline levels for the remaining 19 cytokines and chemokines studied (Table 4.4).

Table 4.4 Baseline levels of chemokines and cytokines on Luminex assay/ELISA stratified by baseline NLR

Baseline Levels			
High NLR > Low NLR	**Low NLR > High NLR**	**No Difference**	
IL-8	IL-16	IFN-α2	Basic FGF
HGF	CTACK	IFN-γ	G-CSF
IL-10	Eotaxin	IL-2Rα	GRO-α
TGF-β	IP-10	IL-3	MCP-3
	MIG	IL-4	MIF
	RANTES	IL-7	MIP-1α
	SCF	IL-9	MIP-1β
	TRAIL	IL-17α	PDGF-BB
		IL-18	SCGF-β
		SDF-1α	

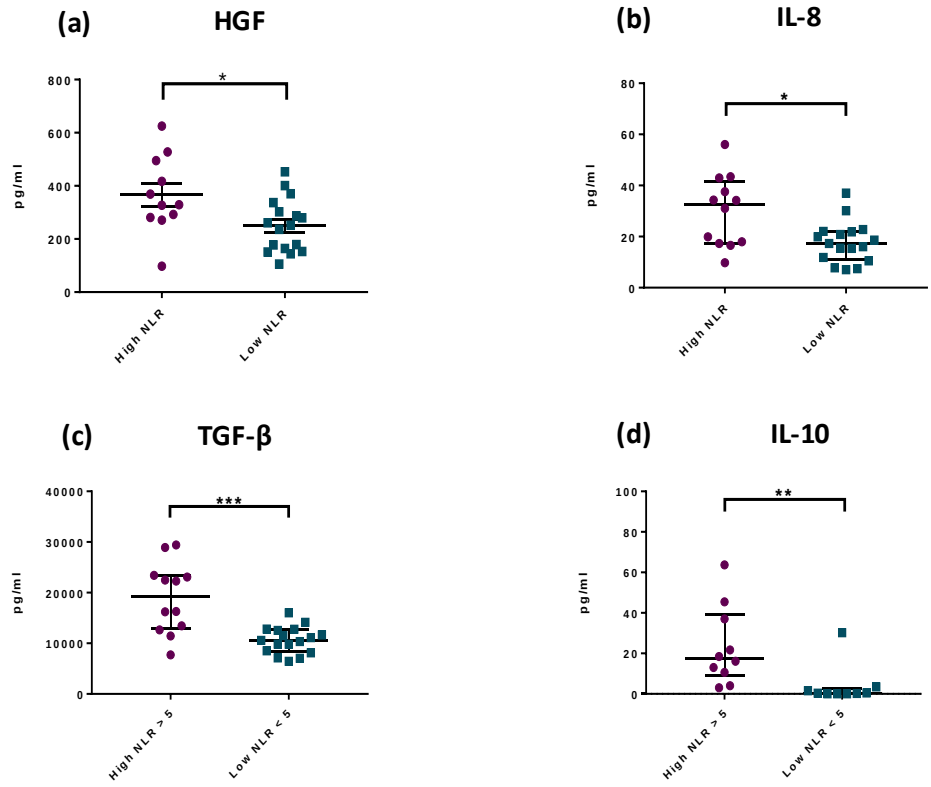


Figure 4.12 Cytokine/chemokine levels stratified by baseline NLR (1)

Cytokine/chemokine analysis of patient plasma was performed and stratified according to baseline NLR. Solutes that were significantly elevated in the high NLR group are shown: **(a)** HGF, **(b)** IL-8 (both n=28), **(c)** TGF-β, **(d)** IL-10 (both n=29).

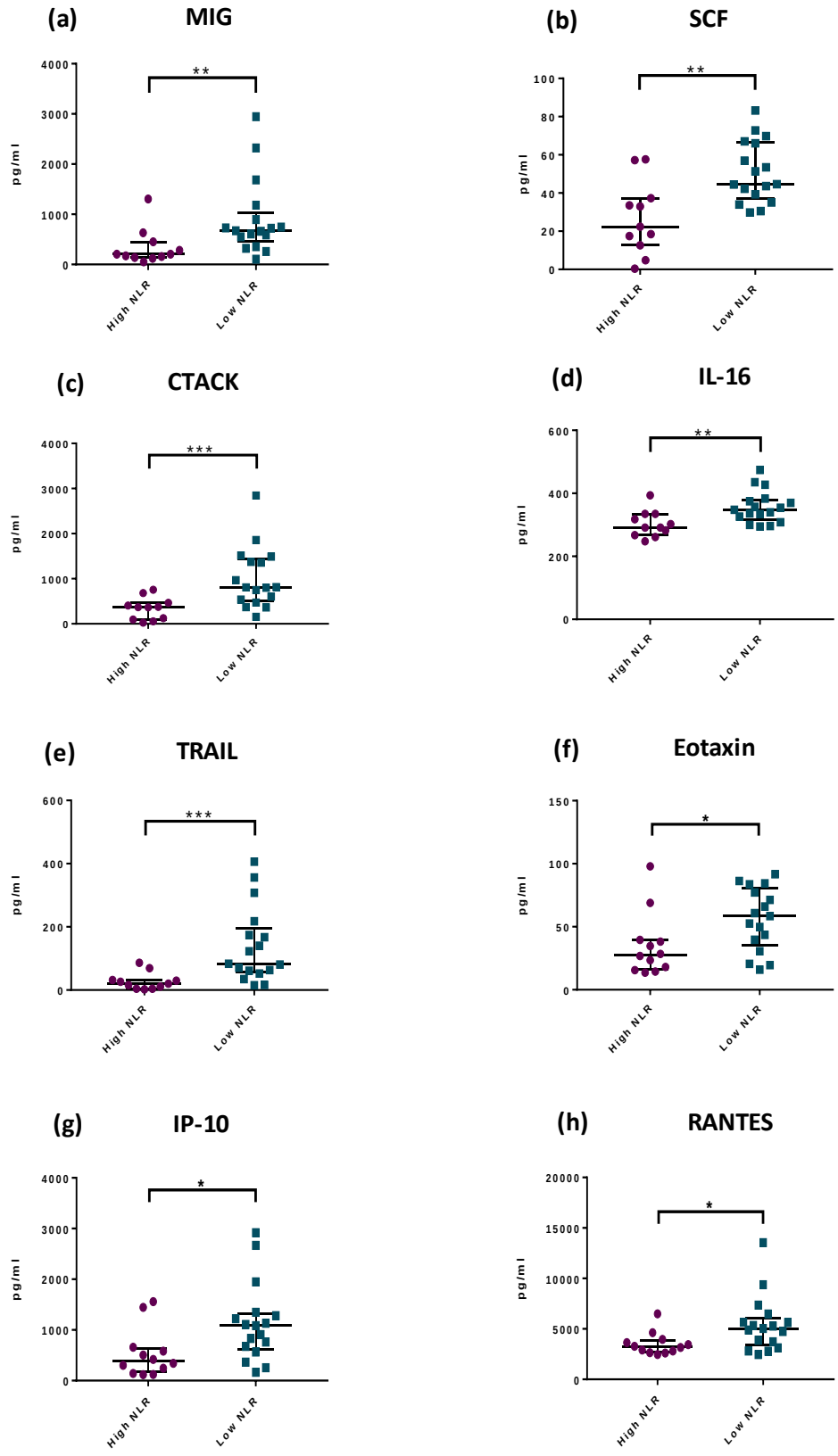


Figure 4.13 Cytokine/chemokine levels stratified by baseline NLR (2)

Cytokine/chemokine analysis of patient plasma was performed and stratified according to baseline NLR. Solutes that were significantly elevated in the low NLR group are shown: **(a)** MIG **(b)** SCF **(c)** CTACK **(d)** IL-16 **(e)** TRAIL **(f)** Eotaxin **(g)** IP-10 and **(h)** RANTES (n=28).

Overall, patients with a high baseline NLR appeared to have a more permissive cytokine profile, with lower levels of pro-immune cytokines and chemokines whilst also demonstrating higher levels of inhibitory cytokines including IL-10 and TGF-β.

4.2.3.2 Change in NLR over time in response to treatment is associated with a distinct chemokine/cytokine profile

There were 5 cytokines/chemokines that demonstrated a significant difference after the first 6 weeks of chemotherapy in both the high and low baseline NLR patients (Figure 4.14). Some were solutes which had previously been shown to differ significantly at baseline between the 2 groups. HGF was the only cytokine in which the fold change at 6 weeks was raised in the low NLR group when compared to the high NLR patients (p=0.0368, Figure 4.14a). For the remaining cytokines, fold change from baseline at 6 weeks was significantly increased in the high NLR group when compared to the low NLR patients, namely SCF (p=0.0048), MIG (p=0.0485), CTACK (p=0.0319) and IL-16 (p=0.0053) (Figure 4.14b-e).

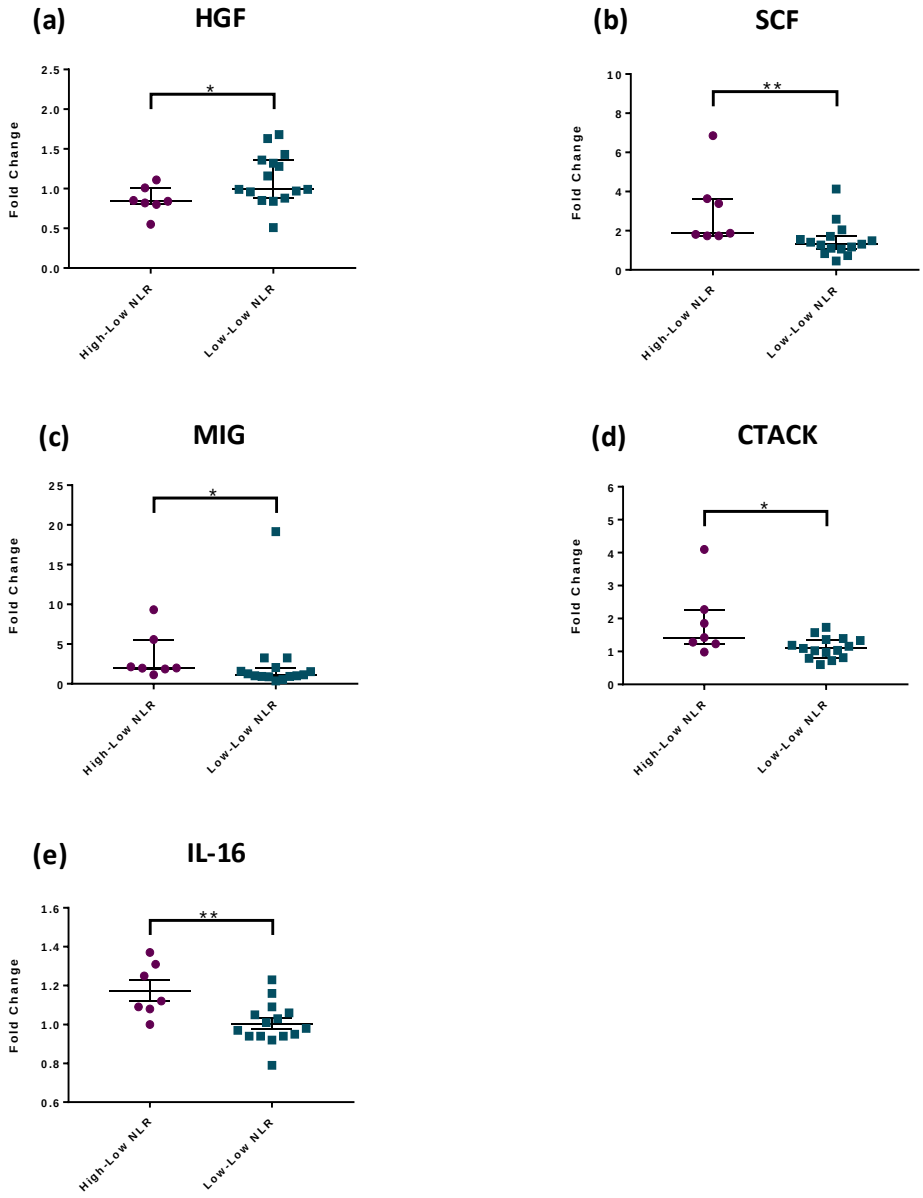


Figure 4.14 Fold change in cytokine/chemokine levels over the first 6 weeks of chemotherapy, stratified by baseline NLR

Data shows fold change of cytokines/chemokines in plasma for NLR patients over the first 6 weeks of treatment. Graphs show: HGF **(a),** SCF **(b),** MIG **(c),** CTACK **(d)** and IL-16 **(e)** (n=22). Fold change in solutes is shown for patients with baseline NLR ≥ 5 which fell to < 5 over 6 weeks (high-low NLR) and those whose NLR remained low over this time period (low-low NLR).

Interestingly, when looking at all patients, there was no demonstrable change in IL-10 levels over the course of chemotherapy at either 6 weeks or 12 weeks (Figure 4.15a). When this data was stratified by NLR and further analysed, there were raised levels of IL-10 seen in the group that had a high NLR at baseline, when compared to the low NLR group. These remained

elevated over the first 6 weeks of treatment whereas the low NLR group continued to have levels of IL-10 that were barely detectable (Figure 4.15b).

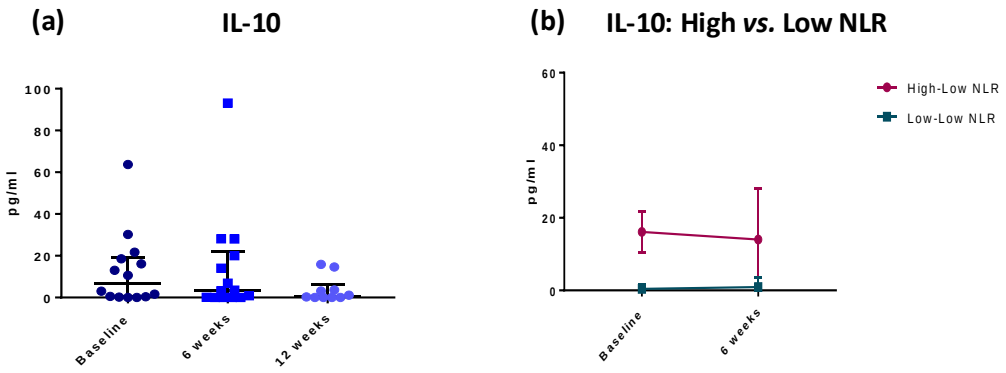


Figure 4.15 Changes in levels of IL-10 over the course of chemotherapy

Plasma levels of IL-10 (pg/ml) are shown for all patients over the first 12 weeks of chemotherapy **(a)**. The change in IL-10, stratified by baseline NLR, after the first 6 weeks of chemotherapy is also shown **(b)** (n=14).

In contrast, levels of TGF-β fell significantly between baseline and 6 weeks (but not between baseline and 12 weeks) across the whole patient cohort (p=0.0135, Figure 4.16a). Subgroup analysis showed that TGF-β decreased in both the high and low baseline NLR groups (Figure 4.16b) but was significant by 6 weeks in the group with a high NLR at baseline (p=0.0234, Figure 4.16c and d)

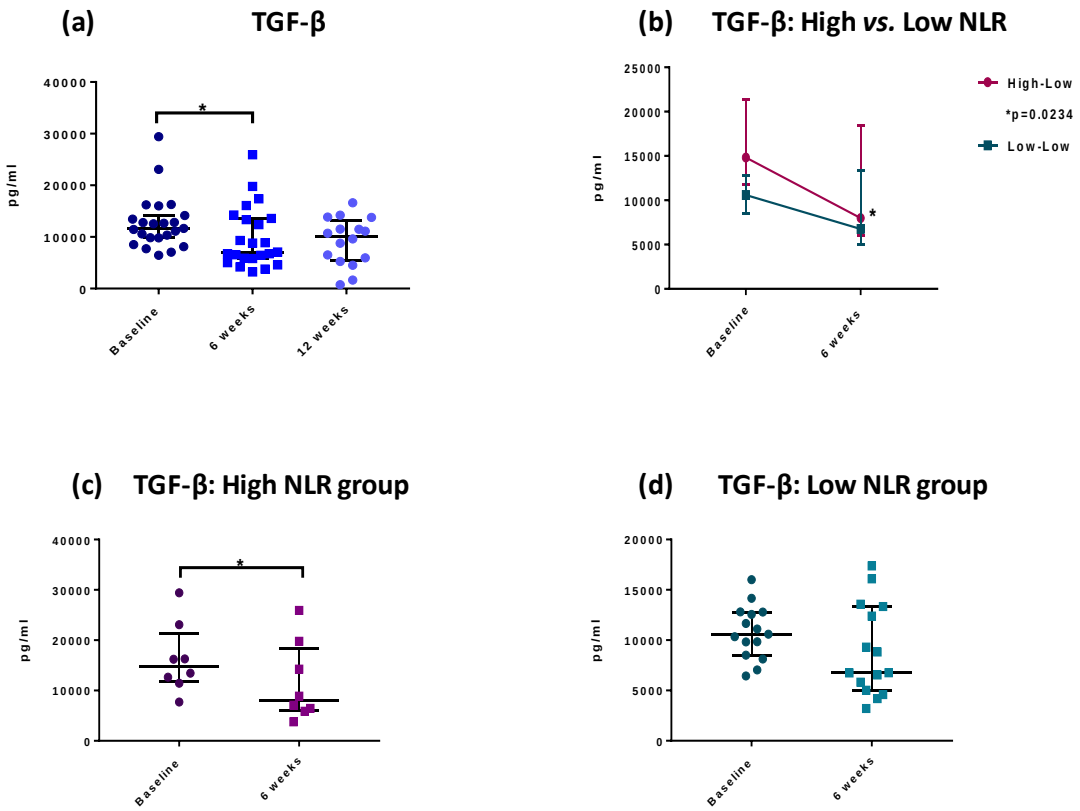


Figure 4.16 Changes in levels of TGF-β over the course of chemotherapy

Plasma levels of TGF-β (pg/ml) are shown for all patients over the first 12 weeks of chemotherapy **(a).** The change in TGF-β, stratified by baseline NLR, during the first 6 weeks of chemotherapy is also shown **(b).** This data is shown for individual patients in **(c)** and **(d)** (n=23).

Taken together, these data demonstrate that the immunosuppressive cytokine milieu seen in patients with a high NLR at baseline can reverse over the first 6 weeks of chemotherapy, with a decrease in TGF-β and increase in other pro-immune cytokines and chemokines including MIG. However, some differences between the two groups remained: IL-10 and HGF remained high in the group of patients with a high NLR at baseline, despite normalisation of the NLR over this time period.

4.2.4 Evaluation of Patient Immune Responses to Chemotherapy Treatment and Association with NLR: Innate Functional Activity

4.2.4.1 High NLR at baseline is associated with decreased NK cell functional activity

When exposed to target tumour cells, degranulation of NK cells (as determined by CD107 expression) was diminished in patients with a high NLR (≥ 5) at baseline. Although this did not reach statistical significance at NLR ≥ 5, as we altered the definition of high NLR, firstly to ≥ 4 then to ≥ 3, the difference in NK cell activity became more marked between those patients with a high or low baseline NLR (shown for different tumour cell targets in Figure 4.17, Figure 4.18, Figure 4.19 (all a-c)). When patients were divided into high NLR ≥ 3 and low < 3, the difference in degranulation between high and low was more marked against all tumour targets; this was significant (p=0.0104) against K562s (a natural target for NK cells) (Figure 4.17c) but was also observed against the CRC tumour cell lines SW480 (Figure 4.18c) and SW620 (Figure 4.19c). Additionally, across the whole patient cohort, there was a negative correlation between levels of CD107 expression prior to chemotherapy and baseline NLR, although statistical significance was not reached (Figure 4.17d, Figure 4.18d, Figure 4.19d).

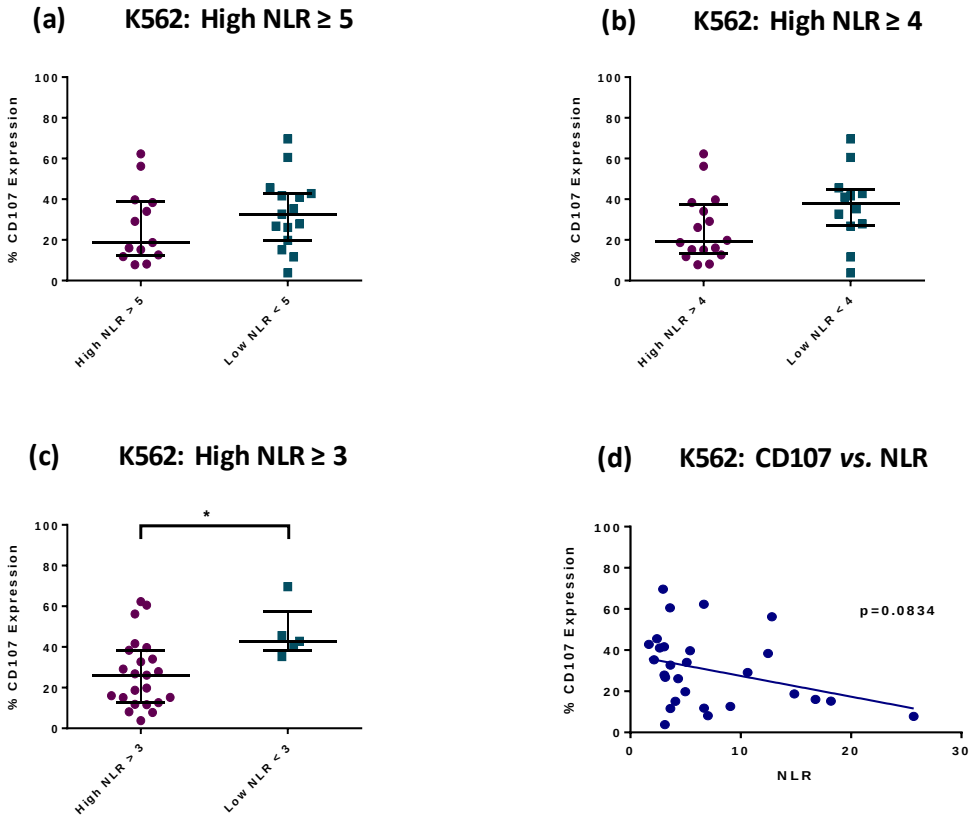


Figure 4.17 Degranulation of patient NK cells, against K562 tumour targets, stratified by baseline NLR

Cryopreserved PBMCs were used in degranulation assays to assess NK cell functional capacity against K562 tumour targets. Percentage CD107 expression on NK cells, as determined by flow cytometry, is shown for different NLR values: **(a)** NLR of greater/less than 5, **(b)** NLR greater/less than 4 and **(c)** NLR of greater/less than 3. The relationship between baseline NLR and CD107 expression, across all patients, is also shown in **(d)** (with linear regression) (n=28).

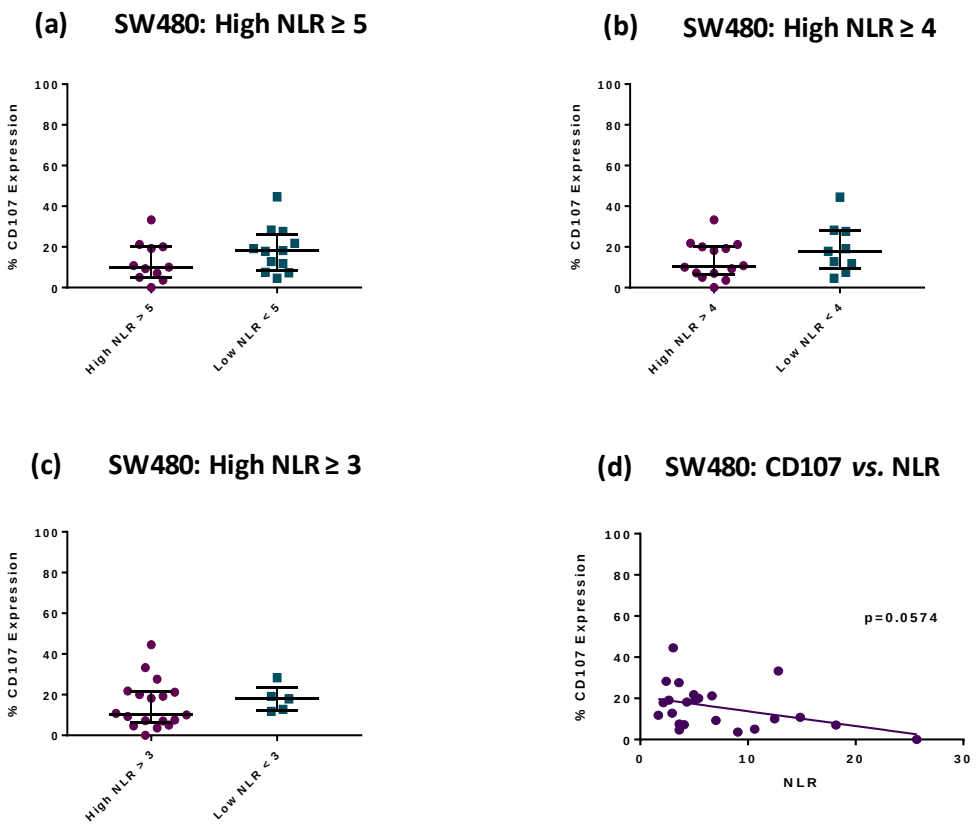


Figure 4.18 Degranulation of patient NK cells, against SW480 tumour targets, stratified by baseline NLR

Cryopreserved PBMCs were used in degranulation assays to assess NK cell functional capacity against SW480 tumour targets. Percentage CD107 expression on NK cells, as determined by flow cytometry, is shown for different NLR values: **(a)** NLR of greater/less than 5, **(b)** NLR greater/less than 4 and **(c)** NLR of greater/less than 3. The relationship between baseline NLR and CD107 expression, across all patients, is also shown in **(d)** (with linear regression) (n=23).

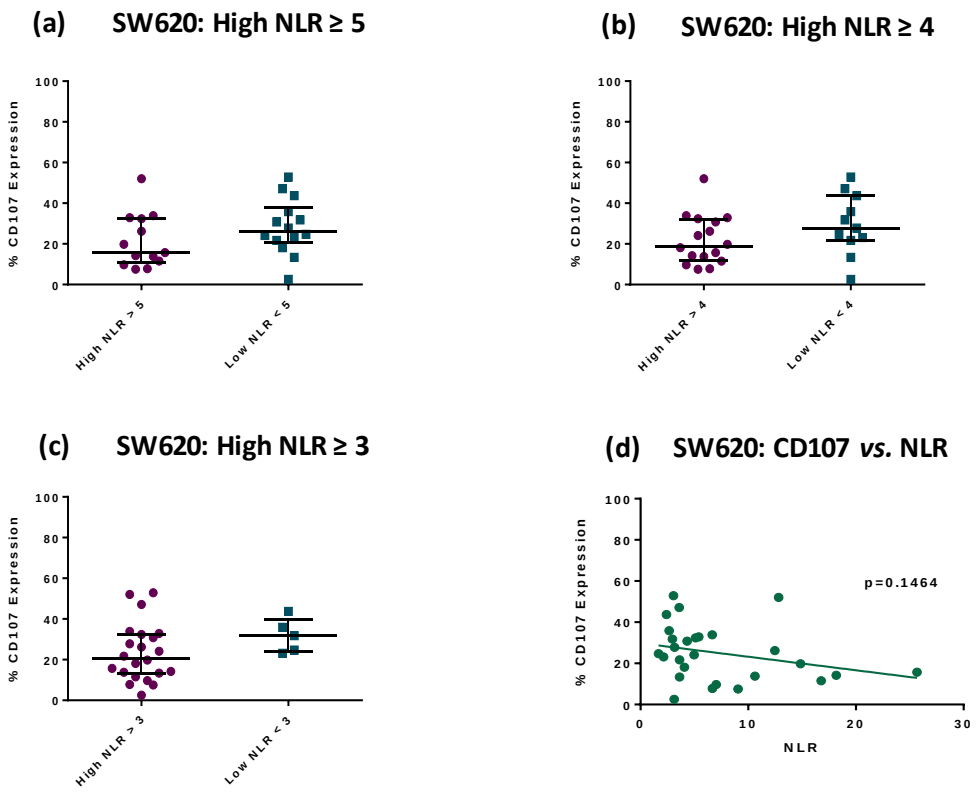


Figure 4.19 Degranulation of patient NK cells, against SW620 tumour targets, stratified by baseline NLR

Cryopreserved PBMCs were used in degranulation assays to assess NK cell functional capacity against SW620 tumour targets. Percentage CD107 expression on NK cells, as determined by flow cytometry, is shown for different NLR values: **(a)** NLR of greater/less than 5, **(b)** NLR greater/less than 4 and **(c)** NLR of greater/less than 3. The relationship between baseline NLR and CD107 expression, across all patients, is also shown in **(d)** (with linear regression) (n=27).

When analysed in a similar way (defining high NLR as ≥ 5, ≥ 4 and ≥ 3), there was also a difference in the cytotoxic potential of NK cells (within the PBMC population) prior to treatment (as determined by ^{51}Cr release assay), although this did not reach statistical significance. Patients with a high NLR prior to chemotherapy trended towards decreased levels of target tumour cell death, when compared to those with a low NLR, against K562 target cells (Figure 4.20a-c), SW480 target cells (Figure 4.21a-c) and SW620 cells (Figure 4.22a-c). As observed previously when evaluating the degranulation of NK cells, there was a negative correlation across the whole patient cohort, between the level of tumour cell death and baseline NLR level tested against all target tumour cells (K562: p=0.0023, r^2=0.3272, Figure 4.20d; SW480: p=0.14226, r^2=0.1705, Figure 4.21d; SW620: p=0.0559, r^2=0.1707, Figure 4.22d).

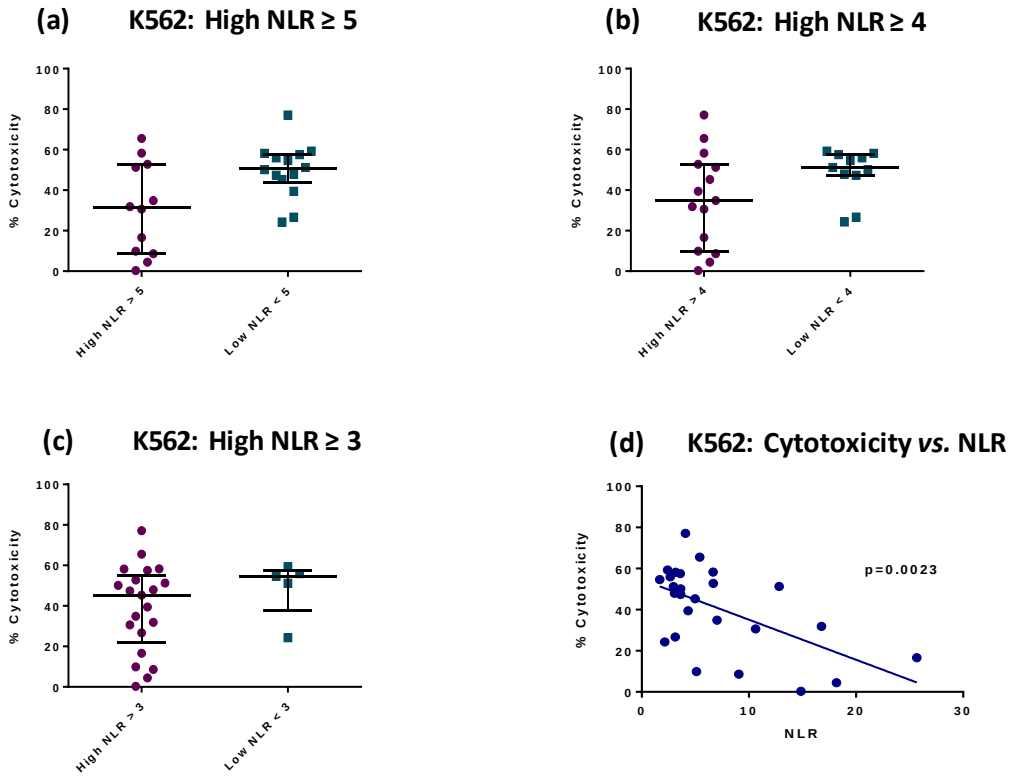


Figure 4.20 Cytotoxicity of patient NK cells, against K562 tumour targets, stratified by baseline NLR.

Cryopreserved PBMCs were used in ^{51}Cr assays to assess NK cell functional capacity against ^{51}Cr-labelled K562 tumour targets. Cytotoxicity of PBMCs at an E:T ratio of 100:1 is shown at baseline (pre-chemotherapy) for decreasing NLR values: **(a)** NLR greater/less than 5, **(b)** NLR greater/less than 4 and **(c)** NLR greater/less than 3. The relationship between baseline NLR and cytotoxicity, across all patients, is also shown in **(d)** (with linear regression) (n=26).

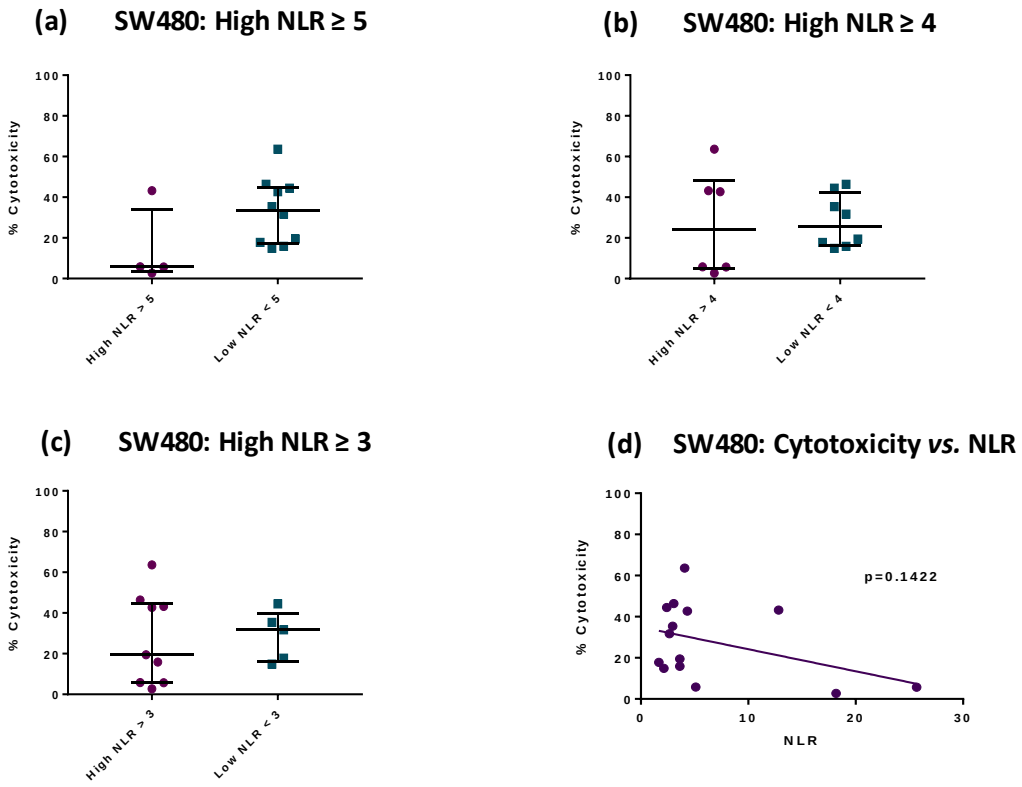


Figure 4.21 Cytotoxicity of patient NK cells, against SW480 tumour targets, stratified by baseline NLR.

Cryopreserved PBMCs were used in ^{51}Cr assays to assess NK cell functional capacity against ^{51}Cr-labelled SW480 tumour targets. Cytotoxicity of PBMCs at an E:T ratio of 100:1 is shown at baseline (pre-chemotherapy) for decreasing NLR values: **(a)** NLR greater/less than 5, **(b)** NLR greater/less than 4 and **(c)** NLR greater/less than 3. The relationship between baseline NLR and cytotoxicity, across all patients, is also shown in **(d)** (with linear regression) (n=14).

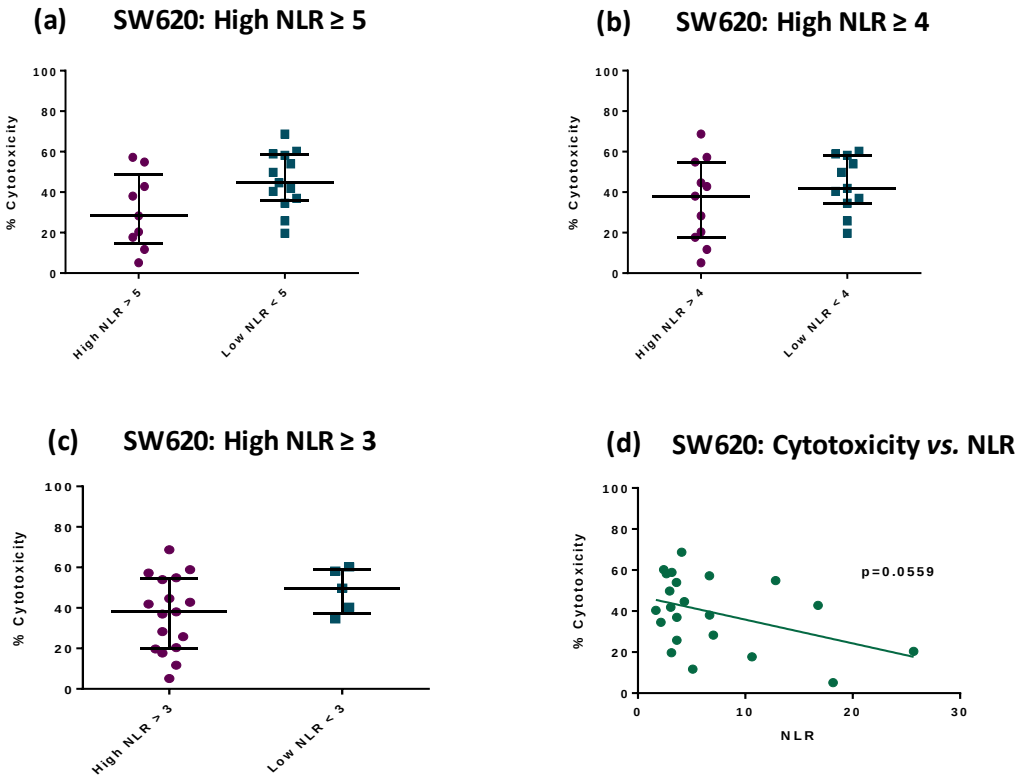


Figure 4.22 Cytotoxicity of patient NK cells, against SW620 tumour targets, stratified by baseline NLR.

Cryopreserved PBMCs were used in ^{51}Cr assays to assess NK cell functional capacity against ^{51}Cr-labelled SW620 tumour targets. Cytotoxicity of PBMCs at an E:T ratio of 100:1 is shown at baseline (pre-chemotherapy) for decreasing NLR values: **(a)** NLR greater/less than 5, **(b)** NLR greater/less than 4 and **(c)** NLR greater/less than 3. The relationship between baseline NLR and cytotoxicity, across all patients, is also shown in **(d)** (with linear regression) (n=22).

4.2.4.2 A lower NK cell functional activity was improved following treatment and was associated with a reduction in NLR

Despite a lower level of CD107 expression on NK cells (in response to a variety of tumour targets) in patients with a high pre-treatment NLR, an increase in CD107 expression was evident when their NLR dropped in response to treatment (Figure 4.23a-c). This was only significant against SW620 target cells (p=0.0041, Figure 4.23c), although the same trend was apparent against K562 and SW480 targets.

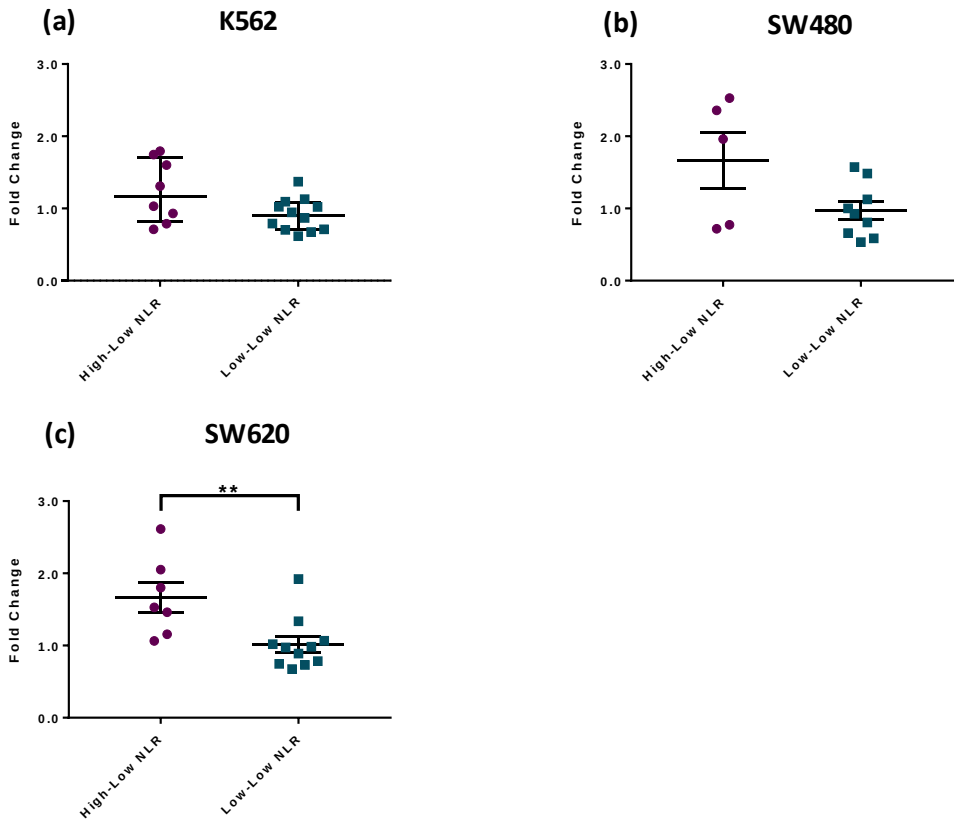


Figure 4.23 Fold change in CD107 expression on patient NK cells in the first 6 weeks of chemotherapy stratified by change in NLR

Cryopreserved PBMCs, taken prior to treatment and following 6 weeks of chemotherapy, were used in degranulation assays to assess NK cell functional capacity against: **(a)** K562 (n=20), **(b)** SW480 (n=14) and **(c)** SW620 (n=18). As determined by flow cytometry, fold change in CD107 expression is shown for patients with baseline NLR ≥ 5 which fell to < 5 over 6 weeks (high-low NLR) and those whose NLR remained low over this time period (low-low NLR).

Once again, despite the low levels of cytotoxicity (as determined in ^{51}Cr assays) associated with a high baseline NLR, this was enhanced by 6 weeks, as NLR decreased (high-low NLR group, Figure 4.24a-c). This was significant against K562 target cells (p=0.0204, Figure 4.24a). Statistical analysis was not possible in the SW480 condition as there was only data from 2 patients in the high-low group (Figure 4.24b).

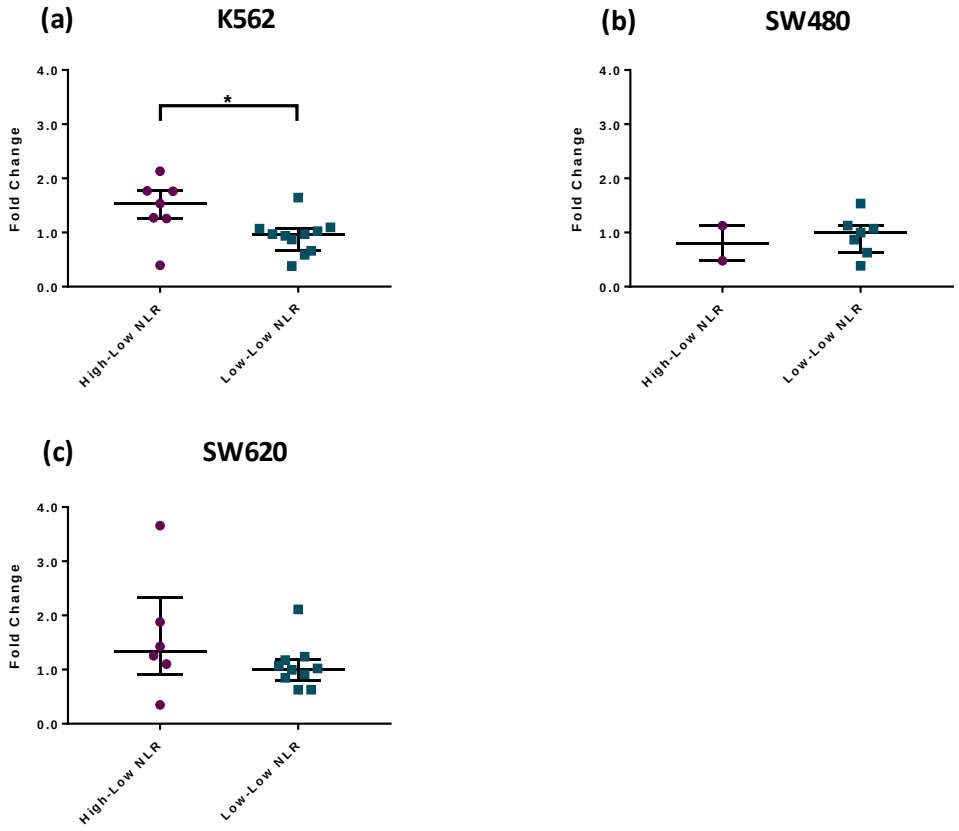


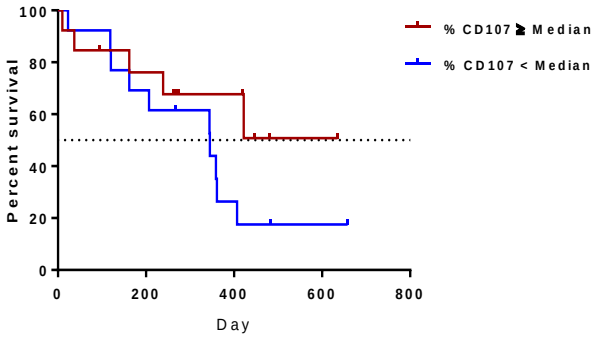
Figure 4.24 Fold change in cytotoxicity (^{51}Cr assays) of patient NK cells in the first 6 weeks of chemotherapy stratified by change in NLR

Cryopreserved PBMCs, taken prior to treatment and following 6 weeks of chemotherapy, were used in a ^{51}Cr-released assay against: **(a)** K562 (n=18), **(b)** SW480 (n=9) and **(c)** SW620 (n=16). Fold change in cytotoxicity is shown for patients with baseline NLR ≥ 5 which fell to < 5 over 6 weeks (high-low NLR) and those whose NLR remained low over this time period (low-low NLR).

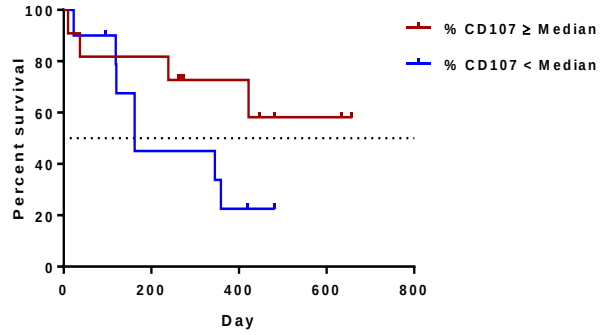
4.2.4.3 Higher CD107 expression on peripheral NK cells prior to treatment is associated with a trend towards increased overall survival

Within the entire patient cohort, those with elevated CD107 expression on NK cells showed a non-significant trend to prolonged OS when results were analysed by median CD107 expression against all tested tumour targets (Figure 4.25). Median OS was not reached in the K562 group with CD107+ NK cells ≥ median *vs.* 345 days in the comparison group (Figure 4.25a). This was also true against SW480 targets (median OS for patients with CD107 expression < median 162 days) (Figure 4.25b) and SW620 targets, where median OS for patients with CD107 expression < median was 344.5 days (Figure 4.25c).

(a) Overall Survival: CD107/K562



(b) Overall Survival: CD107/SW480



(c) Overall Survival: CD107/SW620

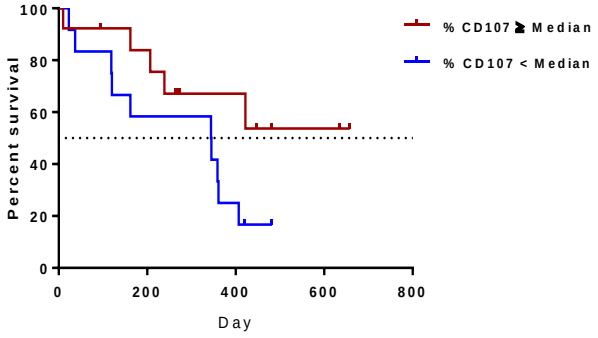


Figure 4.25 Overall survival of patients stratified by median CD107 expression on NK cells against different target tumour cells

All patients were stratified according to CD107 expression on NK cells when incubated with: **(a)** K562 (n=26) **(b)** SW480 (n=21) and **(c)** SW620 (n=25) target cells. Survival was calculated as days from receiving the first dose of chemotherapy. All patients were evaluable in the survival analysis.

4.2.4.4 High cytotoxic potential of PBMCs prior to treatment is associated with increased overall survival

Overall survival was increased in patients whose PBMCs had a higher level of cytotoxicity (as determined by ^{51}Cr-released assay) against both K562 and SW620 target cell lines (Figure 4.26). Against K562 cells, OS was significantly increased in patients where NK cell-mediated cytotoxicity was greater than the median (median OS not reached for high cytotoxicity group *vs.* 344 days, p= 0.0498, HR 2.775, 95 %, CI 0.9496 to 8.108). Against SW620 cells OS was also significantly increased (median OS not reached for high cytotoxicity group *vs.* 359 days, p=0.0404, HR 3.226, 95 % CI 0.9358 to 11.12). There were not enough data points to determine survival against SW480 target tumour cells.

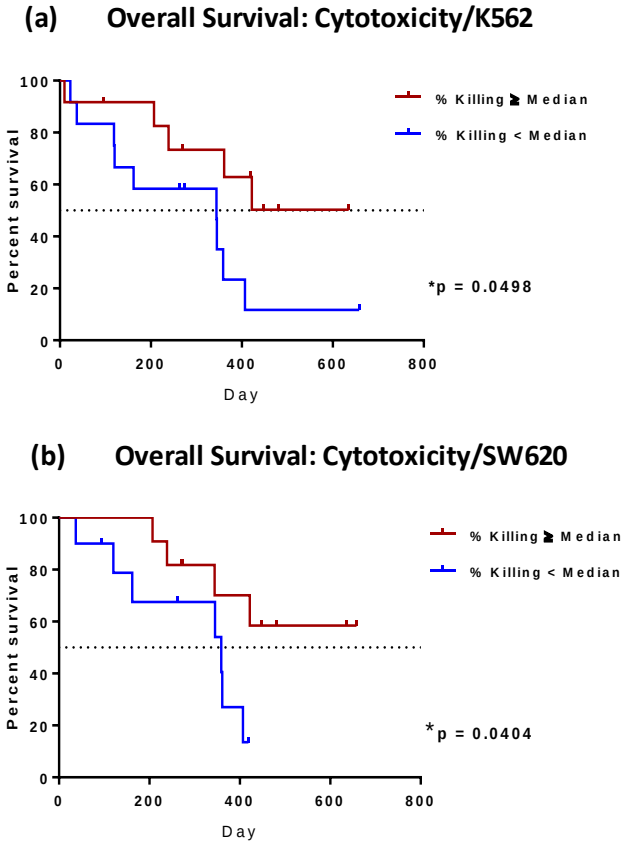


Figure 4.26 Overall survival of patients stratified by median cytotoxicity of NK cells against different target tumour cells

All patients were stratified according to cytotoxicity when incubated with: **(a)** K562 (n=24) and **(b)** SW620 (n=21) target cells. Survival was calculated as days from receiving the first dose of chemotherapy. All patients were evaluable in the survival analysis.

4.2.5 Evaluation of Patient Immune Responses to Chemotherapy Treatment and Association with NLR: ELISpot

4.2.5.1 T cell responses reflect innate immune responses within patients

ELISpot assays were performed to determine the adaptive T cell response of patients undergoing treatment and to monitor any changes which may occur in response to therapy. Once thawed, PBMCs were incubated with either a CRC TAA (CEA) or viral peptides (CEF) and T cell responses were measured by IFN-γ production. As with other previous assays, inherent variability between patients was evident. However, IFN-γ production in response to viral and TAA peptides seemed to reflect the pattern in responses seen in some of the other immune assays (NK cell activation), indicating that despite inter-patient variability, there were similar trends in the innate and adaptive arm of an individual patient's immune response to treatment. As an example, in patient NLR-7, there was an increase in IFN-γ production to CEF peptide (a marker of non-specific T cell recall response) between baseline (pre-chemotherapy) and 6 weeks, which then fell by 12 weeks (Figure 4.27a). This was mirrored in cytotoxicity assays, where the PBMCs taken at 6 weeks showed higher levels of tumour cell killing compared to baseline and immunophenotyping, where expression of CD69 had increased dramatically on both CD4+ and CD8+ T cells by the 6 week sample (Figure 4.27b, c, f, g). Although CD107 degranulation was not noticeably higher at 6 weeks, levels increased over the 12 week period (Figure 4.27d, e).

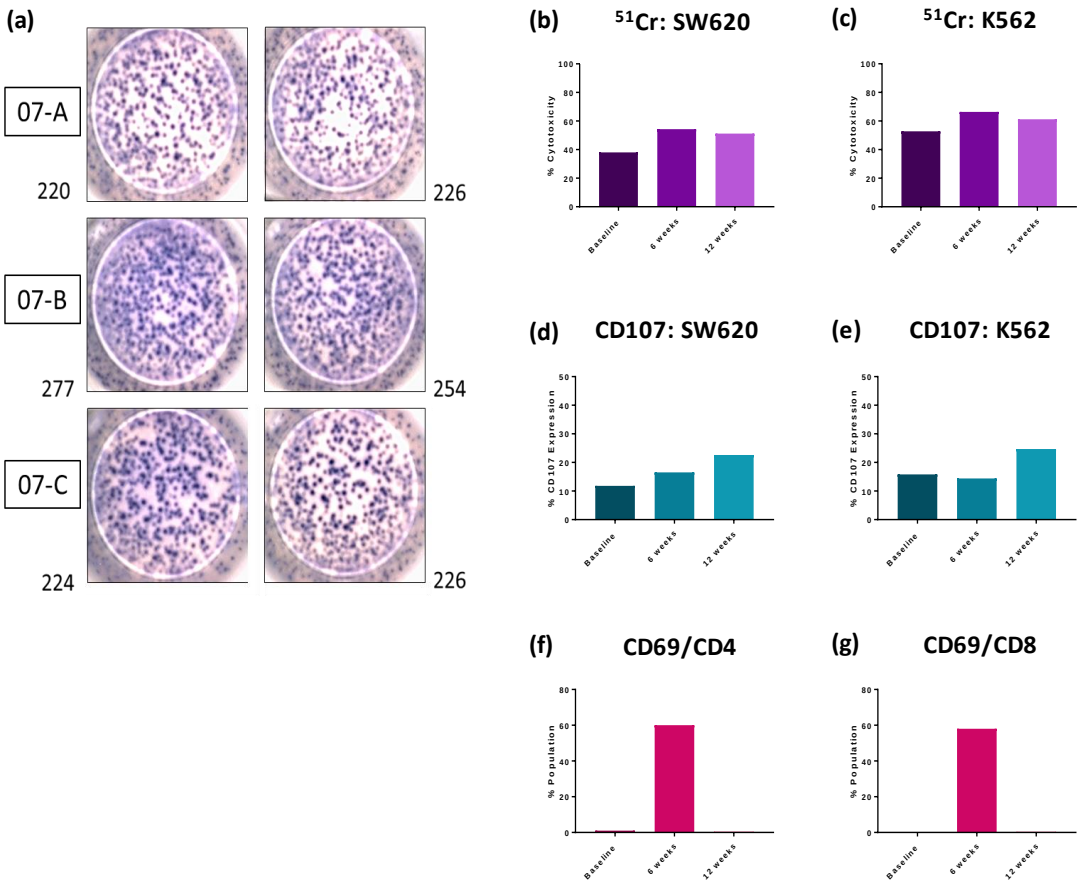


Figure 4.27 Comparison between adaptive T cell responses and innate responses during treatment: Patient NLR-07

Cryopreserved PBMCs were collected from patient NLR-07 and analysed by ELISpot and other innate immune assays. IFN-γ production in response to CEF peptide was aligned with innate responses in the same samples. **(a)** T cell responses (and number of SFU) are shown for baseline, 6 week and 12 week samples in duplicate. Cytotoxicity of PBMCs (determined by ^{51}Cr assay) against SW620 **(b)** and K562 cells **(c)** are also shown. CD107 expression (determined by flow cytometry) is shown against SW620 **(d)** and K562 **(e)**. Also shown is CD69 expression on CD4+ **(f)** and CD8+ **(g)** T cells as determined by flow cytometry.

In contrast, patient NLR-10 did not exhibit any change in T cell responses to CEF peptide (as demonstrated by unvarying IFN-γ SFU over a 12 week period). This was also reflected in cytotoxicity assays, CD107 degranulation assays and in CD69 expression on CD4+ and CD8+ T cells (Figure 4.28).

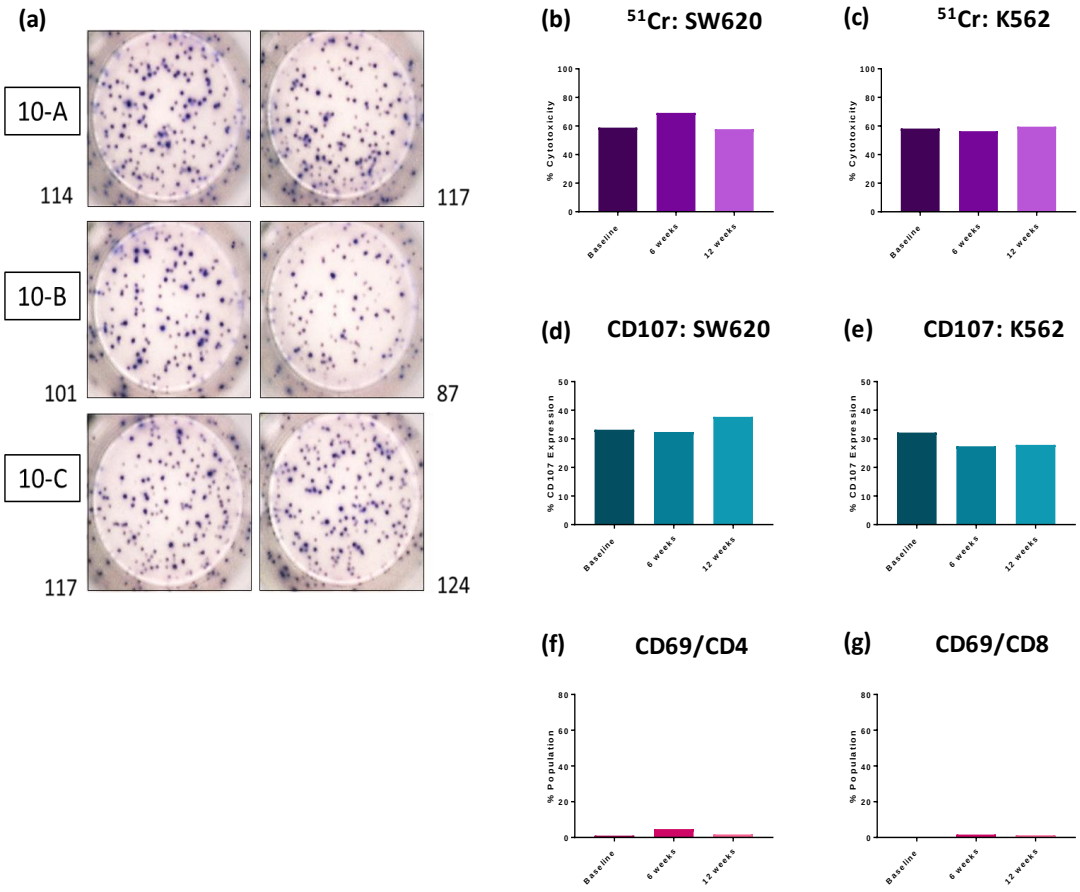


Figure 4.28 Comparison between adaptive T cell responses and innate responses during treatment: Patient NLR-10

Cryopreserved PBMCs were collected from patient NLR-10 and analysed by ELISpot and other innate immune assays. IFN-γ production in response to CEF peptide was aligned with innate responses in the same samples. **(a)** T cell responses (and number of SFU) are shown for baseline, 6 week and 12 week samples in duplicate. Cytotoxicity of PBMCs (determined by ^{51}Cr assay) against SW620 **(b)** and K562 cells **(c)** are also shown. CD107 expression (determined by flow cytometry) is shown against SW620 **(d)** and K562 **(e)**. Also shown is CD69 expression on CD4+ **(f)** and CD8+ **(g)** T cells as determined by flow cytometry.

Finally, in patient NLR-05, there was an increase in T cell ELISpot responses between baseline and 6 weeks and also between 6 and 12 weeks of chemotherapy (Figure 4.29). Once again this was also evident in the chromium-release assay, with a rise in NK-mediated cytotoxicity over the 12 week period of chemotherapy. However, in this patient, CD107 and CD69 expression did not mirror these findings.

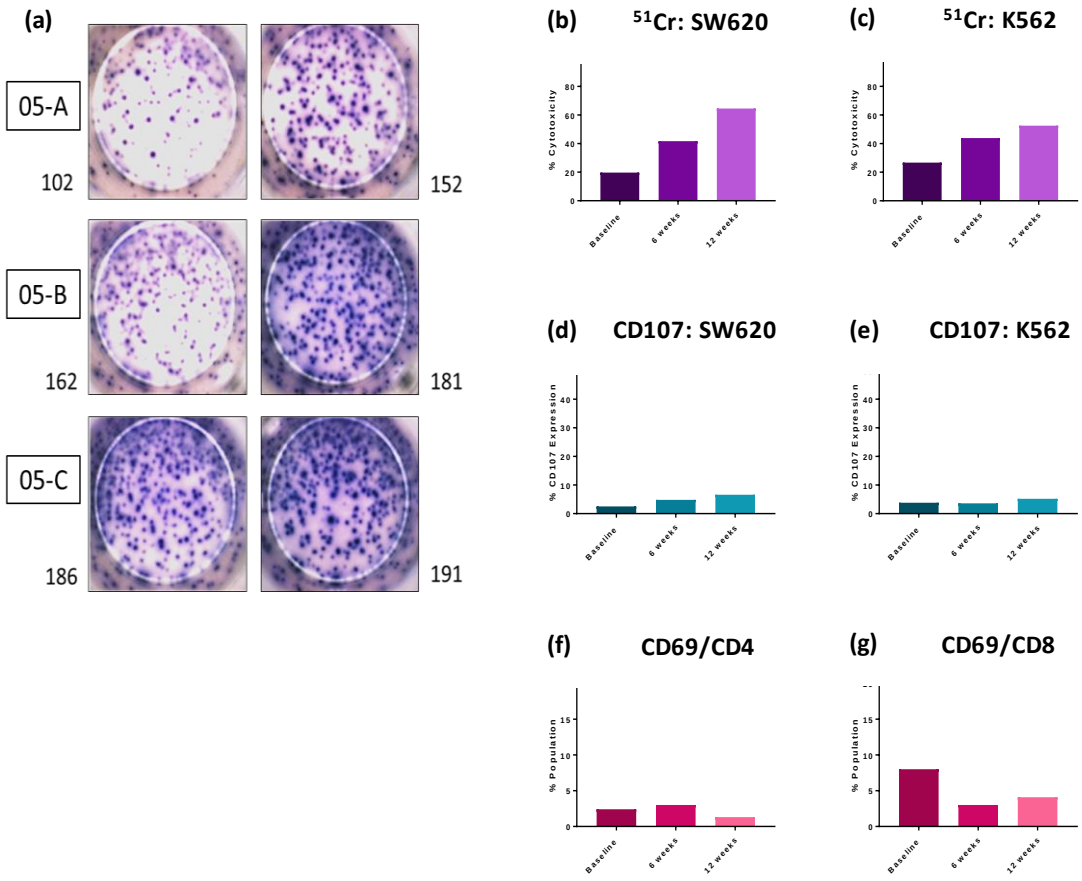


Figure 4.29 Comparison between adaptive T cell responses and innate responses during treatment: Patient NLR-05

Cryopreserved PBMCs were collected from patient NLR-05 and analysed by ELISpot and other innate immune assays. IFN-γ production in response to CEF peptide was aligned with innate responses in the same samples. **(a)** T cell responses (and number of SFU) are shown for baseline, 6 week and 12 week samples in duplicate. Cytotoxicity of PBMCs (determined by ^{51}Cr assay) against SW620 **(b)** and K562 cells **(c)** are also shown. CD107 expression (determined by flow cytometry) is shown against SW620 **(d)** and K562 **(e)**. Also shown is CD69 expression on CD4+ **(f)** and CD8+ **(g)** T cells as determined by flow cytometry.

T cell responses to the TAA peptide CEA were variable across the patient cohort; in some patients there was no change in ELISpot assays over the course of chemotherapy, whereas in others a change in IFN-γ production was detected. In patient NLR-18, there was an increase in SFU to both CEA and CEF peptide, demonstrating a peak in IFN-γ production (and hence T cell response) at the 6 week sample. Interestingly, this was not only mirrored by the increase in NK cell degranulation at the same time point but also by the serum CEA level (Figure 4.30).

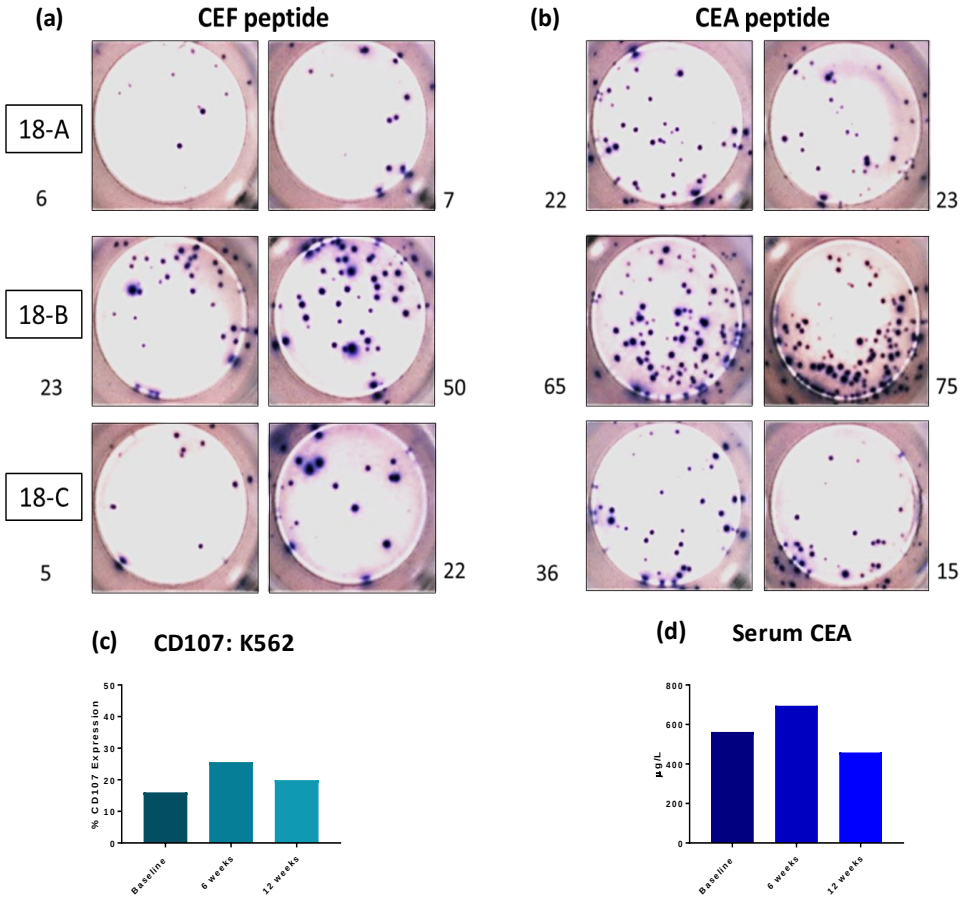


Figure 4.30 Comparison between adaptive T cell responses and innate responses during treatment: Patient NLR-18.

Cryopreserved PBMCs were collected from patient NLR-18 and analysed by ELISpot and other innate immune assays. IFN-γ production in response to CEF and CEA peptides were compared to CD107 degranulation assays and serum CEA measurement. **(a)** T cell responses (and number of SFU) against CEF peptide are shown for baseline, 6 week and 12 week samples in duplicate. Similarly, **(b)** T cell responses (and number of SFU) against CEA peptide are also shown. CD107 expression (determined by flow cytometry) is shown against K562 tumour cells **(c)**. Also shown are serum CEA levels **(d)**.

4.2.5.2 A high or low NLR prior to treatment is associated with a distinct adaptive T cell response

Examination of pre-treatment T cell responses to CEF peptides suggested a decreased response at baseline in the group of patients with a high NLR (Figure 4.31a), although this result was not significant. In addition, mirroring the CD107 and cytotoxicity results, enhanced response to CEF peptide at 6 weeks was observed in patients in whom NLR fell by 6 weeks of chemotherapy (CEF response increased as NLR decreased) (Figure 4.31b). A positive response

to CEF peptide at 6 weeks was defined as the proportion of patients with a 1.5x increase in SFU, minus the negative control.

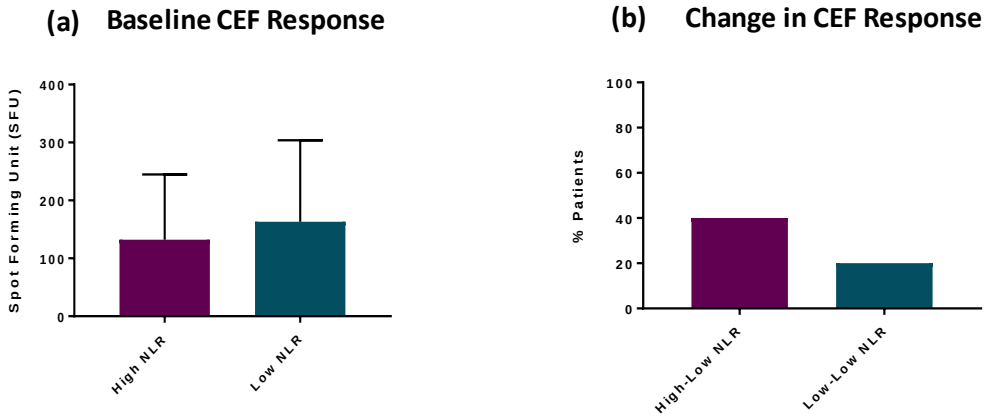


Figure 4.31 T cell responses to CEF prior to treatment, stratified by NLR

Cryopreserved PBMCs were incubated with CEF peptide and analysed by ELISpot assays. T cell responses (SFU) against CEF peptide, prior to treatment, are shown in patients with high or low NLR at baseline **(a)**. Also shown is the positive response to CEF peptide at 6 weeks for patients with baseline NLR ≥ 5 which fell to < 5 over 6 weeks (high-low NLR) and those whose NLR remained low over this time period (low-low NLR) **(b)**.

When looking at response to CEA peptide, there was no appreciable difference prior to treatment when stratified by NLR (Figure 4.32a). However, a higher number of positive responses to CEA peptide was found in low NLR patients (CEA response increased if NLR stayed < 5) (Figure 4.32b).

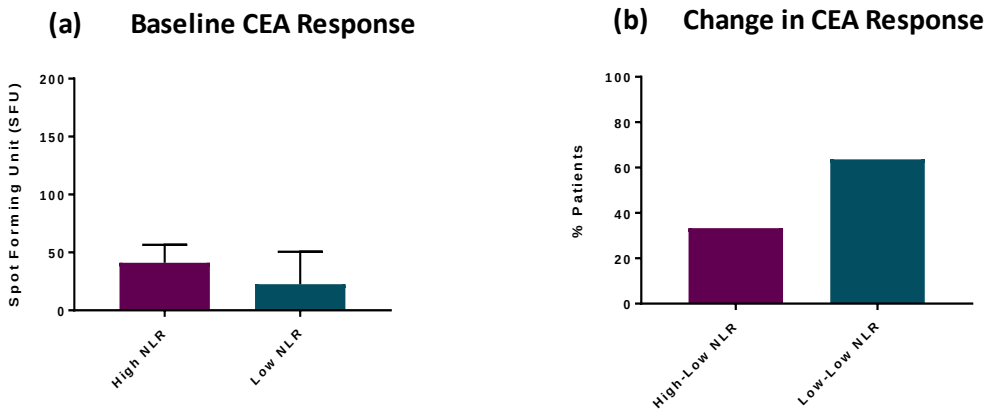


Figure 4.32 T cell responses to CEA prior to treatment, stratified by NLR

Cryopreserved PBMCs were incubated with CEA peptide and analysed by ELISpot assays. T cell responses (SFU) against CEA peptide, prior to treatment, are shown in patients with high or low NLR at baseline **(a)**. Also shown is the positive response to CEA peptide at 6 weeks for patients with baseline NLR ≥ 5 which fell to < 5 over 6 weeks (high-low NLR) and those whose NLR remained low over this time period (low-low NLR) **(b)**.

4.3 NLR Discussion

The data presented in this chapter further delineates the immune response in patients with CRC on first-line chemotherapy and the relationship between NLR and outcome. NLR has been found to be a strong predictive marker in a number of different tumour types and, in CRC, has been associated with pathological stage, response to therapy and survival. The underlying mechanism for this relationship has not been thoroughly investigated. By interrogating this in a CRC patient group, we hoped to reveal underlying immunological correlates which could potentially be exploited in the development of novel treatment strategies.

In order to assess the status of a patient's immune system both at baseline (prior to treatment) and at specific time points during therapy, we performed a number of complementary analyses on patient blood samples. The results suggested differences in immune profiles of those patients with a high baseline NLR and those with a low baseline, such as higher levels of cytokines including IL-10 and TGF-β. Those with a low NLR at baseline had significantly higher plasma levels of cytokines such as RANTES, SCF, TRAIL and MIG. There was also a suggestion that a higher baseline NLR was associated with diminished innate immune responses against tumour cell targets (cytotoxicity and degranulation). With chemotherapy, there was a change noted in this immune profile, with a fall in levels of TGF-β and Tregs. There was also some evidence that T cell responses towards CEF and TAAs altered with chemotherapy.

There was no obvious change in activation markers such as CD69, CCR7 and HLA-DR on immune cell populations including CD4+ and CD8+ T cells, monocytes and B cells (Figure 4.4 and Figure 4.5) over the time period assessed. However, the timings of these samples may not be optimum to reveal such a response to therapy. Due to ethical constraints samples were taken prior to chemotherapy, after 6 weeks and initially also after 12 weeks of chemotherapy; dates which corresponded with planned hospital visits. It is possible, therefore, that transient changes in activation markers were missed. The early activation marker, CD69, is expressed in the first 6-12 hours after T cell stimulation (421). In comparison, HLA-DR and CCR7 are later markers of immune function. Increased HLA-DR expression, for example, has been demonstrated 48 - 72 hours post *ex vivo* stimulation of PBMCs (421). Similarly, *in vitro* stimulation of CD4+ T cells has shown that the proportion of CCR7+ cells increases over a short number of days but the CCR7- subpopulation increased significantly by three weeks

(422). It is possible that taking samples at alternative time points, e.g. prior- and 24 hours post-chemotherapy, may have permitted detection of such changes.

In our data, the proportion of Tregs within the CD4+ population fell through the first 6 weeks of chemotherapy and reduced further by 12 weeks (Figure 4.10). This was independent of the baseline NLR, although it was interesting that, in contradiction to what may have been predicted, peripheral Tregs were present at a higher frequency in the low NLR group compared to the high. Established dogma would predict that patients with a high NLR (and, in our hands, a seemingly depressed immune response) would have a higher proportion of Tregs. However, possible future work could include analysis of paired tumour samples which may reveal that decreased peripheral Tregs reflect a higher proportion of Tregs in the TME. It should also be noted that there are a number of phenotypic classifications of Tregs, with different immunosuppressive properties (naïve, effectors and terminal effectors (50)) which may be further subcategorised by the presence or absence of CD45RO, which can also be used to differentiate naïve and memory T cell populations (50,423). These phenotypically different sub-classifications of Tregs appear to express different levels of IL-10 and TGF-β and changes in the populations of each can be variably associated with PFS and OS in NSCLC patients (50). These differences, which we have not analysed fully, may influence the apparent increase in OS in the population of patients with higher levels of peripheral Tregs (Figure 4.11). Our discovery of fewer Tregs in the patients with a high baseline NLR seems discordant with the higher concentrations of the cytokines TGF-β and IL-10, which are produced by Tregs (50) and contribute to the immunosuppressive milieu. However, other cell types are also known to secrete these cytokines and may be the physiological source *in situ*.

There was a significant difference in the expression of the LN-homing receptor CD62L on Tregs between the high and low NLR groups at baseline: this was diminished in the high NLR group (Figure 4.9b). In the context of a decreased Treg population, this may support the hypothesis that the population of Tregs we are examining in the periphery do not include the CD62L+ Tregs which have already trafficked to LNs or tumour. It is at these sites that Tregs can exert their suppressive effects and contribute to immune tolerance to tumour antigens, presented by APC (424). Again, this may be a factor when considering the differences in OS in patients with varying populations of peripheral Tregs and supports the view that, in addition to the evaluation of peripheral blood cells to therapy, the TME should also be studied. In support of this data, other groups have found that 5-FU-containing chemotherapy (including FOLFOX and FOLFIRI in CRC) is associated with a decreased proportion of Tregs in peripheral blood

(425,426). In a group of gastric cancer patients receiving neo-adjuvant chemotherapy, a reduction in peripheral Tregs over the course of treatment also corresponded to an increased OS, supporting the evidence for an improvement in OS even in patients with a high NLR which falls over the course of treatment (425).

We found that there was no appreciable difference between the populations of PD-1+ and PD-L1+ Tregs at baseline in either the high or low NLR groups. This was the same in many of the other cell populations analysed. However, expression of PD-1 on NK cells, monocytes and B cells (Figure 4.7 - Figure 4.8) increased during chemotherapy, with a significant change from baseline seen by 6 weeks. While this may suggest a potential use for immunotherapy, the efficacy of immune checkpoint inhibitors (anti-PD-1 or anti-PD-L1) should depend on the presence of both receptor and ligand within the immune environment (427). Supporting this theory, 5-FU chemotherapy has been shown to increase expression of PD-L1 on both colorectal and oesophageal cell lines, which was also low pre-chemotherapy (428). It is worth noting, however, that these cell lines were microsatellite instable HCT 116 cells, which are deficient in MSH1 and as already discussed, are more amenable to immune checkpoint inhibitors (324,429). This increase in PD-L1, however, may also indicate a developing immune-resistance to traditional chemotherapy and provide a further rationale for the use of immunotherapy after sequential chemotherapy in gastrointestinal cancers. This upregulation of PD-L1 is not limited to 5-FU and CRC however: increased expression has also been noted following cisplatin- or carboplatin-based neoadjuvant chemotherapy in urothelial cancer (430) and once again may signify a broader mechanism of immune response to chemotherapy-induced cell death, developing immune tolerance and emerging resistance to chemotherapy. Wang *et al.* (431) have previously shown that the colorectal cell line MC38 is resistant to immune checkpoint blockade *in vitro* but that treatment with oxaliplatin stimulated immune cell infiltration into the tumour. Furthermore, treatment with sequential oxaliplatin followed by anti-CTLA-4 and anti-PD-1 blockade led to reduced tumour size and increased survival in mice implanted with MC38 tumours. This is possibly a consequence of combination therapy up-regulating the ligands PD-L1 and PD-L2. As such, there appears to be a rationale for potential sensitisation of CRC to immune checkpoint inhibition following chemotherapy.

We observed that innate immune function was also attenuated in patients who had a higher baseline NLR, as evidenced by reduced NK cell function against tumour cell targets (Figure 4.17 - Figure 4.22); a negative correlation with baseline NLR and NK cell function was evident. Of interest, in patients with a high baseline NLR, these differences seemed to be

abrogated following 6 weeks of first-line chemotherapy, corresponding to a fall in NLR: NK cell function increases as the NLR decreases (Figure 4.23 and Figure 4.24). All patients involved in the current study received chemotherapy containing 5-FU or the pro-drug capecitabine. Treatment with 5-FU has previously been shown to increase both the number of peripheral NK cells (432–434) and their function (as evidenced by increased expression of cell surface CD69). As discussed above, it is likely that any increase in CD69 in our patient cohort was not detected due to the timings of sample acquisition. There is a solid body of evidence that, aside from their frequency within peripheral blood, NK cells themselves may be dysregulated in the context of malignancy. Expression of the activating receptors NKG2D, NKp30 and NKp46 are diminished on peripheral NK cells from patients with CRC compared to healthy controls (379). Expression of NKG2D is also attenuated in patients with metastatic disease compared to healthy controls and both low grade and high grade non-metastatic disease (16). Our results showed an increase in levels of TGF-β in patient plasma (Figure 4.12c), which is itself known to inhibit NK cell function (145). Interestingly, other groups have also shown a correlation between downregulation of NK cell activating receptors and TGF-β (146). Collectively, this supports our data which links abrogated NK cell function with poor-prognosis high NLR CRC. The role of NK cells in immune surveillance and their potential to control the development and growth of malignancy indicates that these findings likely represent a vicious circle of dysfunctional NK cells contributing to the development of metastatic disease and NK function being further suppressed in the context of higher grade, aggressive, metastatic disease.

To further our understanding of the immune response and how it relates to NLR status, an extensive analysis of the cytokine and chemokine profile of patient plasma was performed. Initial comparisons of pre-treatment samples between the high and low NLR groups revealed thought-provoking findings. Although a considerable number of chemokines and cytokines were suppressed prior to treatment in the context of a high NLR, other solutes, specifically TGF-β, IL-10, HGF and IL-8 were elevated in these patients (Figure 4.12 and Figure 4.13). The elevated levels of TGF-β and IL-10 that we observed in the high NLR group was not unexpected, given the data supporting their role in the suppression of anti-tumour immune responses (100,155). Significantly, elevated levels of TGF-β have been observed in patients with Dukes stage C and D CRC compared to healthy controls (141). Although our cohort of patients all had metastatic disease, our data would support the correlation between high levels of immunosuppressive cytokines and increased stage/extent of disease. This was also illustrated in the work by Ivanovic *et al* (435), which showed that plasma levels of TGF-β were

elevated in patients with more extensive stage breast cancer. This translated to decreased two-year survival in patients with plasma TGF-β1 >3 ng/ml compared to those with levels more comparable to the HD group (10 % *vs.* 52 %, respectively) (435). Reflecting similar findings to those already discussed, IL-10 is highly predictive of DFS in patients who have resections for CRC (160). The interesting finding in our patient cohort was that, although levels of TGF-β fell significantly over the first 6 weeks of chemotherapy, levels of IL-10 remained steady (Figure 4.15 and Figure 4.16). This might be unexpected given our observation that the peripheral blood Treg population fell with chemotherapy. However, although Tregs are a major source of IL-10, other immune cell populations, which did not decline with chemotherapy, can also produce IL-10. In the context of HIV infection, it has been shown by Said *et al.* that increased PD-1 expression can induce IL-10 production from monocytes (153). Similarly, the elevated PD-1 expression noted in our data may be one of the potential triggers for ongoing IL-10 production in response to this inhibitory signal. The difference in the plasma levels of these two immunosuppressive cytokines with chemotherapy may also go some way to explaining why patients with a high NLR may have different outcomes than those with low NLR, even if it normalises with therapy (406).

Both IL-8 and HGF can be secreted by cancer-associated fibroblasts (CAFs) (436). CAFs are fibroblasts that are associated with the TME and secrete a range of cytokines and chemokines that can contribute to tumour growth, invasion, metastases, angiogenesis and immunosuppression (436). Through activation of the intra-cellular MAPK pathway, HGF promotes cell growth, survival and metastasis (437). Production of HGF either by CAFs or tumour cells is potentially due to mutations in the HGF promoter gene found in a proportion of CRC patients (438). In keeping with our findings of elevated HGF levels in patients with a high NLR at baseline, HGF levels in peripheral blood and in colonic tumour tissue were higher compared to normal controls (439). Increased levels were also associated with LN and liver metastases and tumour size. Furthermore, elevated HGF correlates with serum CEA and survival in both early stage (440) and later stage disease (441). As such, the raised levels of HGF detected in high NLR patients may contribute to their considerably reduced immune activation potential prior to treatment.

IL-8 plays a role in the chemotaxis of both neutrophils and lymphocytes to sites of inflammation or infection (164,442). IL-8 can also stimulate angiogenesis (82,443) and its production can be induced in the presence of factors such as hypoxia and acidosis (such as is found in the TME of CRC) (443). IL-8 can be produced by tumour cells of various aetiologies,

including but not limited to, breast cancer, lung cancer, HCC, melanoma and CRC. Furthermore, it is associated with tumour progression, stage and outcome (444)(445)(446). Although IL-8 is upregulated in inflammatory bowel disease (such as ulcerative colitis), it is significantly more elevated in CRC specimens (447) and is correlated with the presence of liver metastases. *In vitro*, CRC cell lines can produce endogenous IL-8, which, in turn, stimulates both angiogenesis and cell growth (448). Interestingly, there is evidence that migration of PBMCs, specifically Tregs, into the TME can be induced by IL-6 and IL-8 secretion by tumour cells (449). The mechanism of this is thought to be the upregulation of IL-8 receptors, CXCL1 and CXCL2, on Tregs by IL-6. In summary, increased IL-8 is associated with more advanced stages of CRC, which produce further IL-8, supporting angiogenesis and influx of Tregs into the TME of patients with a high NLR. This data demonstrates that IL-8 is also associated with elevated NLR and immune dysfunction *in vitro*.

A large proportion of cytokines/chemokines that we analysed were elevated prior to treatment in the low NLR group (Figure 4.13). One of these was IL-16 (also known as lymphocyte chemoattractant factor, LCF). As well as migration, IL-16 can also stimulate the growth/proliferation of CD4+ cells. In keeping with our findings that a high NLR may correspond with a depressed immune response, IL-16 has been shown to be differentially expressed in colon cancers. MSI cancers, for example, shown previously to demonstrate a more immunogenic phenotype (370,371,429,450) are associated with higher levels of IL-16 (451).

The chemokine TRAIL can initiate apoptosis of tumour cells. It is produced by T cells, NK cells, monocytes and DCs. Supporting our findings of lower plasma TRAIL in high NLR patients (Figure 4.13), a relatively lower expression of TRAIL has been documented in carcinomas compared to normal epithelium or adenomas (130). This, along with our findings, may suggest a downregulation of TRAIL to avoid apoptosis: in our cohort of high NLR patients, downregulation of TRAIL may be one of the mechanisms contributing to the development of more aggressive/widespread disease. Interestingly (and relevant to our data), CPT-11 (irinotecan) can upregulate TRAIL receptors (135) and combination therapy with 5-FU or CPT-11 can decrease tumour growth *in vivo* (452).

Eotaxin, also known as CCL11, an eosinophil chemokine in the same family as RANTES, has also been implicated in angiogenesis (453) and metastasis of cancer cells through its effects on MMPs (453,454). In our investigation, low NLR patients had more circulating eotaxin (Figure 4.13); levels of eotaxin are known to be elevated in the serum of patients with breast,

lung and CRC (455,456). Similarly, greater concentrations of eotaxin are detected in supernatants of hepatic metastases from CRC tumours, above that found in normal liver (457). In keeping with our other findings, decreased levels of eotaxin suggests a depressed immune response and potentially decreased immune infiltration into the tumour of high NLR patients.

RANTES is also a chemokine which induces the recruitment of monocytes and macrophages into the TME. Subsequently, such immune infiltrates can undergo differentiation into a more tumorigenic phenotype (so-called M2 TAMs and N2 TANs). It may be expected, then, that the high NLR population would have a higher plasma levels if we are postulating that the TME is more immunosuppressive. Comparable with our findings, Johdi *et al.* found that levels of RANTES are lower in the serum of patients with colorectal polyps and CRC (though not significantly so) than healthy controls (456). As with the other pro-inflammatory solutes that were found to be lower in the high NLR groups, it is unclear whether this is a result of a more immunosuppressive environment (immune tolerance to a tumour which should elicit a brisk anti-tumour response) or whether the patients with a high NLR have so because these pro-immune cytokines are decreased. As part of the process resolving inflammation, RANTES can be sequestered by apoptotic neutrophils and T cells through enhanced expression of its receptor, CCR5 (458). Upregulation of CCR5 can be seen in the presence of markers of immune exhaustion such as PD-1, which was also raised in our patient population (458). The processes of sequestering this and other pro-inflammatory cytokines may be further stimulated by the presence of IL-10 (459). This may support the idea of some kind of immune exhaustion/tolerance in the high NLR patients which contributes to lower plasma levels of inflammatory cytokines.

IP-10 was elevated in the group of patients with low pre-treatment NLR (Figure 4.13). One of its many actions is as a chemoattractant for monocytes and T cells (460) and it's production in response to IFN-γ contributes to the migration of activated T cells into sites of inflammation or into tumours. Illustrating this, IP-10 is elevated in CRC patients with MSI tumours compared to those whose tumour demonstrated MSS (451). This would be in keeping with an enhanced immune response in MSI tumours. Although our cohort of CRC patients did not include anyone with confirmed MSI tumours, our other findings support a depressed immune response in patients with high NLR over a number of different parameters. In this context, the lower levels of IP-10 in patients with a high NLR would support our other findings. IP-10, along with MIG, which was also decreased in our cohort of high NLR patients, exhibits angiostatic features. The anti-tumourigenic properties of both IP-10 and MIG have been attributed to

their chemotactic properties: the recruitment of immune cells including T cells and NK cells contribute to the anti-tumour effects of other cytokines, such as IL-12.

Cutaneous T cell-attracting chemokine (CTACK, also known as CCL27), is another T cell attractant. It has been described as having a prominent role in inflammation of the skin and, as such, has been shown to be encourage chemotaxis of immune cells in models of melanoma (461). Following transfection with adenovirus expressing CTACK, the CRC cell line CT26 was injected into BALB/c mice, leading to a reduction in tumour growth compared to CRC transfected with viral vector alone (462). Although this was not significant, it may indicate that the expression of CTACK by CRC cells can contribute to an anti-tumour immune effect and this would be supported by our findings of increased levels of CTACK in the patients with a low NLR (Figure 4.13). Although there is no published data, it may be worth considering whether chemotherapy can increase production of CTACK by tumour cells and increase the chemotaxis of immune cells into the tumour.

While we observed that patients with a low NLR had higher levels of plasma SCF than those with a high baseline NLR (Figure 4.13), other groups have demonstrated the contrary. In a study of 75 patients, levels of SCF in serum were actually lower in patients undergoing surgical resection for CRC compared to healthy controls (463). Although we are postulating that a high NLR is associated with a poorer prognosis and that this could correlate with more advantaged stage of disease, patients with a high NLR may have a higher neutrophil count. As such, patients with a low NLR may be predisposed towards having a low neutrophil count and as SCF is a haematopoietic growth factor, it may be that neutropenia in these patients can stimulate the production of SCF as a compensatory mechanism. Similar phenomena have been seen in mice treated with 5-FU chemotherapy, where SCF was shown to reverse thrombocytopenia in treated mice (464).

Unlike IL-16, MIG and SCF, levels of eotaxin, IL-8 and RANTES did not vary over the first 6 weeks of chemotherapy (Figure 4.14). Again this may contribute to the ongoing differences in prognosis between the high and low NLR groups, despite normalisation of the NLR in the former.

To expand on our understanding of NLR status and response to chemotherapy within the innate immune system, the adaptive arm of the immune system was also investigated; T cell responses were examined by ELISpot. There was an indication that T cell responses in patients with a high baseline NLR were attenuated against the CEF viral peptide pool but this was not

statistically significant (Figure 4.31). Interestingly, in those patients where the NLR dropped over the first 6 weeks of chemotherapy, T cell responses to non-specific viral peptides increased as the NLR decreased. Conversely, in patients with a consistently low NLR, it was the responses to CEA which increased (Figure 4.32).

There is conflicting data as to whether T cell responses against TAAs confer clinical benefit. In another cohort of patients with CRC, responses to TAAs were varied, with a greater proportion of patients with later stage disease registering a response. In this group, there was no survival benefit seen (465). In contradiction to this data, Scurr *et al*. found that CEA-specific responses were associated with a poorer outcome, even when patients were stratified by stage (384). Whether a positive CEA response was actually indicative of occult metastatic disease in earlier stage patients (not included in our study) is unclear.

Given the decline in Tregs over time in our data (independent of baseline NLR), an increase in IFN-γ effector T cell responses during therapy may have been expected. The findings of Clarke *et al.* further support the role of Tregs in suppressing T cell responses: Treg depletion was associated with increased response to the 5T4 tumour antigen (56).

Overall, the data presented in this chapter suggests a depressed immune response in patients with a high baseline NLR, as measured by a number of different parameters. Following 6 weeks of chemotherapy, however, there appeared to be evidence of elevated immune responses comparative to baseline if a patient's NLR decreased to <5. Despite this, OS in the group with a reduction in NLR following chemotherapy is still poorer than those patients whose NLR stayed low throughout. Non-specific (CEF) T cell responses in this group seemed to increase over time but if this is not tumour-specific (similar to the CEA response in the low-low group) this may contribute to the poorer prognosis in this group of patients. These findings support further exploration into the role of immunotherapy following chemotherapy in patients with CRC given the change in the immune milieu which has been detected. Although there may be potential clinical benefit to this strategy in CRC patients with high or low baseline NLR, there may be additional advantages in the former group, who traditionally have a poorer outcome with standard chemotherapy.

Chapter 5 Immune Responses in Melanoma

5.1 Introduction

5.1.1 Melanoma

The incidence of melanoma skin cancer is increasing, with over 15,000 new cases diagnosed in 2014, making it the fifth most common cancer in the UK (466). As incidence increases with age, the burden of disease continues to rise with an ageing population. Despite the rapid improvement in treatment options over the last five years, malignant melanoma still accounts for nearly 2,500 deaths per year (466). Once LN-positive, five-year survival falls to approximately 50 %. When distant metastases are present, prognosis may be only a few months (467) and one year survival for stage IV disease is less than 50 % (468).

Risk factors for the development of melanoma include fair skin which burns easily, red or fair hair, UV exposure (previous history of sunburn or use of sunbeds, especially at less than 35-years of age (469)) and a genetic disposition or family history. Individuals with greater than 50 moles or with dysplastic (abnormal appearing) naevi are also at increased risk as they may be a sign of familial conditions such as atypical mole syndrome or familial atypical multiple mole-melanoma syndrome. Melanoma is more likely to develop on the trunk or limbs, although subtypes include mucosal and uveal melanoma.

Until recently, the mainstay of treatment was dacarbazine chemotherapy but the emergence of novel therapies have altered this paradigm. For the management of melanoma with a mutation of the *BRAF* gene, vemurafenib can increase OS from 9.7 to 13.6 months compared to dacarbazine (470). More recently, use of the anti- CTLA-4 antibody ipilimumab and anti-PD-1/PD-L1 receptor antibodies (immune checkpoint inhibitors) - used either alone or in combination - can increase OS by modulating the immune response (189,471–473).

5.1.2 Pathology and Staging of Melanoma

Melanoma arises from melanocytes, the pigmented cells between the epidermis and dermis. Pigmentation is due to the production of melanin by melanocytes. Initially melanoma spre ads outwards (the radial growth phase) and if detected and treated at this stage, outcomes can be excellent. However, once lesions enter a vertical growth phase (VGP) and begin to grow downwards into deeper layers of the dermis, the risk of metastatic spread increases, at which

point the disease can become essentially incurable (474). Suspicious lesions are subject to the 'ABCDE' assessment: whether they are **a**symmetrical, with irregular **b**orders, have **c**olour change with a **d**iameter over 6 mm or whether they **e**volve over time (increase in size, bleed, itch or form nodules). An excision biopsy of the lesion (with appropriate surgical margins) will then determine the diagnosis.

The pathological subtypes of melanoma include (475):

- Superficial spreading melanoma (SSM)
- Uveal melanoma
- Mucosal melanoma
- Desmoplastic melanoma
- Lentigo maligna melanoma
- Nodular melanoma
- Acral lentiginous melanoma

As well as pathological classification, staging, especially of localised melanomas includes tumour depth (Breslow thickness), number of mitoses (as a measure of tumour proliferation), presence of TILs and the presence of ulceration. The extent of disease is determined using TNM staging classification (Table 5.1) (476). Measurement of serum LDH is also used in metastatic disease and has been shown to be prognostic, with patients having an elevated LDH at baseline having a shorter OS (477). Sentinel node biopsy is used to determine the presence of micrometastatic LN disease, whereas macrometastatic LN disease is detected clinically.

The histological features of melanoma can significantly impact survival. Ten-year survival for T1 disease (less than 1 mm thickness) is 92 %, falling to 50 % for T4 disease (melanoma greater than 4 mm thick) (476). Similarly, the presence of ulceration in the primary tumour can reduce survival: ulcerated T4 disease (T4b) has a five-year survival of 53 %, whereas non-ulcerated disease of the same T stage (T4a) is 71 % (476). OS also diminishes with increasing age: for early stage disease (I and II) five-year survival for patients aged between 40 - 49 is over 80 %, reducing to 60 % in those over 80 years of age (467).

Table 5.1 TNM staging of melanoma (adapted from 7th AJCC) (476)

Stage	Tumour	Thickness (mm)	Ulceration/Mitosis
I	T1	≤ 1 mm	**T1a** Without ulceration and mitosis < 1/mm^2
			T1b With ulceration or mitosis ≥ 1/mm^2
	T2	1.01 – 2 mm	**T2a** Without ulceration
II			**T2b** With ulceration
	T3	2.01 – 4 mm	**T3a** Without ulceration
			T3b With ulceration
	T4	> 4 mm	**T4a** Without ulceration
			T4b With ulceration
	Nodes	**Metastatic Nodes**	**Nodal Burden**
III	N1	1	**N1a** Micrometastasis
			N1b Macrometastasis
	N2	2-3	**N2a** Micrometastasis
			N2b Macrometastasis
			N2c In transit metastasis
	N3	≥ 4 LN or/and in-transit metastases	
	Metastases	**Site**	**Serum LDH**
IV	M1a	Distant skin, LN or lung	Normal
	M1b	Any visceral	Normal
	M1c	Any distant	Elevated

5.1.3 Management of Localised Disease

Surgical management is the cornerstone of treatment in localised disease. Surgical margins for melanomas with a Breslow thickness of up to 2 mm should be 1 cm (for stage I disease) and 2 cm for lesions with a thickness of greater than 2 mm (stage II disease) (478,479). Sentinel LN biopsy (SNB) is considered for patients with lesions greater than 1 mm thick and, if positive, completion lymphadenectomy should be considered (479,480). The presence of ulceration in the primary tumour and higher Breslow thickness both confer a higher risk of positive SNB (481). LN dissection can also be considered for symptom control for patients with palpable disease.

For inoperable local disease of the upper or lower limb, systemic therapy may be considered but local chemotherapy (usually melphalan) can be given by isolated limb perfusion (ILP). During this procedure, the blood supply to the affected limb is isolated and chemotherapy

given in higher doses than would be tolerated systemically. Isolated limb infusion is a similar but simpler process, where chemotherapy is administered through radiologically-placed catheters, rather than ILP where a perfusion machine (similar to a cardiopulmonary bypass machine) is required to keep the limb oxygenated. Using these techniques, CR or PR rates can approach 50 %. (reviewed in (482)).

Adjuvant therapy for melanoma has been the major change over the last few years. In a phase III trial of adjuvant ipilimumab, given until PD, five-year recurrence-free survival (RFS) in the treatment group was significantly increased (40.8% *vs.* 30.3 %) (483). However, serious toxicity (grade 3 or 4) was observed in 54.1 % patients and single agent ipilimumab is currently not approved for use by the NICE. In contrast, trials of single agent anti-PD-1 inhibitors have shown significant benefit without unacceptable toxicity. In patients with resected stage III disease, one year of adjuvant pembrolizumab resulted in an increased one year RFS, compared to placebo, (75.4 % vs. 61.0 %) (484). In patients with mutations in *BRAF*, treatment with one year of combined therapy following resection (dabrafenib and trametinib) not only increased RFS, but also OS (86% in the combination-therapy group *vs.* 77% in the placebo group; 95% CI 0.42 to 0.79; p=0.0006) (485).

5.1.4 Management of Advanced Disease

5.1.4.1 Chemotherapy

Although currently not usually first-line therapy, due to the many other treatment options available, chemotherapy with dacarbazine (DTIC) or carboplatin was the mainstay of treatment for many years. A small randomised phase III trial (61 patients) did not demonstrate a significant improvement in OS when IFN-α was added to DTIC chemotherapy (486). In addition, despite trials of combination therapy, RRs rarely exceed 20%. Trials of carboplatin chemotherapy have demonstrated similar disappointing results. In one phase II trial of carboplatin, the RR of patients with stage IV disease was 16 % (487) and in another the RR was only 11 % with a median OS of 4.7 months (488).

Temozolomide (TMZ) is an oral analogue of DTIC which has shown some activity in melanoma in early phase trials. It has an advantage over DTIC as it can cross the blood brain barrier, so has been investigated in patients with melanoma brain metastases (489). In phase III trials, RR with TMZ alone was 13.4 % (490). When compared with standard-of-care DTIC, TMZ has been shown to be comparable in terms of OS (7.7 months *vs.* 6.4 months, respectively), although

this phase III trial did not include patients with central nervous system (CNS) metastases (491). In a retrospective study of patients receiving either DTIC or TMZ, the incidence of CNS disease was significantly lower in the TMZ group (492).

5.1.4.2 Targeted Therapy

5.1.4.2.1 BRAF Inhibition

The use of targeted treatments has revolutionised the management of mutation-positive melanoma. The most common mutation in melanoma is in BRAF, a RAF kinase, which is found in approximately 40 % of patients with melanoma. Mutations in BRAF lead to continuous activation of the MAPK pathway (493). In the absence of mutations in the pathway, such as BRAF, the MAPK pathway is usually activated by cell surface receptors (receptor tyrosine kinases, RTKs), such as the EFGR. Mutations in BRAF lead to a nucleotide variation at position 600 of the kinase. The most common of these (up to 80 %) is V600E, where valine is exchanged for glutamic acid, although other mutations include V600K (lysine in place of valine).

The BRAF inhibitors (BRAFi) include vemurafenib and dabrafenib and can confer dramatic benefits to patients with BRAF-mutated melanoma in terms of disease control and OS (494). In the BRIM-3 trial of vemurafenib vs. DTIC, there was a significant increase in OS (13·6 months vs. 9·7 months, respectively) with over 50 % of patients treated with BRAFi having an objective disease response (470). Similarly, in another phase III trial comparing DTIC to dabrafenib, OS was prolonged in the BRAFi group (15.6 months vs. 18.2 months, respectively) (495,496). The most common toxicities of BRAFi include pyrexia, arthralgia, diarrhoea and prolonged QT interval on electrocardiogram (470). In a class-effect manner (due to their method of action) possible side-effects also include the development of skin lesions such as squamous cell carcinoma (SCC) (497). This is due to the effect of BRAFi on WT non-melanomatous cells: blocking BRAF in this situation may reveal other mutations in the MAPK pathway which then drive the development of SCC (498). Despite the good responses demonstrated by patients on BRAFi, resistance to therapy will develop in most cases, usually due to the development of secondary mutations in the MAPK pathway or by signalling through alternative mechanisms, such as the PI3K/AKT pathway or upregulation of other cell-surface RTKs (499,500).

5.1.4.2.2 MEK Inhibition

MAPK/ERK kinases (mitogen-activated protein kinase kinase; MEK) are further downstream from BRAF in the MAPK pathway. Combination therapy with a MEK inhibitor (MEKi) and a BRAFi is one way of avoiding resistance to BRAFis: by blocking the MAPK pathway in two different ways and, by the same mechanism, decreasing the incidence of BRAFi-associated skin lesions such as SCC (501,502). Single agent MEKi has some efficacy in patients with mutations in BRAF: in comparison with DTIC, MEKi with trametinib improved PFS from 1.5 to 4.8 months and 6 month OS from 67 % to 81 % in patients with metastatic melanoma (503). Clinical trials of combination therapy with BRAF/MEKi have shown significantly increased OS and PFS compared to single agent BRAFi (502,504–506). In these studies RRs for combination therapy were over 60 % (502,504,506).

5.1.4.3 Immunotherapy

The immune system has been shown to play an important part in the pathogenesis of melanoma, with tumours displaying a large proportion of somatic mutations (507) which have the potential to act as neoantigens to prime an anti-tumour response. This is illustrated by case reports of spontaneous melanoma regression (508,509) but this phenomenon is halted in most patients by the activity of immune checkpoints such as anti-CTLA-4 and anti-PD-1. The presence of PD-1 expression on TILs correlates with decreased OS and increased Breslow thickness (201). There are a number of melanoma TAAs which have been identified, including MART-1 and glycoprotein 100 (gp100) (471) which are prevalent in melanoma tumours (510). As such, they have the potential to be targeted by antigen-specific T cells, if this population can be harnessed therapeutically. Melanoma TILs have been correlated with outcome and the presence of positive sentinel node biopsy (481,511). Similarly, antigen-specific T cells are found in the peripheral blood of over half of patients with melanoma (512). Being a particularly immunogenic tumour, melanoma was one of the first conditions in which immunotherapy was thoroughly investigated and brought into clinical use. As well as the earlier use of IFN, the field has expanded to include immune checkpoint blockade and novel immune therapy such as OVs.

5.1.4.3.1 Checkpoint inhibitors

The principle of immunotherapy in melanoma must be that, although any previous immune response has not been effective in clearing the tumour, the potential for an anti-tumour immune response can be exploited by checkpoint inhibition. Anti-CTLA-4 antibodies, such as

ipilimumab and tremelimumab, block the interaction between the inhibitory molecule CTLA-4 on T cells and CD80/86 on APCs and hence block the downregulation of T cell activation and initiate immune-mediated anti-tumour responses.

A phase III trial demonstrated a significant increase in OS from 6.4 months to 10 months when patients were given ipilimumab compared to gp100 (471). Survival at two years was 23.5 %, compared to 13.7 % (471). This finding suggests that immunotherapy could potentially lead to long-term disease control, even in a small number of patients. Indeed, long-term follow up of 177 patients treated in multiple clinical trials with ipilimumab showed that the patients who had CRs demonstrated ongoing disease control for up to 99 months (ongoing at the time the study was published) (513).

Similarly, the anti-PD-1 antibodies, including nivolumab and pembrolizumab, prevent inhibition of T cell activation at the site of the tumour (and elsewhere) and can enhance anti-tumour T cell responses. Indeed, the expression of PD-L1 on melanoma tumour cells is associated with increased Breslow tumour thickness and poorer OS (201). When nivolumab was given first-line as a single agent to BRAF-WT patients with metastatic melanoma, there was an increase in one year OS compared to DTIC chemotherapy (72.9% *vs.* 42.1 %, respectively) with an objective RR of 40 % (473). Overall survival in the anti-PD-1 treatment group was not reached. Similar findings were observed in a cohort of heavily pre-treated patients receiving nivolumab: one year OS was 62 % with a median OS of 16.8 months (472).

Toxicities of the checkpoint inhibitors are class-driven and usually autoimmune in origin. Diarrhoea, nausea, fatigue and rashes are common with both anti-CTLA-4 and anti-PD-1 antibodies. The concerning toxicities include immune-mediated hepatitis, colitis, pneumonitis and hypophysitis leading to symptoms of adrenal insufficiency. Treatment is supportive, with the use of high-dose steroids or immunosuppression with agents such as the anti-TNF-α drug infliximab (514). In a phase III trial of pembrolizumab *vs*. ipilimumab (both given 3 weekly), the incidence of colitis of grade 3 or greater was reported to be 2.5 % vs. 7 %, respectively (515). The frequency of hypophysitis was 0.4% *vs.* 1.6 %. Pembrolizumab appears to have a higher incidence of hepatitis and pneumonitis (any grade) (515). Consequently, aside from these effects, the PD-1 inhibitors seem to be better tolerated. Whether pembrolizumab was given every two or three weeks, RRs were similar (approximately 33 %) compared to 11.9 % for patients treated with ipilimumab. Survival at one year for 3 weekly treatment was 68.4% in patients given pembrolizumab and 58.2% for those treated with ipilimumab (515). Due to this

and other studies, anti-PD-1 therapy is generally considered first-line prior to anti-CTLA-4 agents in BRAF-WT patients.

Combination therapy with anti-PD-1 and anti-CTLA-4 is only considered in patients with a good performance status, due to the increased risk of toxicity. Combination therapy with nivolumab and ipilimumab had a 7.7 % risk of severe colitis (compared with 0.6 % for nivolumab) and a 55 % incidence of any treatment-related severe adverse event (AE), compared to 16.3 % for nivolumab and 27.3 % for ipilimumab alone) (516). Despite this, PFS was 11.5 months in the combination treatment group, compared to 2.9 months for ipilimumab alone (516).

5.1.4.3.2 Oncolytic viruses

OVs infect, replicate in and are cytotoxic to malignant cells. They were initially thought to exert their effect primarily through this mechanism of direct oncolysis but through further study it has become clear that they can generate an immunogenic anti-tumour response, which in some cases is the more potent effect (section 1.5.4).

T-VEC is a genetically modified HSV encoding the immunomodulatory cytokine GM-CSF. In the phase I trial, T-VEC was given *i.t.* to patients with a range of solid tumours including melanoma, breast and gastrointestinal cancers (517). The main toxicities were local inflammation, erythema and flu-like symptoms or fever. There was some evidence of clinical response, including in melanoma patients and, interestingly, in both injected and uninjected lesions (517). The subsequent phase III trial of T-VEC *vs.* GM-CSF alone confirmed that T-VEC has efficacy in metastatic malignant melanoma. These results demonstrated that not only did T-VEC increase disease RRs (26.4 % *vs*. 5.7 %) but that this translated into improved median OS (23.3 months for T-VEC *vs.* 18.9 months for GM-CSF) (518,519). This further supports the concept of OVs having a systemic, rather than simply localised, anti-tumour effect. As such, T-VEC was the first OV to be recommend by NICE for the management of unresectable disease in metastatic melanoma (479). To further exploit the anti-tumour effects of T-VEC combination studies with checkpoint inhibition are currently ongoing with encouraging preliminary results (520–522).

5.1.4.3.3 Monitoring response in immunotherapy

Radiological responses to treatment, especially in the context of clinical trials are usually reported according to the Response Evaluation Criteria in Solid Tumours (RECIST) criteria. Due to the potential for increased tumour burden as a result of immune infiltration

(pseudoprogression), rather than true PD, the use of RECIST criteria in trials of immunotherapy can have obvious pitfalls. In patients treated with ipilimumab, for example, PD at 12 weeks can then lead to shrinkage of measurable lesions and become either PRs or even CRs over the following number of months (523). This has led to the development of immune-related response criteria (irRC) or immune-RECIST which aim to more accurately evaluate responses in patients receiving immunotherapy and prevent early cessation of treatment (524). In clinical trials of patients receiving pembrolizumab, 7% demonstrated some type of atypical response, including pseudoprogression and 14 % had PD on classical RECIST criteria *vs.* irRC (525). Patients with seemingly PD by RECIST (but not by irRC) had an increased two year OS compared to patients who had PD by both criteria (37.5 % *vs.* 17.3 %, respectively), showing that pseudoprogression does not translate to attenuated responses from immunotherapy (525). Amongst other recommendations in these modified response criteria, seemingly PD should be re-evaluated on repeated imaging to account for pseudoprogression (in the absence of obvious clinical deterioration). In these situations, clinical assessment of response is important in clinical decision making.

5.1.5 Immune Response in Melanoma

5.1.5.1 Innate Immunity

When NK cells from HDs are cultured with melanoma cell lines, the expression of activating receptors (including NKp30, NKp44 and NKG2D) is diminished, suggesting one method by which tumour cells, even with down-regulated MHC-I, can avoid NK cell mediated cytotoxicity (18). NK cells exposed to melanoma cells also have diminished cytotoxic potential against target tumour cells in a manner dependent on IDO (an immunosuppressive enzyme produced by some melanoma cell lines) (18). Interestingly, melanoma cells taken from different anatomical sites within the same patient have variable expression of NK-cell ligands and thus have different susceptibility to NK-mediated killing (526). Malignant cells from LNs were shown to express greater levels of ligands for NKp44 and NKp46 than skin metastases from the same patient (526). In *in vivo* models, mice injected with melanoma cells derived from skin had a shorter survival than those receiving cells derived from nodal metastases, in the presence of small numbers of NK cells (526). When the mice also received large numbers of NK cells, all tumours were controlled and survival was prolonged, underlying the role of NK cells in immune-surveillance and the importance of NK ligand expression in the TME.

Although NK cells exhibit high levels of cytotoxicity *ex vivo*, the efficacy of this potential can change at E:T ratios more in keeping with that seen in the TME. Indeed, when NK cells are cultured with melanoma cell lines at such ratios, the decrease in target tumour cell viability was seen to slow and cease after two days (527). This phenomenon was due to upregulation of MHC-I molecules on melanoma cells, mediated by NK cell production of IFN-γ, which, in turn, inhibited NK cell function (by engaging with inhibitory cell surface receptors). This illustrates that, while NK activity may be enough to contain smaller tumours, once the tumour burden increases, NK-mediated cytotoxicity is diminished through a number of mechanisms, leading to tumour escape (527). As well as lower frequencies of activating receptors, NK cells in patients with melanoma express higher levels of inhibitory receptors than HDs (25). For example, higher levels of TIM-3, which gradually increased on the population of peripheral NK cells with stage of disease, is associated with poor prognostic factors such as ulceration of the primary tumour and Breslow thickness > 1mm (25). Although these cells are functionally impaired and displayed this exhausted phenotype, TIM-3 antibody blockade increased NK cytotoxicity against melanoma cell lines and IFN-γ production (25). Interestingly the BRAFi vemurafenib has been shown to modify the immunogenicity of NK cells by increasing expression of the ligand for NKp30 but also increasing inhibitory NK cell receptor ligands such as MHC-I and decreasing other activating ligands for NKG2D (528). Treatment of melanoma cells with vemurafenib also diminishes NK cell degranulation and IFN-γ expression *in vitro* (528), although there is conflicting data around this (529). This data should not be taken in isolation, however, given the direct cytotoxic effect of BRAFi on mutated melanoma cells and the influence they have over the cytokine profile. As such, the inhibitory effect of MEKi (by the modulation of NK activating receptors) can be attenuated by cytokines which would be found in the TME such as IL-15 and IL-18 (529).

5.1.5.2 Adaptive Immunity

Circulating melanoma antigen-specific CD8+ T cells have been detected in the peripheral blood of patients with stage III/IV melanoma; however, they were found to be functionally poor (530). Although a majority of cells were phenotypically consistent with memory T cells, they were unable to either express CD69 in response to stimulation, effectively lyse melanoma cell lines and/or produce IFN-γ and TNF-α (530). Other studies have seemingly confirmed this finding in patients with high levels of MART-1-specific CD8+ cells. While the presence of antigen-specific memory T cells was not associated with an increased survival, patients who had PD in the first two months of chemotherapy (DTIC or TMZ) had a higher

proportion of MART-1-positive T cells (531). This was thought, in part, to be attributable to both a high antigen load in later-stage disease and loss of HLA-A molecules on matched tumour cells, thus inhibiting effective antigen presentation and activation of a CTL response. Antigen-specific T cells in peripheral blood express PD-1 and target patient-specific neoantigens (532). Examination of the peripheral blood of patients with metastatic melanoma has revealed the presence of antigen-specific Tregs which express FoxP3 and secrete IL-10 on stimulation with antigen-peptides (such as NY-ESO-1) (533). These Tregs were also able to suppress T cell proliferation *in vitro*.

It has been postulated that BRAF-mutated melanoma may be more immunogenic than BRAF-WT; examination of PBMCs from BRAF-mutated melanoma patients has revealed populations of BRAF-mutant specific CD4+ and CD8+ T cells (534,535). Treatment with BRAFi could, therefore, stimulate a direct cytotoxic effect as well as an immunogenic one by increasing the proportion of antigen-positive T cells and diminishing the immunosuppressive influence on the background of an already 'primed' immune response (536,537).

The effect of checkpoint inhibition on both the innate and adaptive immune response is discussed in detail in section 1.5.2.

5.1.5.3 Tumour Infiltrates

The prognostic implication of TILs is well documented in many cancers, including melanoma, where it is seen to be a surrogate marker of an anti-tumour immune response. The phenotype of these cells is also important in determining the balance between pro-tumour and an anti-tumour TME (538). In melanoma, infiltration of TILs is subdivided into absent, non-brisk (local infiltration) or brisk (extensive infiltration throughout the VGP). Perhaps as expected, the absence of TILs in the primary melanoma have also been shown to predict for positive SNB: a brisk TIL infiltrate was associated with a 3.9 % chance of positive SNB, compared to 26.2 % for melanomas with absent TILs (481). Data on the prognostic value of TILs in melanoma is conflicting, with some studies showing a strong advantage with the presence of brisk TILs in VGP (5 year OS 77 % *vs.* 37 % for absent TILs) (511) and others showing no advantage (five year OS 86 % for presence of TILs vs. 90.1 % with absent TILs) (539). The phenotype of TILs is obviously important and so even melanomas with a brisk TIL infiltrate may have a poorer prognosis if the T cells are exhausted and anergic. As such, the presence of peritumoural T cells positive for the activation markers CD25 and OX40 is associated with decreased metastatic potential and increased OS (212). Similarly, while the presence of Fox P3+ Tregs in

the TME suppress the anti-tumour responses of CD4+ and CD8+ T cells (540), higher expression of T_H1 cytokines (such as IL-2) in primary melanoma samples are associated with tumour regression (541).

BRAFi can alter immune cell infiltration into melanoma: CD8+ TILs were seen to increase following treatment (536) and diminished again on PD (537). Greater infiltration of CTLs is also associated with elevated melanoma antigen presentation in tumour biopsies (536). This was also true for patients receiving dual BRAFi/MEKi. Interestingly, one patient from this study who developed PD on single agent BRAFi and had decreased CTLs on tumour biopsy at that time was commenced on dual therapy with the addition of a MEKi. Further tumour biopsy demonstrated an increase in CD8+ infiltrate and positive antigen staining (536). As expression of TIM-3 and PD-1 rise after BRAFi (presumably as a marker of exhaustion), there is a rationale for combined BRAF/checkpoint inhibitor therapy. Phase I/II trials of dual BRAFi/MEKi plus anti-PD-1 antibody (NCT02130466) or anti-PD-L1 antibody (NCT02027961, NCT01656642) are ongoing.

In a cohort of patients receiving treatment with the anti-PD-1 antibody nivolumab (the majority being patients with melanoma), evaluation of archival tissue revealed that PD-L1 expression on tumour cells was associated with an enhanced tumour immune cell infiltrate and increased expression of both PD-1 and PD-L1 on immune cells (325). This correlated with both clinical benefit (disease response/stability for >6 months) and objective response by RECIST (325).

5.1.5.4 Cytokine Production

The cytokine profile of the TME and the immune response can influence the pathophysiology of melanoma and its response to therapy. Cultured melanoma cell lines express low levels of IFN-γ, IL-2 and TNF-α but higher levels of IL-8, IL-6 and TGF-β (446). Although normal melanocytes can produce low levels of TGF-β, levels of the isoforms TGF-β2 and TGF-β3 increase in progression from melanocytes to *in situ* melanomas and finally, to metastatic melanoma (542). These findings suggest a possible role for TGF-β in the metastatic potential of melanoma, presumably contributing to the suppression of an anti-tumour response. Similarly, expression of IL-10 by melanocytes/melanoma cells has been observed to correlate to tumour stage (543). Interestingly, increased levels of IL-10 corresponded to a shift between the radial and vertical growth phases in melanoma tissue samples (543).

Higher serum levels of MCP-1 are associated with a lower RECIST response to treatment with BRAFi (161). Once again, as a chemokine for macrophages, this may represent an increase in M2 TAMs, which are associated with poorer prognosis (section 1.2.6).

IL-8 is a potent chemokine for granulocytes (particularly neutrophils) and thus contributes to the phagocytosis of pathogens. It is also pro-angiogenic and has been implicated in tumour development (544). Transfection of melanoma cells with IL-8 promoted both tumourigenic and metastatic potential by upregulating MMP-2 in *in vivo* models (545). As well as IL-8, levels of other angiogenic cytokines, such as VEGF, are noted to be elevated in patients with melanoma (546). Higher serum levels of both IL-8 and VEGF are associated with poor prognosis and lower PFS (546). Treatment of melanoma cell lines with a MEKi *in vitro* led to a reduction in levels of both IL-8 and VEGF (547). Similarly, in patients treated with BRAFi, IL-8 decreased significantly over the course of therapy, which, in turn, correlated with a rise in CD8+ CTLs in paired tumour biopsies (161). Surprisingly, a larger decline in serum levels of IL-8 corresponded to a reduction in OS (161). Higher baseline IL-8 levels were associated with enhanced tumour burden, potentially identifying a group of patients with a poorer prognosis compared to the group with lower baseline IL-8 levels. Although treatment with BRAFi/MEKi can potentially initiate anti-tumour responses through the release of TAAs, these data suggest that they can also manipulate the TME and systemic immune response in other ways. Similarly, a decrease in IL-8 with anti-PD-1 antibody therapy correlates with disease response and an early fall in IL-8 (within the first few weeks of therapy) with improved OS (548). Interestingly, in the three non-melanoma patients in this study who exhibited pseudoprogression on imaging, IL-8 levels remained low (548), suggesting the potential for IL-8 as a biomarker for checkpoint inhibitor therapy.

5.1.6 Rationale for Current Study

The current study aims to further delineate the immune response in patients receiving immune checkpoint inhibitors for treatment of metastatic melanoma. We aimed to investigate the response of patients receiving first-line treatment, as previous data from early phase trials likely included heavily pre-treated patients, in whom immune responses may be skewed due to the advanced nature of the disease and previous lines of treatment.

Peripheral blood from patients receiving first-line checkpoint inhibitors for metastatic melanoma (ipilimumab or pembrolizumab) was analysed to determine the immune response to these therapies, specifically:

1. Further validation of the panel of immune assays, showing that the protocols we have developed are robust in different cohorts of patients.
2. Immune cell activation potential using a novel immunophenotyping panel, NK degranulation and ^{51}Cr-release assays and analysis of the adaptive immune response through the assessment of IFN-γ production to specific melanoma TAAs and viral peptides in ELISpot assays.
3. Analysis of patterns of cytokine production using Luminex assays.
4. Comparison of the changes in immune response between patients receiving the anti-CTLA-4 antibody ipilimumab and the anti-PD-1 antibody pembrolizumab.
5. Identification of patterns in the immune response between patients with melanoma receiving immunotherapy and patients with CRC receiving chemotherapy.

5.2 Melanoma Results

5.2.1 Patient Recruitment and Demographics

Seven patients due to receive standard-of-care ipilimumab were consented for blood sample collection between April 2015 and March 2016. A dose of 3mg/kg was administered every three weeks until progression or unacceptable toxicity, for a maximum of four cycles (12 weeks). Sequential blood samples at baseline, 6 weeks and 12 weeks were collected and analysed from three patients (43%) (Figure 5.1). A further three patients had only baseline samples collected. One patient had two samples collected. Where samples were not collected, this was usually due to clinical deterioration, patients not returning to clinic or declining blood sampling. Only patients who had multiple samples taken (including a baseline sample) were included in the analysis of change in immune function over time.

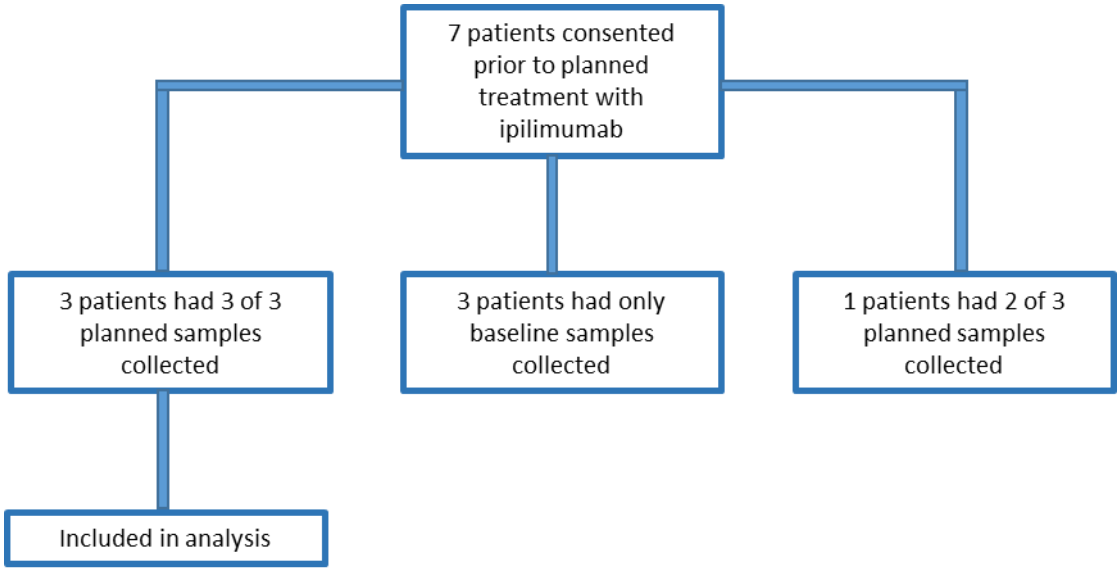


Figure 5.1 Recruitment of patients prior to ipilimumab therapy

During the period of the study, treatment with pembrolizumab became the standard-of-care for first line treatment of melanoma as an alternative to BRAFi. Seventeen patients due to receive pembrolizumab were consented for blood sample collection between December 2015 and April 2017. Treatment was administered every three weeks at a dose of 2mg/kg until disease progression or toxicity. One patient was subsequently discovered to have an additional diagnosis of B cell chronic lymphocytic leukaemia (CLL) and so was excluded from

the analysis. Single baseline samples were received from two patients and one patient only had samples taken at baseline and 12 weeks. Four patients had samples taken at baseline and 6 weeks only. A total of nine patients (53%) had all three planned samples collected (Figure 5.2). Once again, where samples were not collected, this was due to clinical deterioration or death.

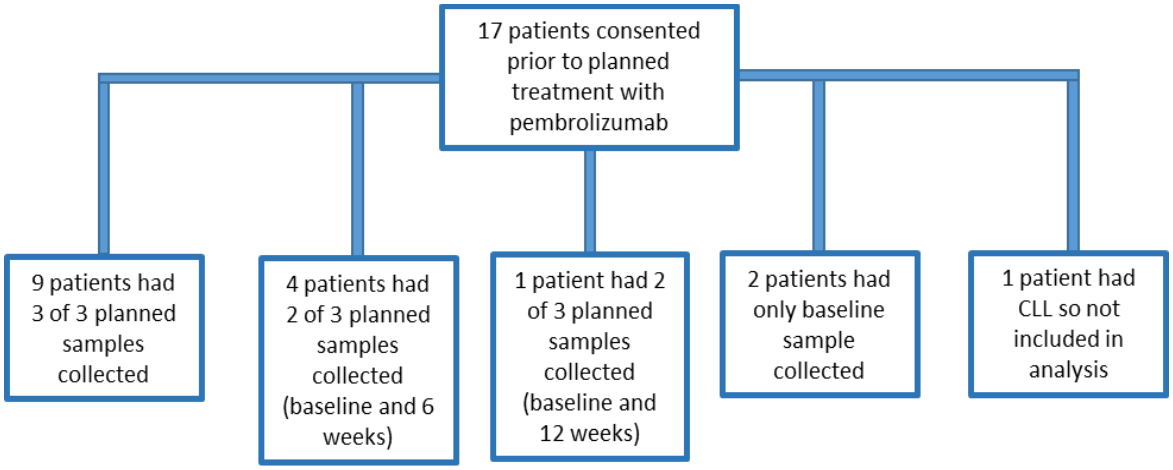


Figure 5.2 Recruitment of patients prior to pembrolizumab therapy

As previously, only patients who had multiple samples taken (including a baseline sample) were included in the analysis of change in immune function over time. Sample collection for all patients is shown in Appendix Tables 8.7 and 8.8. Patient demographics for those being treated with ipilimumab are shown in Table 5.2 (n=3). Patient demographics for those being treated with pembrolizumab are shown in Table 5.3 (n=16).

Table 5.2 Patient demographics for those receiving treatment with ipilimumab.

Patient Demographics (n/%)							
Sex		**Mean Age**		**ECOG Performance Status at Baseline**			
Male	3 (100 %)	Male	70.3 years	0		1 (33 %)	
Female	0	Female	0	1-2		2 (67 %)	
		Both	70.3 years	3-4		0	
Primary Tumour Site				**Previous Therapy**			
Upper limb		1 (33 %)		IFN		1	
Lower limb		1 (33 %)		BRAFi		0	
Torso		0		MEKi		1	
Head/Neck		1 (33 %)		Temozolomide		0	
Unknown primary		0		Ipilimumab		0	
				No previous therapy		1	
Histology of Primary Tumour		**Number of Metastatic Sites**		**Mutational Status**		**Breslow Thickness of Primary Tumour**	
SSM	1 (33 %)	1	2 (67 %)	BRAF	0	≤ 1 mm	0
Nodular	0	2	0	NRAS	1 (33 %)	1.01 – 2 mm	1 (50 %)
Ocular	0	≥3	1 (33 %)	No mutation	2 (67 %)	2.01 – 4 mm	0
Unknown	2 (67 %)					> 4 mm	1 (50 %)

Table 5.3 Patient demographics for those receiving treatment with pembrolizumab.

Patient Demographics (n/%)							
Sex		**Mean Age**		**ECOG Performance Status at Baseline**			
Male	13 (81 %)	Male	58.2 years	0	5 (31 %)		
Female	3 (19 %)	Female	56.3 years	1-2	11 (69 %)		
		Both	57.9 years	3-4	0		
Primary Tumour Site				**Previous Therapy**			
Upper limb		2 (12.5 %)		IFN	0		
Lower limb		6 (37.5 %)		BRAFi	4		
Torso		3 (19 %)		MEKi	0		
Head/Neck		1 (6 %)		Temozolomide	1		
Unknown primary		4 (25 %)		Ipilimumab	1		
				No previous therapy	11		
Histology of Primary Tumour		**Number of Metastatic Sites**		**Mutational Status**		**Breslow Thickness of Primary Tumour**	
SSM	6 (37.5 %)	1	4 (25 %)	BRAF	6 (37.5 %)	≤ 1 mm	2 (18 %)
Nodular	5 (31.5 %)	2	8 (50 %)	NRAS	4 (25 %)	1.01 – 2 mm	3 (28 %)
Ocular	1 (6 %)	≥3	4 (25 %)	No mutation	6 (37.5 %)	2.01 – 4 mm	2 (18 %)
Unknown	4 (25 %)					> 4 mm	4 (36 %)

Peripheral blood subsets and clinical markers are shown in Table 5.4.

Table 5.4 Patient peripheral blood parameters prior to therapy

Patient Demographics (n/%)		
	Ipilimumab (n=3)	**Pembrolizumab (n=16)**
Baseline Platelet Count		
≥ULN	1 (33 %)	1 (6 %)
<ULN	2 (67 %)	15 (94 %)
Baseline Neutrophil Count		
≥ULN	1 (33 %)	2 (12.5 %)
<ULN	2 (67 %)	14 (87.5 %)
Baseline Lymphocyte Count		
≤LLN	0	2 (12.5 %)
>LLN	3 (100 %)	14 (87.5 %)
Serum LDH		
≥ ULN	1 (33 %)	10 (62.5 %)
< ULN	2 (67 %)	6 (37.5 %)

5.2.1.1 Survival outcomes

Patients were stratified to determine if any survival advantage was apparent. No patients were lost to follow-up for survival data. Data was censored on 4th September 2017. Median OS for the whole group (those treated with ipilimumab and those treated with pembrolizumab) was 202.5 days. There was no survival difference seen with either Breslow thickness at baseline or BRAF mutational status (data not shown). There was increased survival in patients with an LDH level of less than the ULN but likely due to the small number of patients, this did not meet statistical significance (Figure 5.3). This had been shown in other analyses of patients with melanoma (477,549).

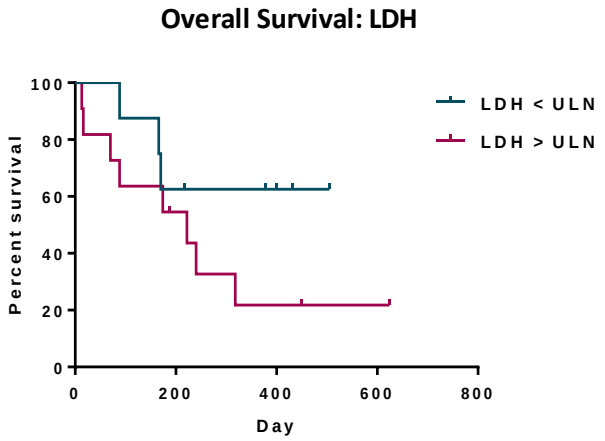


Figure 5.3 Overall survival of melanoma patients stratified by baseline LDH levels

All patients were grouped according to peripheral blood LDH prior to starting checkpoint inhibitor (n=19, 16 patients prior to starting pembrolizumab and 3 prior to starting ipilimumab).

5.2.2 Evaluation of Patient Immune Responses During Therapy with Immune Checkpoint Inhibitors: Immunophenotyping

5.2.2.1 Immune cell activation status was altered following pembrolizumab treatment

PBMCs collected from patients being treated with checkpoint inhibitors were immunophenotyped using Duraclone tubes to evaluate the effect of ipilimumab or pembrolizumab on the immune system. Specific cell populations were analysed for expression of both markers of cellular activation and checkpoint receptor molecules. In the pembrolizumab-treated group CD69 remained unchanged by treatment on CD4+ and CD8+ T cells and B cells (Figure 5.4a-c). However, CD69 expression on the NK and γδT cell populations was more pronounced at baseline and declined over time; the decrease was significant between baseline and 6 weeks (p=0.0461 and p=0.0398, respectively) (Figure 5.4d, e). Furthermore, CD69 expression on γδT cells continued to fall up to 12 weeks (p=0.0078) (Figure 5.4e). In contrast, there was no appreciable difference in other activation markers, such as HLA-DR and CD86 (monocytes and B cells), CCR7 (NK/NKT cells, γδT cells, CD4+ and CD8+ T cells) and CD25 (CD4+ and CD8+ T cells) Appendix Table 8.9.

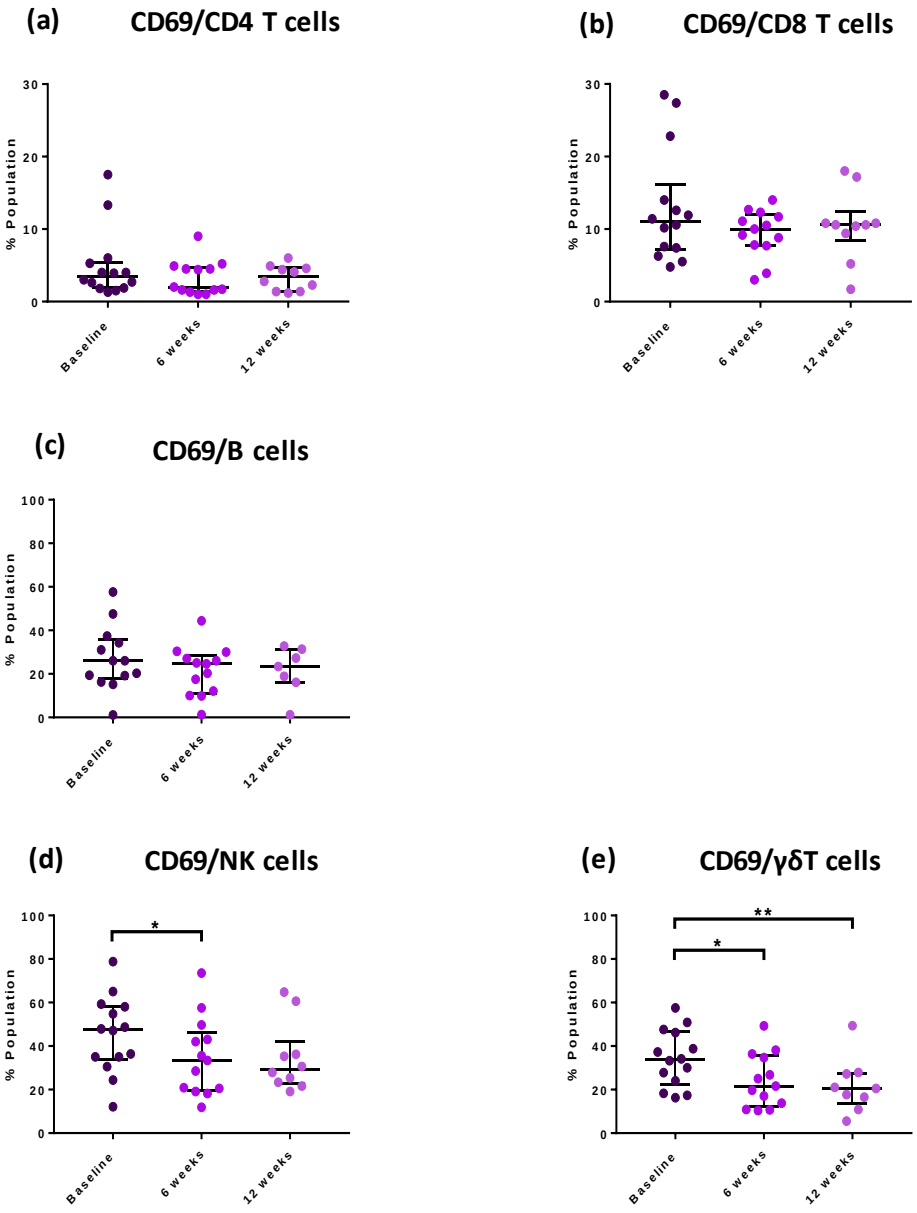


Figure 5.4 CD69 expression on immune cell sub-populations over the course of treatment with pembrolizumab

Cryopreserved PBMCs were immunophenotyped and CD69 expression on immune cell sub-populations determined by flow cytometry. The percentage expression of CD69 over the course of immunotherapy with pembrolizumab is shown on: CD4+ T cells **(a),** CD8+ T cells **(b)**, B cells **(c)**, NK cells **(d)** and γδT cells **(e)** (n=13 for B cells, all other graphs show n=14).

In the ipilimumab treated group (n=3), overall, there was no significant difference over the course of treatment for any of the markers investigated on any cell populations. This included CD69 on CD4+ and CD8+ T cells, NK cells and B cells (data not shown). Additionally, after resting overnight, the monocyte population in these three patients was too small to gain any meaningful data.

5.2.2.2 Checkpoint inhibitors are efficient at blocking target molecules

Following treatment, immune cells were also analysed for changes in expression of relevant immune checkpoint receptors and their respective ligands. As expected, due to binding of pembrolizumab to its receptor PD-1, expression fell over the course of treatment (Figure 5.5).

In patients treated with pembrolizumab, PD-1 expression on CD4+ and CD8+ T cells was reduced at both 6 and 12 weeks (CD4+ cells p=0.0002 and p=0.0488, respectively; CD8+ cells p=0.0015 and p=0.0117) (Figure 5.5a and b). This was also observed on γδT cells between baseline and 6 weeks (p=0.0410) (Figure 5.5c). Treg PD-1 expression was also attenuated over the course of treatment (p=0.0215 at 6 weeks and p=0.0098 at 12 weeks) (Figure 5.5d). There was no change demonstrated in PD-1 expression on NK cells, NKT cells, B cells or monocytes. Similarly, there was no significant change in the proportion of immune cells expressing PD-L1 over the 12 weeks of treatment. No significant changes were seen in any other cell population Appendix Table 8.9.

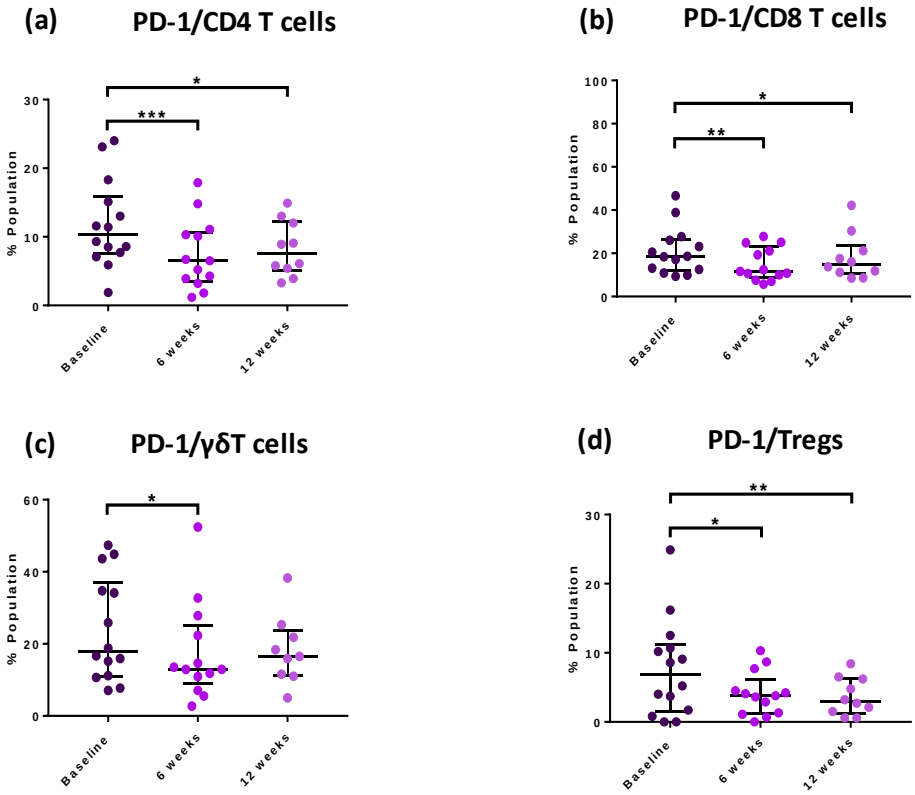


Figure 5.5 PD-1 expression on immune cell sub-populations over the course of treatment with pembrolizumab

Cryopreserved PBMCs were immunophenotyped and PD-1 expression on immune cell sub-populations determined by flow cytometry. The percentage expression of PD-1 over the course of immunotherapy with pembrolizumab is shown on: CD4+ T cells **(a)**, CD8+ T cells **(b)**, γδT cells **(c)** and Tregs **(d)** (all n=14).

In the patients treated with ipilimumab, expression of CTLA-4 on CD4+, CD8+ T cells and NK cells also fell over the course of treatment (due to binding of CTLA-4 by ipilimumab). Whereas the reduction in PD-1 expression with pembrolizumab treatment was significant, this was not the case for CTLA-4 expression with ipilimumab (Figure 5.6)

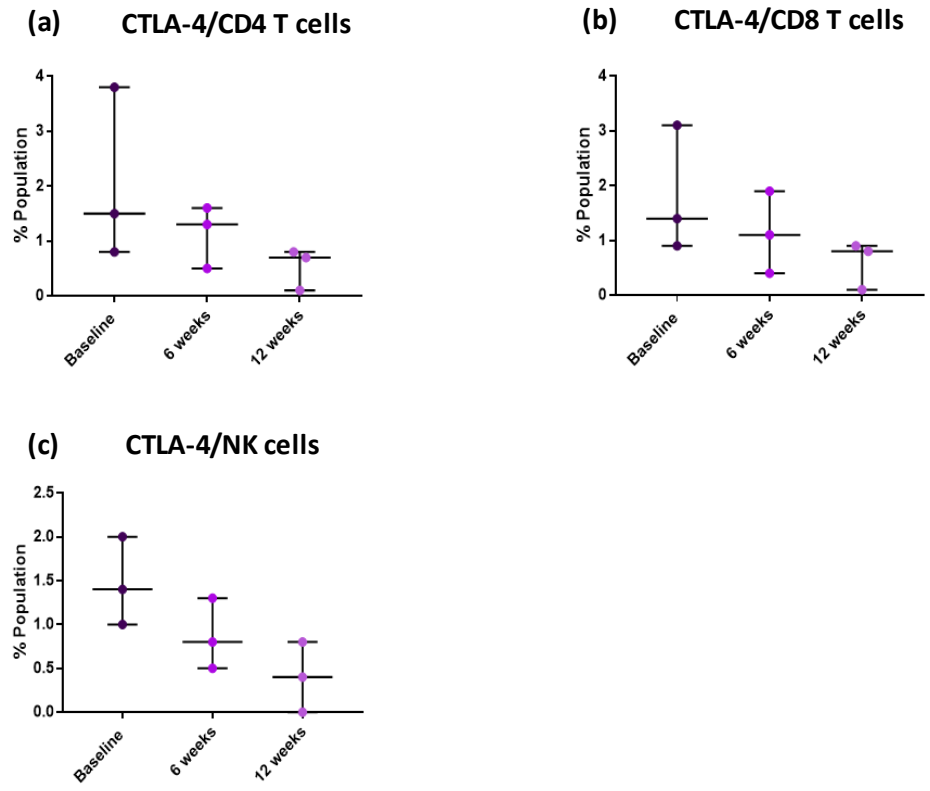


Figure 5.6 CTLA-4 expression on immune cell sub-populations over the course of treatment with ipilimumab

Cryopreserved PBMCs were immunophenotyped and CTLA-4 expression on immune cell sub-populations determined by flow cytometry. The percentage expression of CTLA-4 over the course of immunotherapy with ipilimumab is shown on: CD4+ T cells **(a)**, CD8+ T cells **(b)** and NK cells **(c)** (all n=3).

Finally, when looking at both patient groups, there was no appreciable difference in the expression of CTLA-4 on immune cell subsets from patients treated with pembrolizumab. The same was true for PD-1 expression in those treated with ipilimumab (data not shown).

5.2.2.3 Expression of PD-1 on immune cell populations at baseline correlates with overall survival

Overall survival was calculated for all melanoma patients prior to receiving treatment with a checkpoint inhibitor (pembrolizumab or ipilimumab). Patients were stratified according to baseline PD-1 expression on immune cells and whether this was less than or greater than the median levels within the treatment group (Figure 5.7). Greater PD-1 expression on both CD8+ T cells (Figure 5.7a) and B cells (Figure 5.7b) correlated with significantly decreased OS (222 days *vs.* median OS not met, p=0.0476, HR 4.19, 95 % CI 1.128 to 15.56 and 174 days *vs.* median OS not met, p=0.0482, HR 4.213, 95 % CI 1.134 to 15.65, respectively). There was no relationship between PD-1 expression on CD4+ T cells and OS (data not shown). Conversely, PD-1 expression on Tregs conferred an increased OS but this did not reach statistical significance (Figure 5.7c).

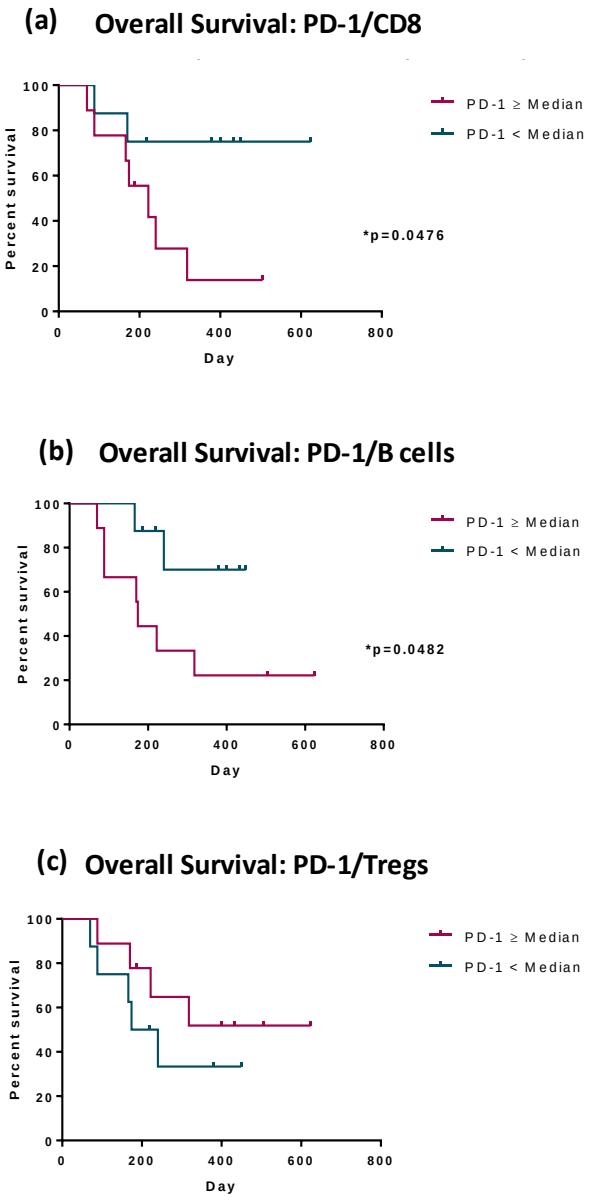


Figure 5.7 Overall survival of all melanoma patients who received an immune checkpoint inhibitor, stratified according to baseline PD-1 expression

Patients were stratified according to the median expression of PD-1 on immune cell populations prior to therapy. Survival was calculated as days from receiving the first dose of immunotherapy. All patients were evaluable in the survival analysis. This is shown for: CD8+ T cells **(a)**, B cells **(b)** and Tregs **(c)** (n=17: 14 patients prior to starting pembrolizumab and 3 prior to starting ipilimumab).

There was no relationship between OS and baseline PD-1 expression on any other immune cell populations. Similarly, CTLA-4 expression did not correlate with OS on any of the cell populations at baseline (data not shown).

5.2.2.4 Immune checkpoint inhibition does not affect Treg frequency, although L-selectin expression is enhanced

Treg frequency and other activation markers were analysed for their response to treatment. In patients treated with pembrolizumab, there was no significant change in the frequency of FoxP3+ Tregs over time, either as a definitive population nor as a proportion of CD4+ T cells (Figure 5.8a and b). However, the expression of CD62L on Tregs increased between baseline and 12 weeks (p=0.0254) (Figure 5.8c).

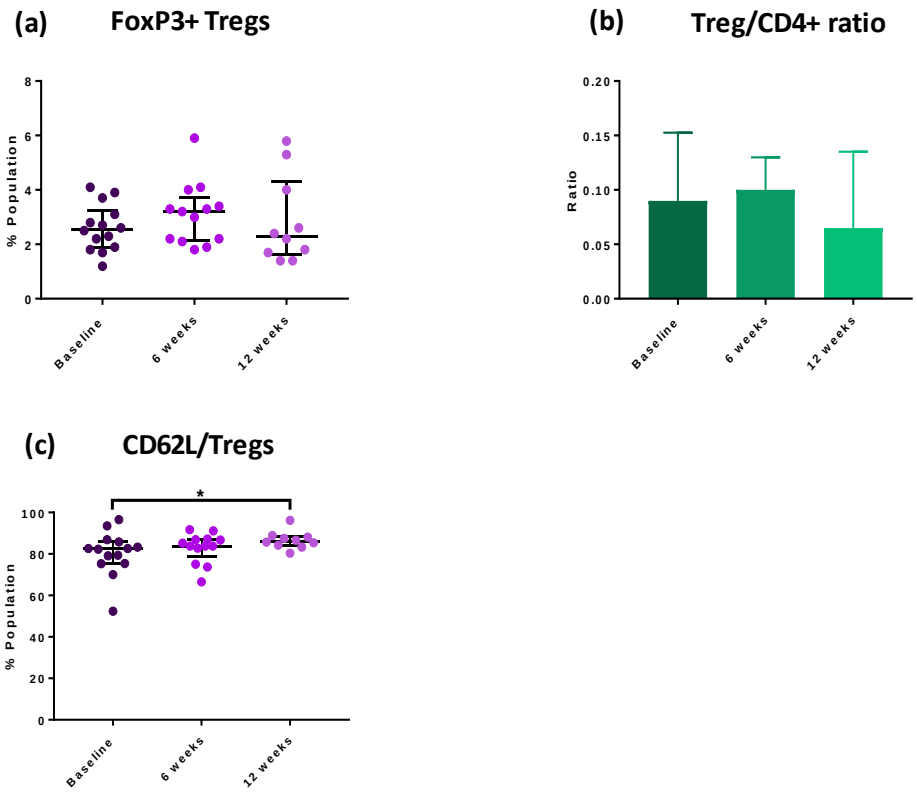


Figure 5.8 Changes in Treg populations in patients over the course of treatment with pembrolizumab

Cryopreserved cells were immunophenotyped for Tregs. The percentage of FoxP3+ Tregs **(a)** and the Treg:CD4 ratio **(b)** were determined by flow cytometry. The percentage expression of CD62L on Tregs was also calculated **(c)** (all n=14).

In patients treated with ipilimumab, there was no change in the population frequency of Tregs nor expression of CD62L on Tregs (data not shown).

5.2.2.5 Immunophenotyping fresh PBMCs reflects the results of cryopreserved PBMCs

During the latter stages of sample collection, it was possible to perform immunophenotyping on freshly isolated (non-cryopreserved) PBMCs from patients being treated with pembrolizumab. A total of seven pembrolizumab patients had samples collected and analysed as fresh PBMCs.

As seen when immunophenotyping cryopreserved PBMCs (Figure 5.4), there was a trend towards a fall in CD69 expression on CD4+, CD8+ T cells, NKT cells and γδT cells, particularly over the first 6 weeks of pembrolizumab therapy (Figure 5.9). When evaluating NKT cells, this difference was significant (p=0.0469). There was no significant changes in CD69 expression on NK cells, monocytes or B cells (data not shown).

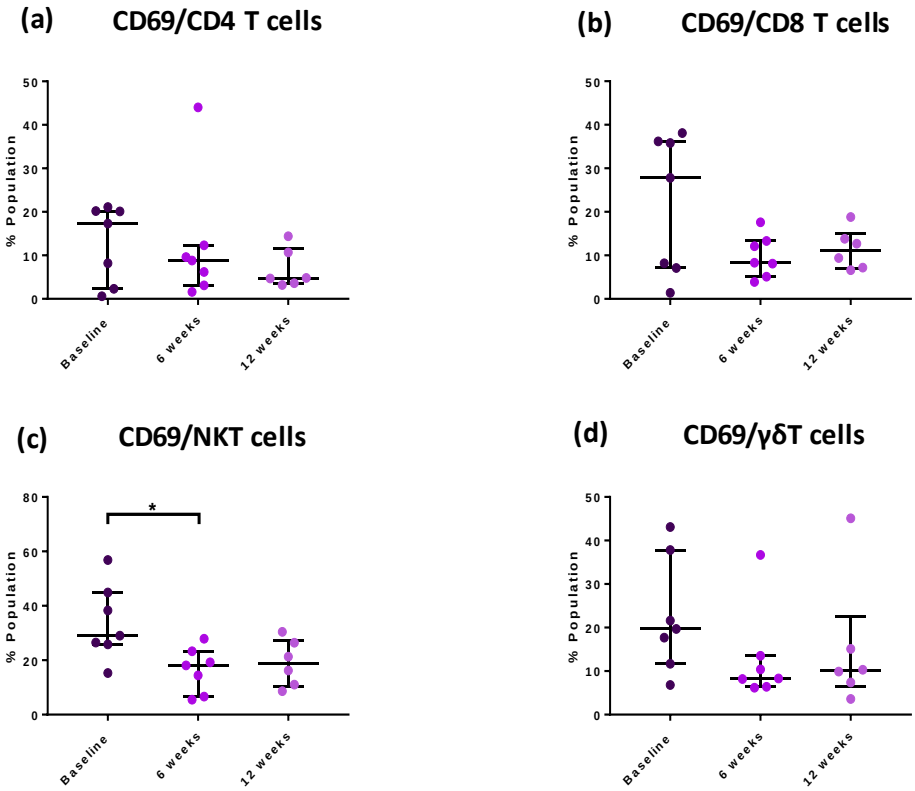


Figure 5.9 CD69 expression on fresh immune cell sub-populations over the course of treatment with pembrolizumab

Fresh PBMCs were immunophenotyped and CD69 expression on immune cell sub-populations determined by flow cytometry. The percentage expression of CD69 over the course of immunotherapy with pembrolizumab is shown on: CD4+ T cells **(a)**, CD8+ T cells **(b)**, NKT cells **(c)** and γδT cells **(d)** (all n=7).

In addition to the decrease in CD69, interrogation of fresh blood revealed a significant reduction in CD25 expression on both CD4+ and CD8+ T cells between baseline and 6 weeks (p=0.0313 and p=0.0156, respectively) (Figure 5.10). Of note, this was not demonstrated in cryopreserved cells (Appendix Table 8.9).

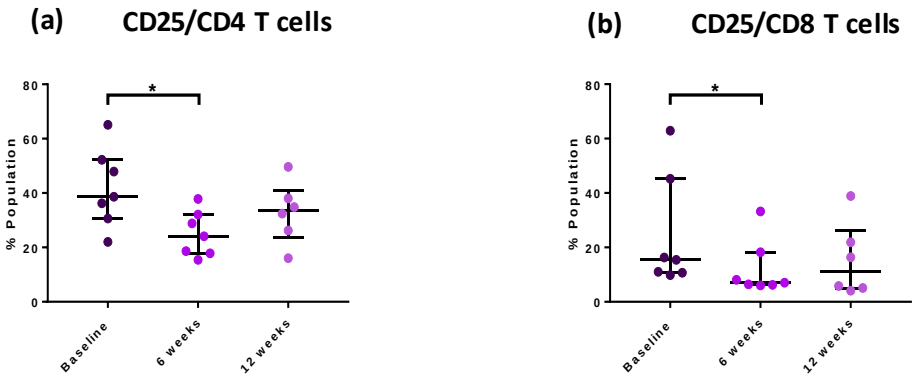


Figure 5.10 CD25 expression on fresh immune cell sub-populations over the course of treatment with pembrolizumab

Fresh PBMCs were immunophenotyped and CD25 expression on immune cell sub-populations determined by flow cytometry. The percentage expression of CD25 over the course of immunotherapy with pembrolizumab is shown on: CD4+ T cells **(a)** and CD8+ T cells **(b)** (n=7).

As already demonstrated with cryopreserved PBMCs, treatment with pembrolizumab lowered levels of PD-1 on immune cell populations in fresh whole blood. This decrease was significant between baseline and 6 weeks on CD4+ T cells, CD8+ T cells, NKT cells, γδT cells and Tregs (p=0.0156, p=0.0156, p=0.0156, p=0.0313 and p=0.0156, respectively) (Figure 5.11). Between baseline and 12 weeks, there was a significant reduction in PD-1 expression on CD4+ T cells, CD8+ T cells, NKT cells and Tregs (all p=0.0313). Additionally, PD-1 expression on NKT cells fell significantly between 6 weeks and 12 weeks (p=0.0313). There was no significant change in PD-1 expression on NK cells, B cells or monocytes (data not shown).

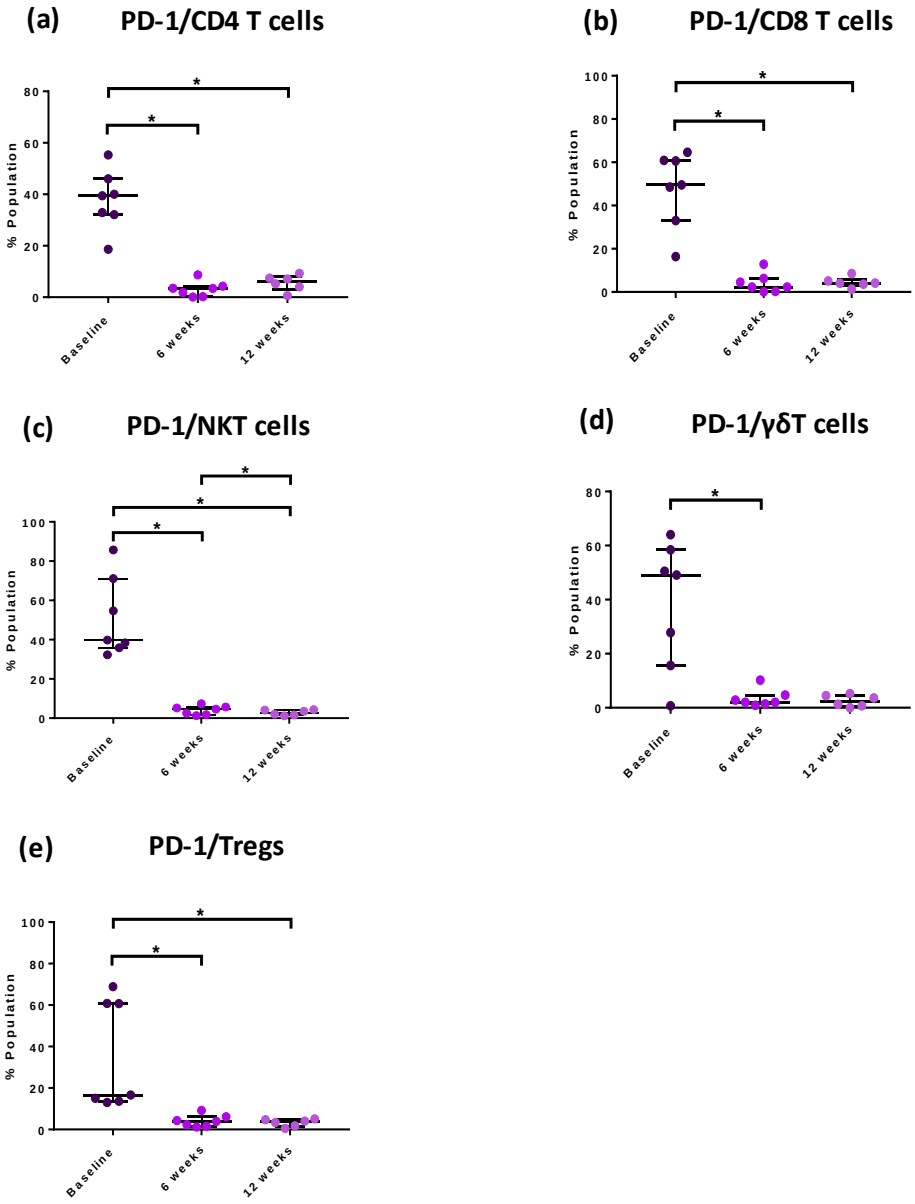


Figure 5.11 PD-1 expression on fresh immune cell sub-populations over the course of treatment with pembrolizumab

Fresh PBMCs were immunophenotyped and PD-1 expression on immune cell sub-populations determined by flow cytometry. The percentage expression of PD-1 over the course of immunotherapy with pembrolizumab is shown on: CD4+ T cells **(a)**, CD8+ T cells **(b),** NKT cells **(c)**, γδT cells **(d)** and Tregs **(e)** (all n=7).

As seen when immunophenotyping cryopreserved PBMCs, when fresh PBMCs were analysed, there was no significant change in the other activation markers investigated, including PD-L1, HLA-DR, CD86 and CD11c on monocytes and B cells and CCR7 on the immune cell populations described in Appendix Table 8.9 (data not shown).

Also reflecting the results from cryopreserved PBMCs, there was no change in the proportion of FoxP3+ Tregs over treatment. In contrast to the cryopreserved PBMCs, no change in CD62L positivity was observed in the fresh Treg population (data not shown).

As shown in previous chapters, analysis of cryopreserved PBMCs is comparable to that of fresh immune cell populations. Although there may be some change in the magnitude of the positive populations, the pattern of expression between time points seem to be retained.

5.2.3 Evaluation of Patient Immune Responses During Therapy with Immune Checkpoint Inhibitors: Cytokine/Chemokine Profile

5.2.3.1 The cytokine/chemokine profile of patients receiving pembrolizumab changes during treatment

Evaluation of plasma levels of cytokines and chemokines over the course of therapy with checkpoint inhibitors revealed a unique profile in patients receiving pembrolizumab. While there were increases in a number of pro-immune cytokines and immune cell chemokines, a fall in some T_H2 cytokines was also demonstrated.

A total of 48 chemokines and cytokines were evaluated by Luminex in patients receiving pembrolizumab. Of these, nine were below the lower detection limits of the assay (Appendix Table 8.10). Additionally, in this patient group, there were 29 chemokines and cytokines that did not demonstrate an appreciable or significant difference over the 12 week investigational period (Appendix Table 8.11). Three of the evaluable cytokines significantly decreased in concentration over the first 6 weeks of therapy: the T_H1 cytokine IFN-γ and the T_H2 cytokines IL-4 and IL-5 (p=0.0398, p=0.0381, p=0.0398, respectively) (Figure 5.12).

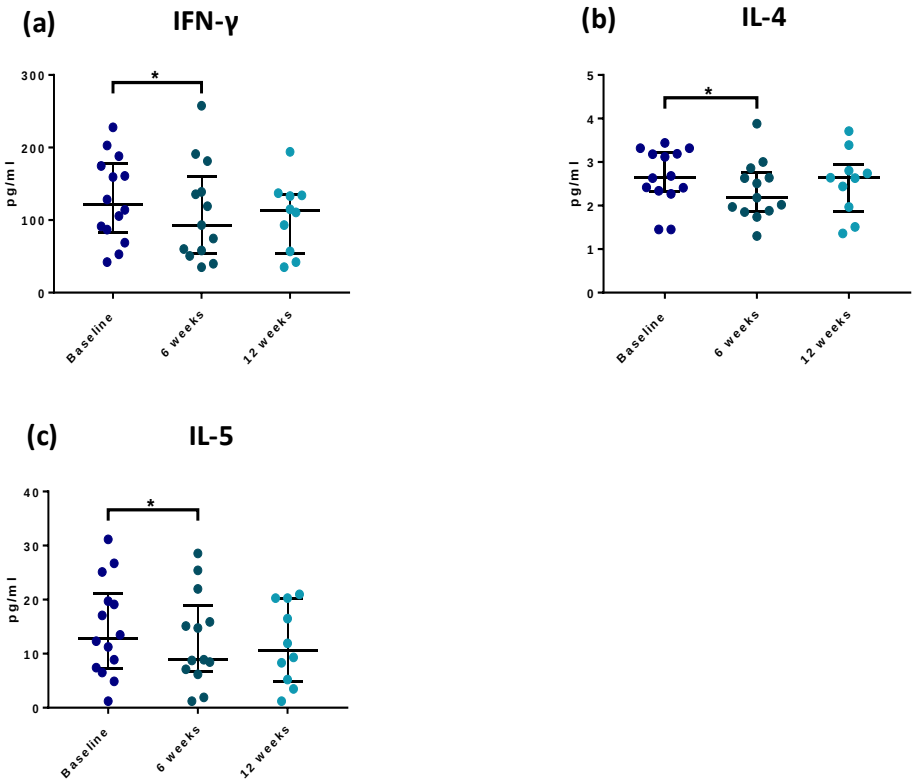


Figure 5.12 Change in IFN-γ, IL-4 and IL-5 levels over the course of treatment with pembrolizumab

Cytokine analysis was performed using Luminex assays and the change in concentration (pg/ml) of IFN-γ **(a)**, IL-4 **(b)** and IL-5 **(c)** were compared over time (all n=14).

Seven cytokines were seen to elevate over the course of treatment with pembrolizumab. Over the first 6 weeks, there was a significant increase in levels of IL-16 (p=0.0327), IP-10 (p=0.0081), MIG (p=0.0002), SCF (p=0.0498) and SCGF-β (p=0.0171) (Figure 5.13a-e). In addition, levels of IL-16 (p=0.0098), MIG (p=0.002), SCF (p=0.0195), IL-3 (p=0.0488) and IL-18 (p=0.0273) also increased over the 12 weeks of treatment (Figure 5.13a, c, d, f, g).

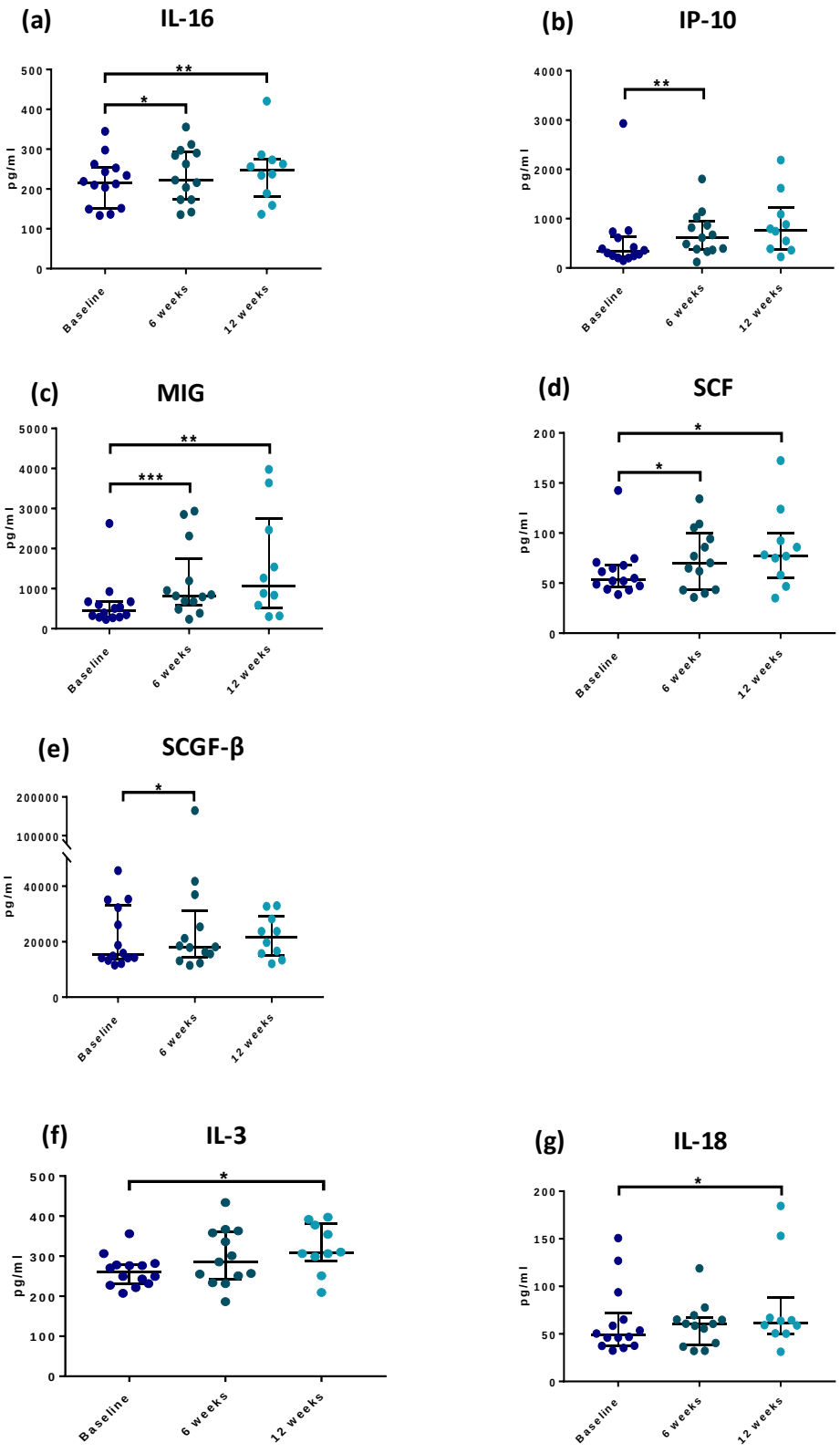


Figure 5.13 Change in chemokine and cytokine levels over the course of treatment with pembrolizumab

Cytokine analysis was performed using Luminex assays and the change in concentration (pg/ml) of IL-16 **(a)**, IP-10 **(b)**, MIG **(c)**, SCF **(d)**, SCGF-β **(e)**, IL-3 **(f)** and IL-18 **(g)** were compared over time (all n=14).

5.2.3.2 The cytokine/chemokine profile of patients receiving ipilimumab has varying responses to treatment

The same Luminex panels were used to analyse plasma from the three patients being treated with ipilimumab. There were 12 cytokines and chemokines that were not detected or that were below the standard curve for these samples (Appendix Table 8.12). Of the remaining solutes, none were seen to change significantly over the course of the 12 week treatment period. This is likely due to a combination of sample size (n=3) and patient number one seemingly being an outlier, when evaluating levels of cytokines such as IFN-γ, IL-4 and IL-5 (Figure 5.14).

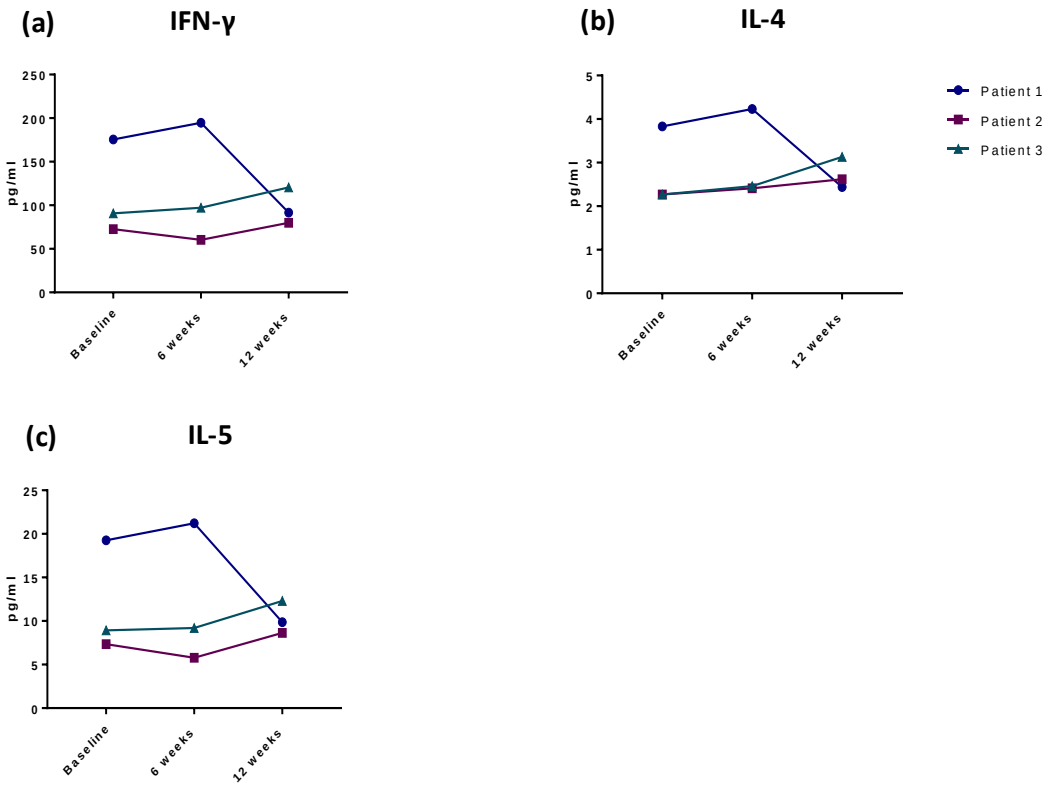


Figure 5.14 Changes in plasma levels of IFN-γ, IL-4 and IL-5 over the course of treatment with ipilimumab

Cytokine analysis was performed using Luminex assays and the change in concentration (pg/ml) of IFN-γ **(a)**, IL-4 **(b)** and IL-5 **(c)** were compared over time (n=3).

Although there were no significant differences in cytokine and chemokine levels with ipilimumab administration, there was a noticeable trend that reflected changes seen in the cohort of patients receiving pembrolizumab for some solutes. Of note, levels of IL-3, IL-18, MIG and SCF increased between baseline measurement and 12 weeks (Figure 5.15).

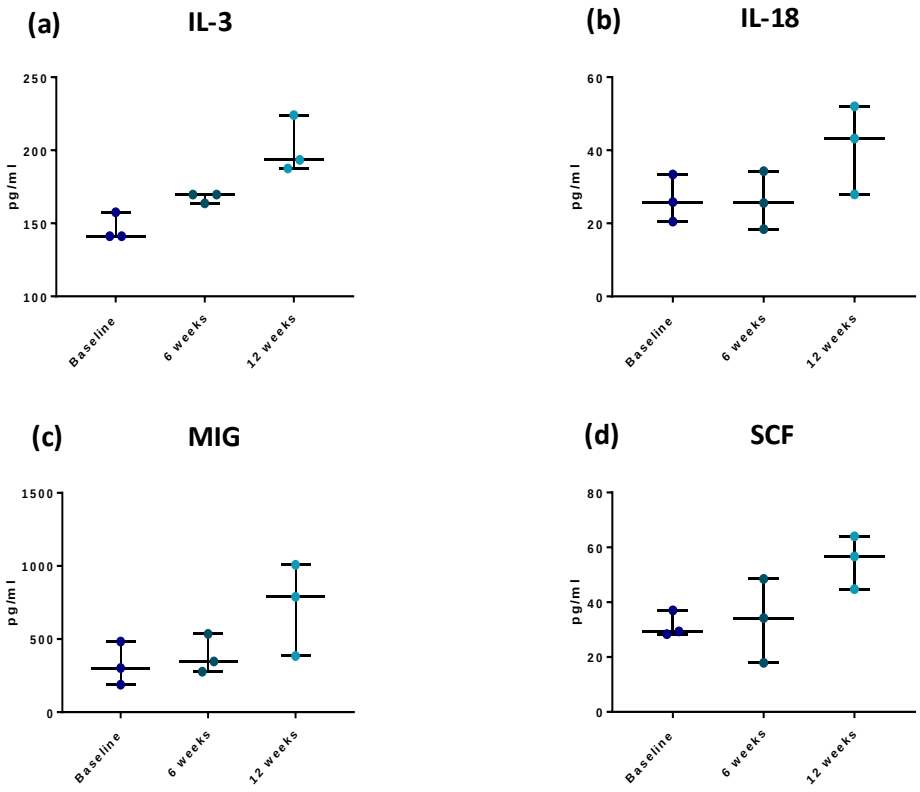


Figure 5.15 Change in chemokine and cytokine levels over the course of treatment with ipilimumab

Cytokine analysis was performed using Luminex assays and the change in concentration (pg/ml) of IL-3 **(a)**, IL-18 **(b)**, MIG **(c)** and SCF **(d)** were compared over time (all n=3).

5.2.4 Evaluation of Patient Immune Responses During Therapy with Immune Checkpoint Inhibitors: Innate Functional Activity

5.2.4.1 NK cell function is not significantly altered over time in patients being treated with pembrolizumab.

Although checkpoint inhibitors were initially used to target and moderate T cell activity, evidence shows that NK cells can be a potential target also. As such, the effect of checkpoint inhibition on NK cell activity was assessed in our patient cohort. NK cell function was measured by CD107 degranulation assays against tumour targets. There was a significant decrease in CD107 expression on NK cells against K562 tumour cells between baseline and 12 weeks (p=0.0059) (Figure 5.16a). However, the change in percentage population was modest (median CD107 expression at baseline 23%, at 12 weeks 16.5 %). NK cell degranulation

against melanoma target cells (Mel624 and Mel888) did not change significantly over the 12 weeks of treatment with pembrolizumab (Figure 5.16b, c).

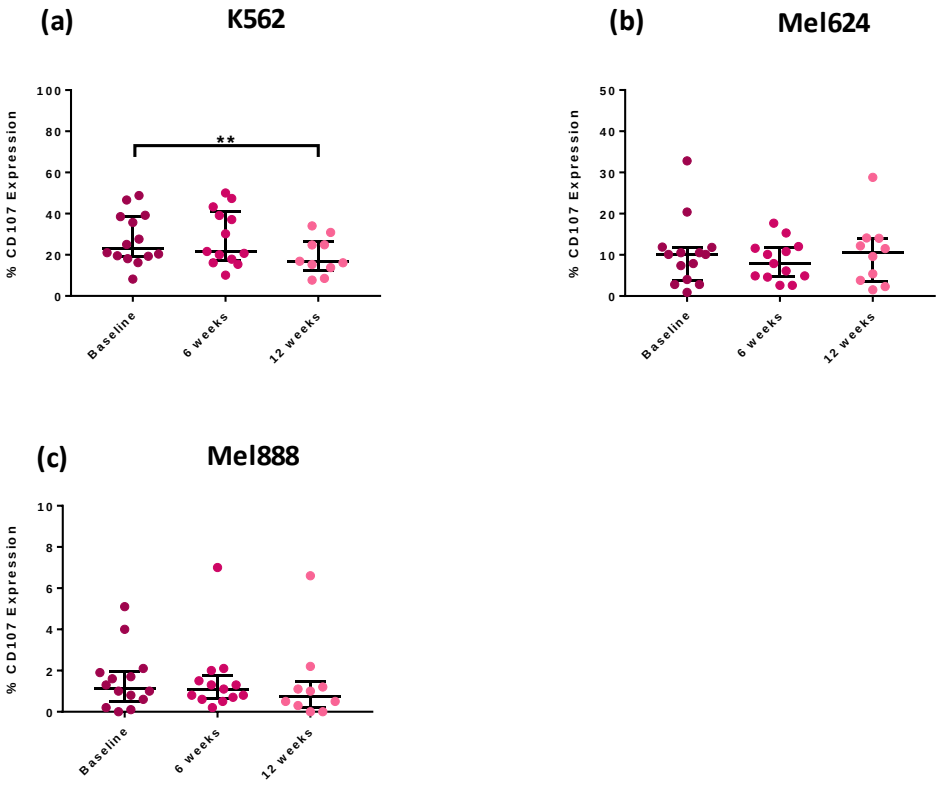


Figure 5.16 Degranulation of patient NK cells against tumour targets over the course of treatment with pembrolizumab

Cryopreserved PBMCs were used in degranulation assays to assess NK cell functional capacity against tumour targets. Percentage CD107 expression on NK cells over treatment, as determined by flow cytometry, is shown against: K562s **(a)**, Mel624s **(b)** and Mel888s **(c)** (n=11).

Chromium-release assays did not demonstrate a significant change over 12 weeks of pembrolizumab therapy (Figure 5.17). As seen in the degranulation assays, cytotoxic potential of PBMCs was lower against melanoma cells lines (Mel624 and Mel888) when compared to the K562 tumour cell line.

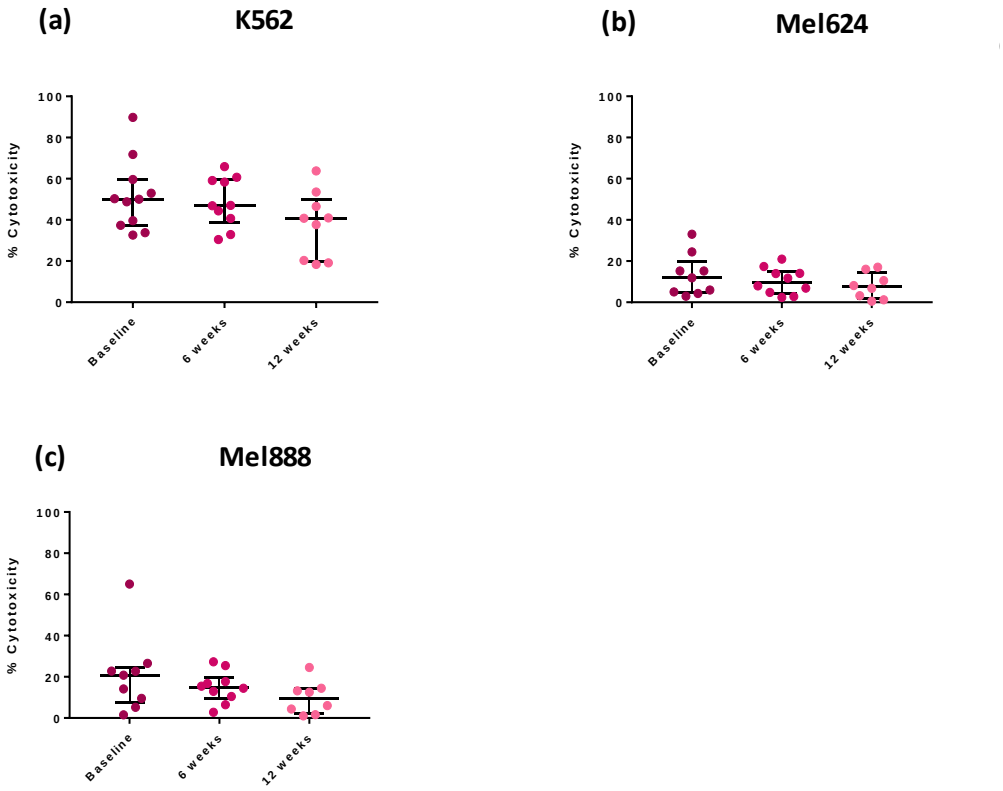


Figure 5.17 Cytotoxicity of patient NK cells against tumour targets over the course of treatment with pembrolizumab

Cryopreserved PBMCs were used in ^{51}Cr-release assays to assess NK cell functional capacity against ^{51}Cr-labelled tumour targets. Cytotoxicity at an E:T ratio of 100:1 is shown over treatment is shown against: K562s **(a)**, Mel624s **(b)** and Mel888s **(c)** (n=11).

5.2.4.2 Innate immune function in patients treated with ipilimumab mostly reflects that of patients treated with pembrolizumab

In patients receiving ipilimumab, the levels of both NK cell degranulation (Figure 5.18) and cytotoxicity of PBMCs (Figure 5.19) were considerably lower against Mel624 and Mel888 target cells. Against K562 cells, both CD107 expression and PBMC-mediated cytotoxicity trended toward a decrease between baseline and 12 weeks but this was not significant (Figure 5.18a and Figure 5.19a). Once again, the small patient population (n=3) limited the statistical significance of these findings.

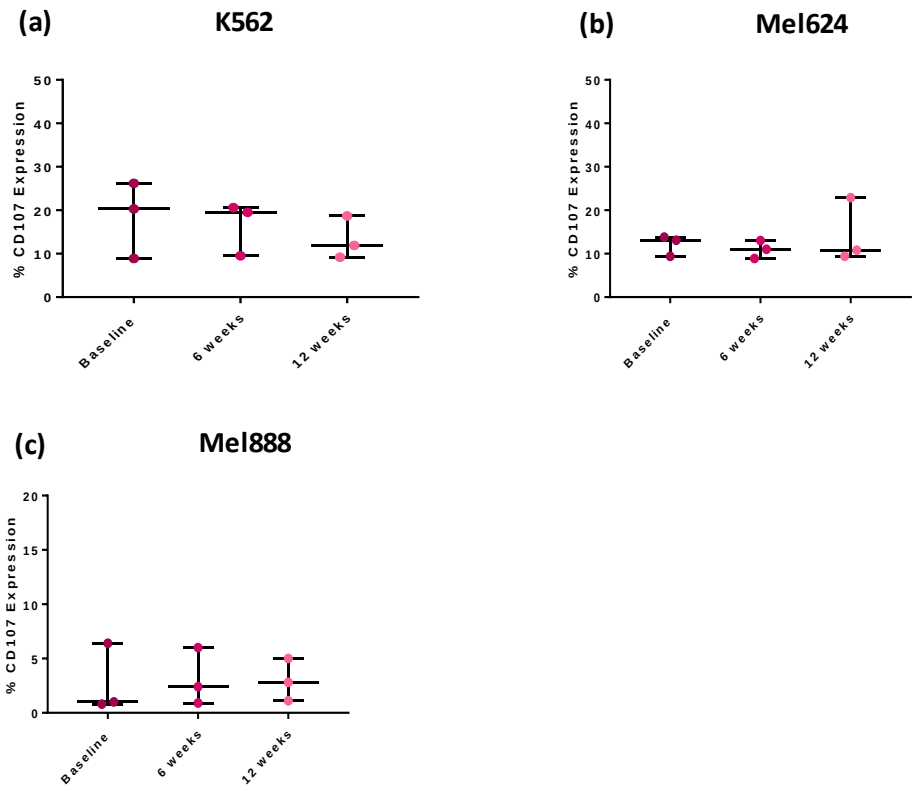


Figure 5.18 Degranulation of patient NK cells against tumour targets over the course of treatment with ipilimumab

Cryopreserved PBMCs were used in degranulation assays to assess NK cell functional capacity against tumour targets. Percentage CD107 expression on NK cells over treatment, as determined by flow cytometry, is shown against: K562s **(a)**, Mel624s **(b)** and Mel888s **(c)** (n=3).

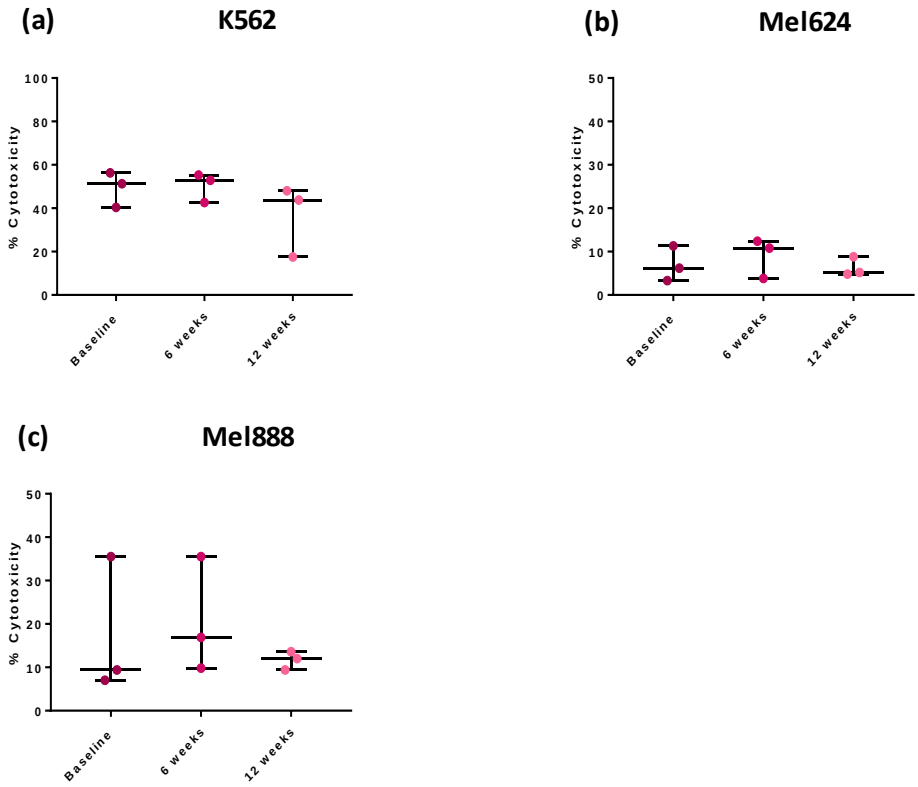


Figure 5.19 Cytotoxicity of patient NK cells against tumour targets over the course of treatment with ipilimumab

Cryopreserved PBMCs were used in ^{51}Cr-release assays to assess NK cell functional capacity against ^{51}Cr-labelled tumour targets. Cytotoxicity at an E:T ratio of 100:1 is shown over treatment is shown against: K562s **(a)**, Mel624s **(b)** and Mel888s **(c)** (n=3).

In a pooled analysis of patients who were about to start treatment with either ipilimumab and pembrolizumab, there was no appreciable OS benefit according to baseline NK cell activity, either by evaluating cytotoxicity or CD107 expression in response to target cells (data not shown). Once again, patient numbers were too small to evaluate OS in individual groups (only ipilimumab or only pembrolizumab groups).

5.2.5 Evaluation of Patient Immune Responses During Therapy with Immune Checkpoint Inhibitors: Adaptive T cell Response

ELISpot assays were performed to assess antigen-specific T cell responses of patients receiving checkpoint inhibitors over a 12 week investigational period. Cryopreserved PBMCs were

thawed and incubated with the MART-1 TAA or CEF viral peptide pool. T cell responses were measured by production of IFN-γ (SFU).

5.2.5.1 T cell responses to viral peptides in patients treated with checkpoint inhibitors

A viral peptide pool (CEF) was utilised to determine pan T cell antigen recall responses in these patients. As expected, the response of patients was highly variable. One patient had a uniform T cell response to CEF peptide over the ipilimumab treatment period (Figure 5.20a) and one patient had a similar magnitude of response at baseline and 6 weeks, with a slight decreased by the 12 week time point (Figure 5.20b). The third patient treated with ipilimumab had a very high response to CEF across all time points, that was too high to count (data not shown).

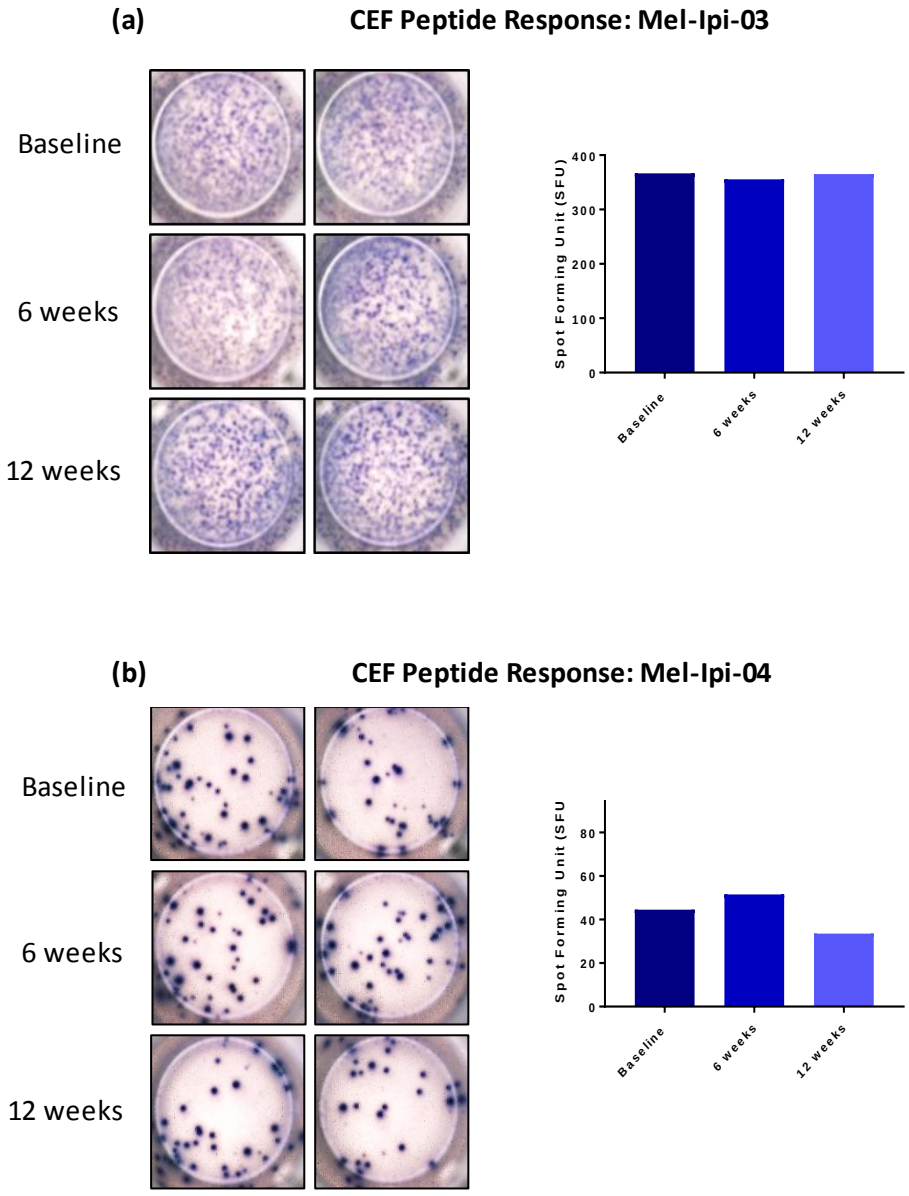


Figure 5.20 T cell responses to CEF viral peptide pool in patients treated with ipilimumab

Cryopreserved PBMCs were collected from patients treated with ipilimumab and analysed by ELISpot. Images, in duplicate, show IFN-γ production in response to CEF peptide for two example patients, alongside quantification of SFU over the treatment period: Mel-Ipi-03 **(a)** and Mel-Ipi-04 **(b)**

Again, as expected, there was also considerable inter-patient variability in the responses to viral peptides across patients receiving pembrolizumab therapy. There was no definitive pattern across time points, with some patients demonstrating a decrease in response to CEF (Figure 5.21a), an increase in response to CEF (Figure 5.21b) or no change during therapy (Figure 5.21c).

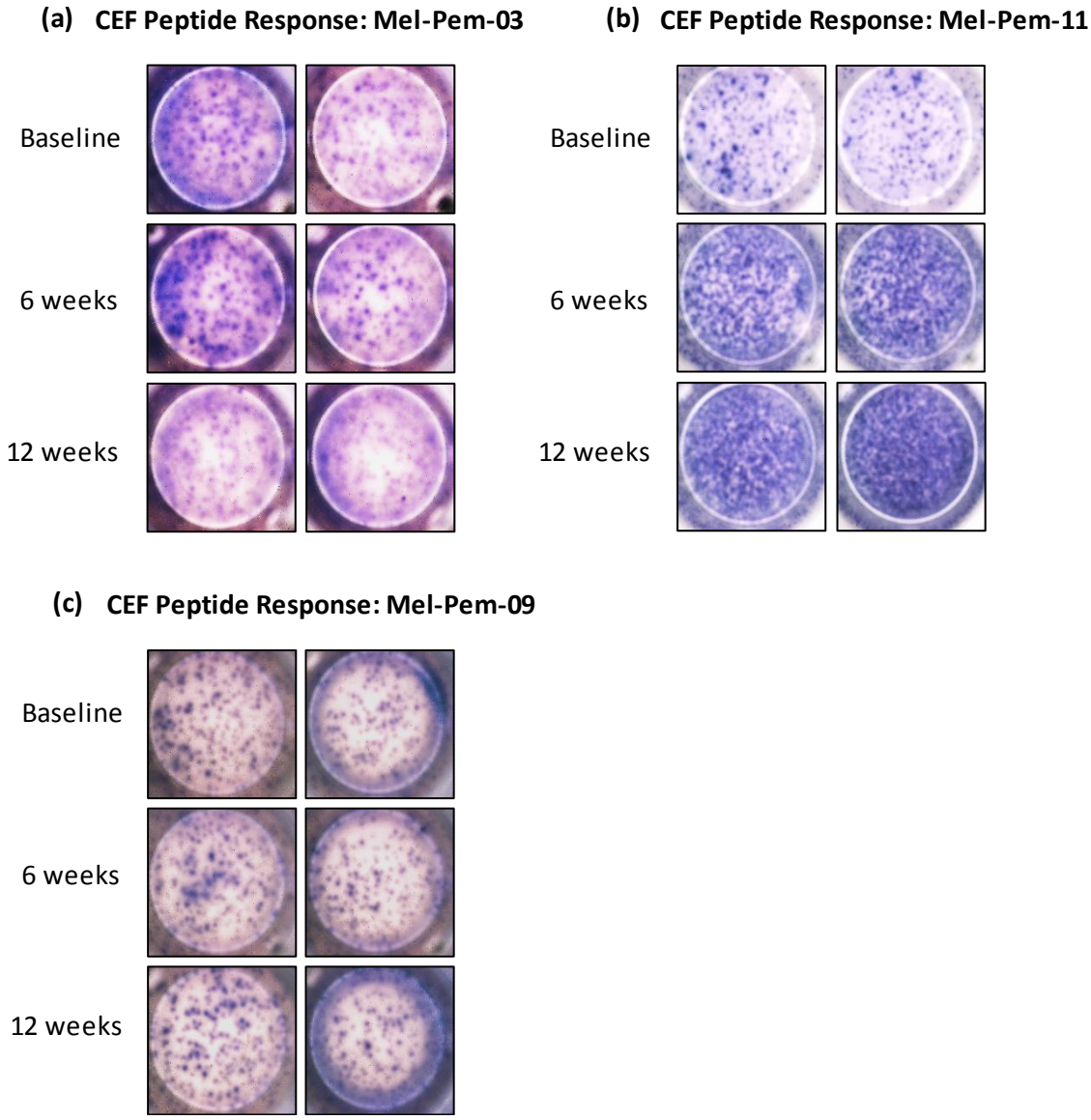


Figure 5.21 T cell responses to CEF viral peptide pool in patients treated with pembrolizumab

Cryopreserved PBMCs were collected from patients treated with pembrolizumab and analysed by ELISpot. Images, in duplicate, show IFN-γ production in response to CEF peptide for three example patients over the treatment period: Mel-Pem-03 **(a)** and Mel-Pem-11 **(b)** and Mel-Pem-09 **(c).**

5.2.5.2 T cell responses to TAAs in patients treated with checkpoint inhibitors

MART-1 was used as a representative melanoma TAA to assess tumour-specific T cell responses in patients treated with immune checkpoint inhibitors. There was a much less-pronounced response to MART peptide compared to CEF in all three ipilimumab-treated patients. In one patient there was no appreciable T cell response to MART at all (data not

shown). In the remaining two patients, one had a response which increased during the course of ipilimumab therapy (Figure 5.22a) and one had a higher baseline response which diminished over the investigational period (Figure 5.22b). Only one patient treated with ipilimumab (Mel-Ipi-03) had evaluable responses to both CEF and MART peptides: no change over time to CEF but an increased T cell response to MART during treatment (Figure 5.20a and Figure 5.22a)

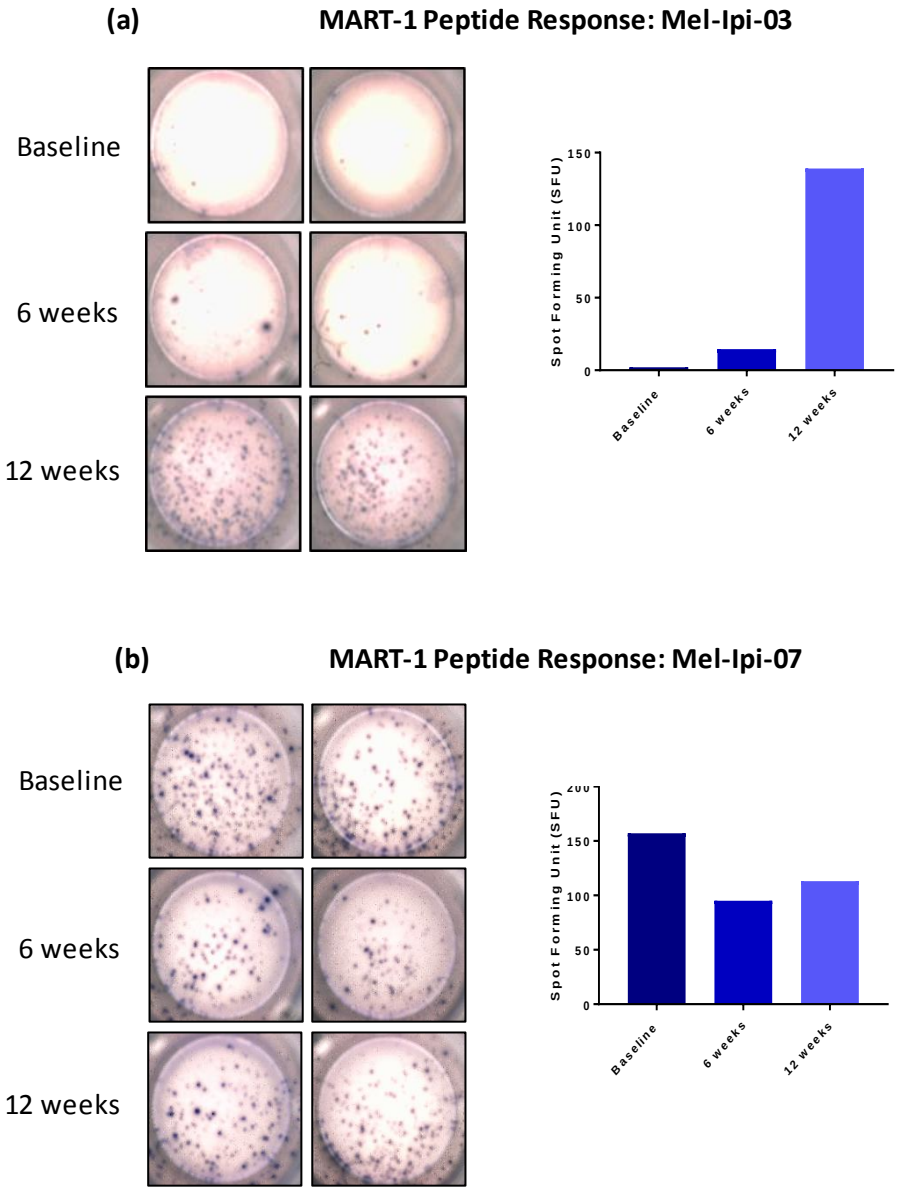


Figure 5.22 T cell responses to MART-1 TAA in patients treated with ipilimumab

Cryopreserved PBMCs were collected from patients treated with ipilimumab and analysed by ELISpot. Images, in duplicate, show IFN-γ production in response to MART-1 peptide for two example patients, alongside quantification of SFU over the treatment period: Mel-Ipi-03 **(a)** and Mel-Ipi-07 **(b)**.

As previously discussed, the adaptive T cell response of all patients receiving pembrolizumab varied greatly across the whole patient group. Correlation with CEF peptide responses also varied across the patient group. There were generally a greater number of T cell responses to CEF peptide than to MART-1, as would be anticipated.

As observed with CRC patients on chemotherapy (section 4.2.5), there was some concordance between innate and adaptive immune assays within individual patients. As an example, in Mel-Pem patient 12, there was an apparent decrease in NK cell activity over the 12 weeks of pembrolizumab therapy, as shown by a fall in both cytolytic activity and NK cell degranulation against K562 target cells (Figure 5.23a and b). Reflecting this, T cell responses against CEF peptide also decreased (Figure 5.23c and d), as did CD69 expression on both CD4+ and CD8+ T cells (Figure 5.23e and f). Taken together, these data indicate that the entirety of a patient's immune system (innate and adaptive), may respond in a similar way to disease and treatment strategies.

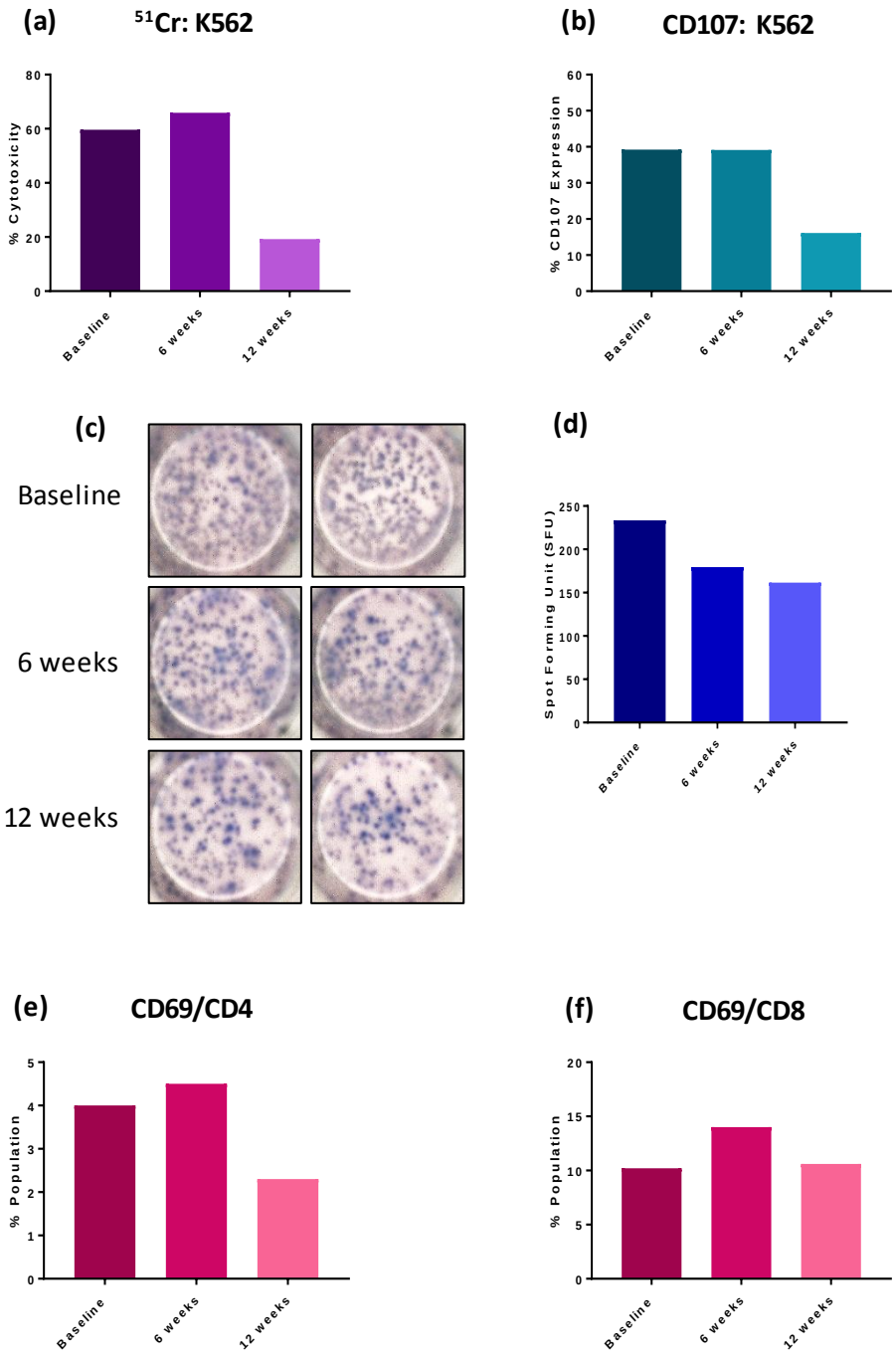


Figure 5.23 Comparison between adaptive T cell responses and innate responses during treatment: Patient Mel-Pem-12

Cryopreserved PBMCs were collected from patient Mel-Pem-12 and analysed by ELISpot and other innate immune assays over the treatment period. IFN-γ production in response to CEF peptide was aligned with innate responses in the same samples. Cytotoxicity of PBMCs (determined by ^{51}Cr assay) against K562 cells is shown in **(a)**, CD107 expression (determined by flow cytometry) against K562s is shown in **(b)**. T cell responses to CEF peptide **(c)** and number of SFU **(d)** are also shown. Finally, CD69 expression is shown on CD4+ T cells **(e)** and CD8+ T cells **(f)** as determined by flow cytometry.

5.2.6 Relationship between NLR and Immune Function in Patients Receiving Checkpoint Inhibitors

Given our previous studies (Chapter 4) we sought to investigate any potential relationship between NLR and immune function in patients receiving immunotherapy. Although there were only a small number of patients within the pembrolizumab treatment group with a high baseline NLR (n=4), a further two patients in the ipilimumab group had a baseline NLR of greater than 5. As shown in Figure 5.24, when both treatment groups were pooled for analysis, there was an increase in OS in the low NLR group (median OS not met in the low NLR group *vs.* 129 days in the high NLR group, HR 0.257, 95 % CI 0.05454 to 1.212). This supported our findings in the CRC group, where OS was also diminished in those with a baseline NLR of greater than 5 (Figure 4.3). There was no appreciable difference in other functional immune assays between melanoma patients with a high or low NLR prior to treatment (data not shown).

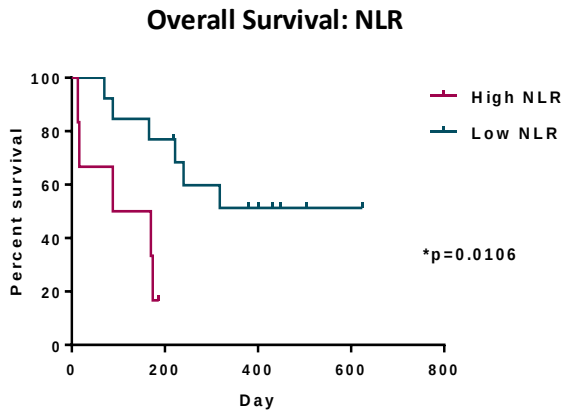


Figure 5.24 Overall survival for melanoma patients prior to checkpoint inhibitor therapy, stratified by NLR

Patients receiving checkpoint inhibitors were stratified according to NLR prior to treatment. Survival was calculated as days from receiving the first dose of chemotherapy. All patients were evaluable in the survival analysis (n=19; 3 patients prior to ipilimumab and 16 patients prior to pembrolizumab).

Although there was a smaller number of melanoma patients being treated with immunotherapy, there are some signals of immune modulation within the group. This data can serve as a useful comparison to the CRC cohort of patients receiving chemotherapy and act as a discussion point for further investigation.

5.3 Melanoma Discussion

This work aimed to further define the immune response in patients receiving treatment with checkpoint inhibitors for metastatic melanoma. Although the use of immune check point inhibitors in melanoma has increased dramatically over the last few years, a limited number of studies have looked specifically at immune responses to therapy *in vivo*.

Sequential samples were taken from patients receiving either ipilimumab or pembrolizumab every 6 weeks between baseline treatment and 12 weeks. Analysis of these samples, using our panel of validated immune assays, was able to provide insight into the different immune responses to anti-cancer therapy when compared to patients receiving traditional cytotoxic chemotherapy. After the project was commenced, pembrolizumab was approved for first-line use in patients with metastatic melanoma. As a consequence, treatment options changed in favour of pembrolizumab, limiting the number of patients in our ipilimumab group. This meant we could only collect a full set of samples from three patients. Subsequently, the focus shifted to collecting samples from patients being started on pembrolizumab.

Although fewer patients were recruited to this study than CRC patients in the previous chapter, they represented a range of patients with metastatic disease, across a range of age groups with different initial histology and site of origin (where this could be determined). Although not significant, these data show that in this group of melanoma patients, a raised LDH at baseline conferred reduced survival: median OS was 222 days in patients with an LDH over the ULN. Median OS was not met in the patients with an LDH within the normal range (Figure 5.3). LDH is associated with prognosis and disease progression in a number of different patient groups and forms part of the AJCC staging criteria (476). As an enzyme involved in cell metabolism it participates in cell growth and tumour bulk. Supporting our data, in a group of 97 patients with metastatic melanoma, those with elevated LDH levels had significantly poorer OS compared to those who did not (5 months *vs*. 16 months, $p<0.0001$) (550). Similar findings were shown in a larger cohort of 284 patients receiving treatment as part of early phase trials: in this dataset, lower LDH also corresponded to prolonged OS (11.8 *vs.* 5.8 months, $p=0.0001$) (477). Further supporting these findings, in a study of 71 stage IV melanoma patients, LDH was found to be a highly specific marker of progressive disease in patients receiving systemic therapy (including chemotherapy, immunotherapy and radiotherapy) (551). The lack of statistical significance in our cohort likely reflects the smaller patient numbers in the study. Interestingly, further studies into the prognostic ability of LDH

have shown that even patients with a 'high normal' LDH can have poorer outcomes than those with a truly low LDH and that even increases in LDH which still fall within the 'normal' range can affect survival.

In our patients, the activation status and potential to respond to immune stimuli was assessed using various validated assays, each of which examined different aspects of the immune system. Immunophenotyping was used to determine the expression of activation markers on various cell types. Evaluation of CD69 (an early activation marker) over the course of pembrolizumab treatment, revealed a slight decline in expression between baseline and 6 week samples, which was significant on NK cells and γδT (Figure 5.4). These changes could be due to a multitude of factors: the extended period of time between the samples or the general natural day-to-day variability in the immune system, without the added complications of tumour burden/treatment. It is possible that any stimulatory effect on the immune system may have been missed in this window and if samples had been taken at an earlier time point, e.g. 24 or 48 hours post-treatment, we may have observed a different result. Unfortunately this was not ethically possible as it would have required further patient visits out-with their treatment schedule. Reduced peripheral CD69+ T cells may actually represent an increase in trafficking of activated T cells out of the periphery, particularly in light of the observed rise in levels of chemokines such as MIG and IP-10 (Figure 5.13). CD69 expression on monocytes could not be determined due to the small population of cells present following overnight resting of PBMCs prior to analysis. Peripheral blood immune cells from patients with melanoma have been shown to have downregulation of IFN-stimulated genes when compared to healthy controls (552). This corresponded with decreased expression of activation markers such as CD69 and illustrated how changes in CD69 can reflect a suppressed immune response. It is also supportive of our data, which demonstrated a reduction in both CD69 and CD25 expression and decreasing levels of IFN-γ over the investigational period (Figure 5.4, Figure 5.10 and Figure 5.12, respectively).

In fresh PBMCs isolated from our patients being treated with pembrolizumab, there was reduced expression of CD25 on CD4 and CD8+ T cells between baseline and 6 weeks of therapy (Figure 5.10). Diminished CD25 expression on T cells is associated with a higher incidence of metastases (553) (although not statistically significant). Conversely, enhanced peritumoural infiltration of CD25+ T cells is associated with prolonged patient survival (553). Although the fall in CD25 in our samples was unexpected (given that treatment with checkpoint inhibitors should increase T cell activation and response), we were unable to take

matched patient biopsies for these peripheral blood samples. It may be, therefore, that peripheral blood levels of CD25 on T cells was diminished as this population was instead in the TME. Interestingly, lower levels of CD25 on CD4+ T cells have also been found in a proportion of patients treated with ipilimumab (554).

The current study did not show a significant change in the levels of Tregs over the course of therapy with either ipilimumab or pembrolizumab (Figure 5.8). There is some conflicting evidence to the effect of both anti-CTLA-4 and anti-PD-1 antibodies on levels of circulating Tregs. In a study of 95 patients receiving ipilimumab, a stable or decreasing peripheral Treg population at week 12 was associated with disease control (CR, PR or SD) (555). This was also significantly associated with prolonged survival compared to patients in whom Tregs increased during the course of treatment (15.8 months *vs*. 5.3 months, respectively, p=0.03) (555). Similarly, in patients treated with the anti-PD-1 antibody nivolumab, disease response was associated with a decline in peripheral Tregs (556). Conversely, patients who did not respond to therapy had a higher population of circulating Tregs at 12 weeks (556). Although some studies have shown a reduction in peripheral Tregs over the course of anti-CTLA-4 therapy (554,557), others have not (558,559), despite CTLA-4 being constitutively expressed on Tregs. While there is conflicting evidence as to whether anti-CTLA-4 therapy affects circulating Tregs, the positive effect that checkpoint inhibition has on the immune function of T cells is the more pertinent in creating an effective anti-tumour response.

The data presented in this chapter also demonstrates an increase in CD62L expression on Tregs in patients treated with pembrolizumab (Figure 5.8). Although no difference in CD62L expression was seen in the small group of ipilimumab patients, analysis of a larger cohort of patients treated with high dose ipilimumab and fotemustine showed enhanced CD62L expression on CD3+ CD4+ memory T cells, likely representing homing of these cells to LNs (560). The observed increase we observed in CD62L+ Tregs (which may be more immunosuppressive than CD62L^{low} Tregs due to their increased ability to home to LNs and suppress antigen-specific priming of T cells) may be part of a feedback mechanism reflecting heightened immune responses in these patients following pembrolizumab.

In contrast to other studies, we were unable to demonstrate any significant differences in the expression of other immune activation markers such as HLA-DR, which has been shown to increase in response to tremelimumab (559) and PD-L1 blockade with atezolizumab (427). Additionally, we did not observe changes in other cell surface markers, such as lower CCR7 expression on peripheral CD4 and CD8+ T cells following ipilimumab therapy that have been

seen in larger patient cohorts (561). Fresh immunophenotyping on all samples may have revealed these more subtle changes in expression of activation markers: changes in CD25 expression were detected in fresh PBMC samples but not in cryopreserved samples (Figure 5.10 and Appendix Table 8.9).

As expected, detection of PD-1 on immune cells was abrogated following treatment with pembrolizumab. This was observed irrespective of whether PBMCs had been analysed fresh or had been cryopreserved prior to immunophenotyping (Figure 5.5 and Figure 5.11). A similar trend for decreased CTLA-4 detection on CD4+, CD8+ T cells and NK cells was seen in patients treated with ipilimumab but once again, due to the small number of patients (n=3) and the low levels of CTLA-4-positive cells (less than 5 % of T cells and less than 1 % of NK cells), these findings were not significant (data not shown). During *in vitro* studies we were unable to find a FACS antibody with a clone that differed from those of the clinical antibodies, so the apparent reduction in the expression of these surface molecules may be due to the inability of the flow cytometry antibody to bind it's epitope on the cell surface in the presence of either ipilimumab or pembrolizumab. The expression of CTLA-4 and PD-1/PD-L1 on cell subsets can be enhanced in malignancy (representing an exhausted and ineffective anti-tumour response) (200) and may be associated with changes in disease-related outcomes (201,562–564). As such, expression of CTLA-4 and PD-L1 on tumour cells (or tumour-infiltrating immune cells) correlates with greater efficacy of PD-1/PD-L1 blockade (321,427,565).

While there was no demonstrable relationship between PD-1 expression on CD4+ T cells and survival, a lower level of PD-1 expression pre-treatment on both CD8+ T cells and B cells was significantly associated with prolonged survival in our patient cohort (Figure 5.7). The reverse was seen in Tregs, where higher PD-1 expression at baseline trended towards an increase in survival (although not statistically significant; Figure 5.7). Unfortunately, there was insufficient data to perform an accurate assessment of treatment effect on changing levels of PD-1 expression (calculation of OS stratified by fold change in PD-1 expression). Without taking treatment effect into consideration, the prolonged survival in patients with lower PD-1 expression on immune cells before treatment is likely a consequence of exhausted immune effector cells in the context of metastatic disease. Conversely, exhausted (PD-1+) Tregs presumably exhibit a lesser degree of immunosuppression. As such, the higher levels of PD-1 expression that we detected at baseline, although associated with poorer survival independent of therapy, confer increased susceptibility to PD-1 blockade. Although in our data we evaluated peripheral immune cells, similar findings have been demonstrated in

tumour biopsies of patients receiving anti-PD-1 therapy for metastatic melanoma: patients who responded to therapy (CR, PR or SD) demonstrated significantly higher pre-treatment PD-1 expression on intratumoural T cells compared to non-responders (565).

Changes in cytokine and chemokine levels in the plasma of our pembrolizumab- and ipilimumab-treated patients showed some degree of concordance. Despite smaller patient numbers in the ipilimumab-treated group, there was a demonstrable increase in levels of IL-3, IL-18, MIG and SCF over the 12 week investigational period for both groups. This change was significant in the pembrolizumab-treated group (Figure 5.13 and Figure 5.15).

IL-18 is a cytokine secreted by immune cells including APCs, T cells and NK cells. IL-18 works with IL-12 to stimulate the production of IFN-γ from T cells and NK cells and thus contributes to a T_H1 immune response (117). It is possible, therefore, that the increase in IL-18 seen in our patient cohort may in some way reflect a negative feedback mechanism to the reduction demonstrated in plasma levels of IFN-γ. In the absence of IL-12, however, production of IFN-γ would have been limited. The production of IFN-γ in response to IL-12 and IL-18 is inhibited by IL-4 *in vivo* (118). The increase in IL-18 and decrease in IL-4 seen in our patient cohort therefore seems to contradict the fall in IFN-γ that was also evident. Indeed, treatment with checkpoint inhibitors is generally observed to elevate levels of IFN-γ, representing up-regulation of anti-tumour immune responses (427,566,567). Additionally, an upregulation of IFN-γ production corresponds to an increase in the expression of immune checkpoint molecules such as PD-1/PD-L1 and CTLA-4, which should further confer greater susceptibility to antibody blockade (125). IL-18 also increases in response to anti-PD-L1 therapy with atezolizumab in patients with cancers including melanoma (427).

As possibly anticipated with checkpoint inhibition, levels of the T_H2 cytokines IL-4 and IL-5 declined during the first 12 weeks of pembrolizumab therapy in our patient cohort. *Ex vivo* evaluation of cytokine production by WBC from melanoma patients treated with anti-PD-1 antibody demonstrated a rise in T_H1 cytokines and a reduction in T_H2 cytokines (568). Although the changes in IFN-γ, IL-4 and IL-5 observed in our pembrolizumab patients were not fully replicated in the cohort of patients receiving ipilimumab, two of the three patients evaluated seemed to follow the same trend (Figure 5.12 and Figure 5.14). One again, due to the small number of patients it is difficult to draw any definite conclusions in this regard.

As also demonstrated in patients receiving chemotherapy for CRC, levels of both MIG and SCF increased during both ipilimumab and pembrolizumab treatment (not significant in the

ipilimumab group due to small number of patients) (Figure 5.13 and Figure 5.15). In addition, elevated IP-10 was evident following pembrolizumab treatment. As the production of both MIG and IP-10 are induced by IFN-γ, it again seems contradictory that both were, in fact, enhanced in the context of decreasing levels of IFN-γ. The window in which we may have detected higher levels of IFN-γ could have been missed due to the timings of the sample collection. Lower IFN-γ levels, however, would fit with the finding of reduced NK cell activity over time as demonstrated by decreasing CD107 expression in pembrolizumab-treated patients (Figure 5.16).

Treatment of melanoma cell lines with SCF induces a more immunogenic phenotype, with reduced production of immunosuppressive cytokines IL-10 and TGF-β alongside enhanced HLA-DR expression, which could potentially contribute to priming of an anti-tumour T cell response against melanoma-associated antigens (569). In the context of our patient cohort, therefore, the higher levels of SCF detected could represent an increase in immune response as a consequence of checkpoint inhibition.

IP-10 and MIG are closely related and are chemokines for monocytes and T cells, attracting immune cell populations into sites of infection and, potentially, into the TME (460). As well as their chemotactic properties, both exhibit a number of pro-immune functions that would support an enhanced anti-tumour response in our cohort of patients treated with checkpoint inhibitors. Through *in vivo* models of NSCLC, treatment with MIG has also been shown to inhibit tumour growth and metastasis through the inhibition of angiogenesis and tumour vascularity (165). Supporting the role of MIG and IP-10 in T cell recruitment, biopsies of melanoma metastases confirmed higher expression of both chemokines in samples with enhanced T cell infiltration (461). In a variety of studies, levels of both MIG and IP-10 have been shown to rise in response to checkpoint inhibition: increasing levels of both MIG and IP-10 were demonstrated in melanoma patients receiving nivolumab as part of a phase II clinical trial (570). Similarly, increased plasma levels of IP-10 were seen in patients receiving both anti-CTLA-4 and anti-PD-1 antibodies (571).

Our data shows a rise in levels of IL-3, IL-16 and SCGF-β over the first 12 weeks of pembrolizumab therapy. IL-16 is another cytokine which is not only secreted by immune cells but acts as a chemoattractant for CD4+ cells, including T cells and APCs. As with the previously discussed chemokines, IL-16 also exerts a number of immune functions that would be in keeping with finding a greater level of this cytokine in the plasma of patients exposed to immune checkpoint inhibitors (Figure 5.13). Like SCF, IL-3 is a haematopoietic cytokine which,

in addition to GM-CSF, M-CSF and G-CSF, can differentiate stem cells into immune cells including lymphocytes, granulocytes and macrophages. Data about the role of IL-3 in patients receiving checkpoint inhibition is limited but it has been used in some clinical trials to try and reduce the rate of cytopenia following chemotherapy. It is currently not used in clinical practice due to the poorer efficacy in phase III trials compared to other treatments such as G-CSF. *In vitro*, combination therapy with IL-3 and IFN-β can help to generate DCs which are able to effectively prime T cells against TAAs (572). These antigen-specific T cells were effective against target cells lines with similar efficacy to those primed by GM-CSF and IL-4 and the increased levels in our patient group suggests another function for IL-3 in priming immune responses of patients on checkpoint inhibitors (572).

In summary, elevated levels of immune-stimulatory cytokines such as IP-10, MIG and IL-18, coupled with a reduction in the T_H2 cytokines IL-4 and IL-5 would be in keeping with an augmented immune response in patients receiving checkpoint inhibitors and are indicative of an immune inflammatory milieu.

NK cell function was limited against melanoma cell lines, thereby potentially masking any changes in response to therapy (Figure 5.17, Figure 5.18 and Figure 5.19). However, CD107 expression against K562 cells (a natural NK cell target) was seen to diminish over time in patients receiving pembrolizumab (Figure 5.16). Although the ipilimumab patient group was too small to reach statistical significance, a similar trend was also apparent. This may have been due, in part, to the low expression of PD-1 on NK cells (< 5 % in patients treated with pembrolizumab, data not shown) (Figure 5.18). If both PD-1 and CTLA-4 were expressed on NK cells, checkpoint inhibition should increase their cytotoxic potential. Elevated expression of both CTLA-4 and PD-1 on NK cells has been shown in patients with cancer and is associated with functionally inhibited cells (25,26,30). It should also be noted that CTLA-4 and PD-1 are not the only inhibitory receptors: expression of KIRs and other checkpoint inhibitors such as TIM-3 can be upregulated on the surface of NK cells from patients with melanoma (25). Decreasing functionality in our patient cohort could be due to upregulation of these and other inhibitory receptors in response to therapy. Further investigations into these markers (TIM-3, LAG-3 and NK cell receptors) may reveal additional information about NK cell function in melanoma during checkpoint therapy.

In addition to investigating the innate immune response to checkpoint inhibition, the adaptive T cell response was also examined using ELISpot assays. The patients included in this study demonstrated variable T cell responses to either CEF viral peptide pool or MART-1 peptide,

although other groups have shown evidence of MART+ CTLs in the peripheral blood of melanoma patients (575). Due to this variability, there was not enough data here to accurately gauge the effects of either pembrolizumab or ipilimumab on T cell response to antigen, as demonstrated by IFN-γ production. Outside this cohort, an increase in MART-specific T cells has been shown to correspond to disease response in a phase I study of patients receiving nivolumab (with or without a peptide vaccine) following ipilimumab therapy in a phase I trial (556).

As seen in the group of patients receiving chemotherapy for CRC, a number of patient T cell responses in ELISpot assays reflected results seen in other immune assays. In patient 12 in particular (pembrolizumab group), a reduced T cell response to CEF peptide was echoed in both decreased CD69+ activated T cells and in innate immune function of NK cells (Figure 5.23). As previously discussed, given the complicated but tightly interwoven nature of both innate and adaptive immune responses, this is perhaps unsurprising. Larger cohorts would be needed to evaluate whether these responses meaningfully correlated to disease outcome or survival.

Although we attempted to match peripheral blood with biopsy samples in our study, this proved prohibitive in the recruitment of patients. Matched tumour and peripheral samples in other data sets have revealed that PD-1 expression on peripheral T cells can be significantly raised when compared to those within the TME (200,571). Moreover, treatment with nivolumab can successfully abrogate PD-1 expression on peripheral T cells, while a higher proportion of TILs will continue to express PD-1 and not be saturated by binding of therapeutic antibody (571). Interestingly, a similar phenomenon has been noted in regard to antigen-specific T cells in peripheral blood *vs.* tumour (200) and may explain the low levels of T cell responses to the MART antigen that was demonstrated in our patients. In keeping with the observation that TILs express higher levels of PD-1 than peripheral blood lymphocytes, MART antigen-specific TILs from patients with melanoma also demonstrate diminished effector function and lower levels of IFN-γ production in response to immune stimulation (200). It may be, therefore, that the small number of patients who had circulating peripheral MART-specific T cells in this study (Figure 5.22) had functional cells, as demonstrated by the production of IFN-γ in ELISpot assays. The majority, however, did not have circulating peripheral antigen-specific cells but may have had exhausted T cells within the tumour, or be primed against different melanoma-associated antigens (other than MART-1).

Finally, as also seen in the cohort of patients receiving chemotherapy for colon cancer, there was a significantly shortened OS in melanoma patients who had a high NLR prior to treatment (Figure 5.24). Unfortunately, once again, due to the smaller number of melanoma patients and the availability of PBMCs for immune assays, it was not possible to draw any meaningful conclusions regarding NK cell function or cytokine production at baseline stratified by NLR. Further assessment of other immune parameters according to baseline NLR did not show any appreciable difference. This remained the case if the NLR threshold was moved to NLR ≥ 4 or ≥ 3 and no correlation was seen between baseline immune function and NLR even with these parameters (data not shown). NLR has been shown in a number of other datasets to correlate with prognosis and survival in patients with melanoma (576,577). In cohorts of patients receiving ipilimumab, an NLR of less than 5 (as in our data) was associated with improved OS and PFS (576). As with the data from our patients with CRC, there are variations in what is deemed to be the cut-off value for NLR: a larger study of 720 patients receiving ipilimumab for metastatic melanoma deemed the optimal NLR to be less than 3 (although this was a derived rather than absolute NLR) (392). Once again, there was shown to be a significantly prolonged OS and PFS in patients with a dNLR of < 3 (392).

In summary, overall, these data illustrate some of the changes in immune response that are observed in patients receiving treatment with checkpoint inhibitors. Although some of the findings are underpowered due to the small number of patients assessed, it can offer a unique insight into the parameters by which anti-tumour immune responses can be exploited and enhanced by treatment with either ipilimumab or pembrolizumab, as well as serving as an interesting comparison to patients receiving chemotherapy for CRC.

Chapter 6 Conclusion

Overall, these data have demonstrated that it is possible to evaluate the peripheral blood immune response to anti-cancer therapy in a robust and reproducible manner. We have successfully developed a panel of immune assays that can be performed on the peripheral blood of patients, detecting differences in the responses to cytotoxic chemotherapy and immunotherapy. As the assays have been optimised to allow for batched analyses, following cryopreservation of PBMCs, they are amenable to being used in groups of patients undergoing a variety of systemic therapies, for a range of different cancers. Despite the recognition that the magnitude of some responses may be slightly diminished in cryopreserved cells, their reaction to immune stimuli appear to be comparable across time points and within patient samples. This allows the identification of trends in immune function, while being able to ensure the high quality, reproducibility of the results, which have been batch-analysed under identical conditions to maintain quality control. While other groups have shown the ability to analyse varying aspects of the immune system in patients, this assay panel is novel in being able to examine multiple aspects of the immune system and how it changes over time within a patient population. Although immune responses are widely variable across individual patients, we have shown that this assay panel could readily be incorporated into clinical trials in order to evaluate responses to treatment. In an era when the use of immunotherapy is rapidly expanding, being able to improve our understanding of immune responses to immunotherapy, chemotherapy, radiotherapy, targeted therapy and viral therapy, would allow us to exploit this when developing new anti-cancer treatment strategies.

In patients with CRC, we have shown that NLR ≥ 5 before chemotherapy is associated with an attenuated immune response, when compared to patients with NLR < 5, supporting our original hypothesis. High peripheral blood NLR before treatment was associated with a cytokine profile that differed from patients with a low baseline NLR: amongst others, higher levels of the immunosuppressive cytokines IL-10 and TGF-β were observed. Similarly, there was a suggestion of differences between innate immune responses, as determined by CD107 expression and ^{51}Cr cytotoxicity assays, in patients with a higher pre-treatment NLR. In support of these data, increased OS was seen in patients with a more robust immune response, i.e. low baseline NLR.

When interrogating the adaptive immune response to viral antigens and TAAs, it appeared that a greater proportion of patients with a high baseline NLR had responses to CEF viral

peptides (as a general recall response). Over the first 6 weeks of chemotherapy, peripheral blood immune responses were seen to activate as the NLR fell to < 5. Alongside this, a decline in TGF-β secretion was seen, as well as a fall in peripheral Tregs.

There were also ongoing differences between the patients with a high and a low pre-treatment NLR that would warrant further study and may explain the persisting difference between the groups even after chemotherapy. Levels of IL-10, for example, did not alter during treatment and remained elevated in the patients who had a high NLR at baseline, irrespective of a fall in NLR over the first 6 weeks of therapy. Once again, differences remained between the two groups, in that more patients with a low NLR over the first 6 weeks of chemotherapy demonstrated greater T cell responses against TAAs.

While NLR has been shown to correlate with outcome in CRC, the rationale behind this relationship is not understood. The current data may, therefore, go some way into understanding this, by demonstrating a diminished immune response. Although, to some extent, this appears to reverse with chemotherapy, differences between the groups remain (high *vs.* low baseline NLR) even if NLR falls. This also appears to correlate with other data suggesting that even normalisation of NLR does not improve OS to the same extent as patients with a low NLR at the outset.

Immunotherapy has only been shown to be effective in a small proportion of CRC patients; that is, patients with dMMR tumours. The data presented here could therefore prompt further study into the potential role for immunotherapy after an initial period of chemotherapy. Although this would appear to be a valid area of further study in all patients, as those with a high baseline NLR continue to have poorer outcomes in terms of OS, effectiveness of sequential therapy in this sub-group of patients would be of particular interest.

Our study also evaluated the immune response to checkpoint inhibitors in patients with melanoma. Firstly, the data confirmed that treatment with ipilimumab or pembrolizumab blocked the expression of CTLA-4 and PD-1, respectively. Although the melanoma patient group was smaller than the CRC patient group, similar trends were noted. In the pembrolizumab-treated patients, a fall in CD69 and CD25 expression was evident on some populations of circulating immune cells. These differences were enhanced when PBMCs where immunophenotyped before cryopreservation. More obviously, the cytokine profile in patient plasma revealed an apparent fall in IFN-γ, IL-4 and IL-5. While the decrease in IL-4 and

IL-5 might be expected in the context of immune stimulation by checkpoint inhibitors, the concurrent decline in levels of IFN-γ seem discordant. In contrast to IFN-γ, plasma levels of other pro-immune cytokines, including IL-3, IL-16, IL-18, IP-10 and MIG increased over the course of treatment. Once again, this supports the theory of an elevated anti-tumour immune response over the course of immunotherapy in patients with melanoma. Although these findings were significant in the patients receiving pembrolizumab, a similar trend was seen in the small group of patients receiving ipilimumab.

There are comparisons to be made between the CRC group treated with chemotherapy and the melanoma patient cohort treated with immune checkpoint inhibitors. Due the different number of patients in each group, variable baseline characteristics, as well as different underlying pathology, these are limited to observations that could be taken forward for further study. In contrast to the CRC patient cohort, changes in innate immune responses, mediated by NK cells, were not demonstrated in the melanoma patient cohort. This may be explained by the finding that less than 5 % of the population of NK cells expressed PD-1 at baseline and, hence, the effect of anti-PD-1 antibodies on NK cell responses may be limited. Future work could therefore also include analysis of the activating and inhibitory receptors on NK cells which would further inform their function during anti-cancer treatment. There are inherent differences between the immune responses evidenced in the groups of patients with CRC and those with melanoma. One explanation of this may be the reason why immunotherapy is generally less effective in patients with CRC (except for the small cohort with dMMR). That is, due to differences in mutational load/neoantigens, the anti-tumour immune response of patients with melanoma may be already 'primed' to some extent. In contrast, our data may suggest that chemotherapy can prime an immune response in patients with CRC. This is supported by the finding that PD-1 increases over the course of treatment on some immune cell populations in patients with CRC. Changes in expression of PD-1 (or CTLA-4) over time in the melanoma cohort, however, was unable to be determined due to blockade of the receptor by pembrolizumab (or ipilimumab), respectively. Future studies would, therefore, include a broader spectrum of immune checkpoints, such as TIM-3, LAG-3 and BTLA-4 in order to be able to determine a fuller picture of immune activation in patients out-with the effect of anti-CTLA-4 and anti-PD-1.

In many of our immune assays, very little change was observed in response to therapy and this is probably due to the timing of samples taken. As these studies used a 'window of opportunity', blood samples were taken at scheduled patient visits and any extra were

ethically prohibited. For this reason, data may not have been captured if the window of response was missed. Hence, in future studies we would aim to not only expand the number of patients in order to get a meaningful data set but also change the timings of the samples to fully interrogate the response. In addition, the local ethics consent which allowed us to collect patient samples did not allow us to collect samples from healthy controls for comparison. For validation assays, a small number of HD blood was used but we were unable to increase these numbers to a level whereby an accurate comparison with an aged-matched patient population would be possible.

While these data utilised the simplicity of peripheral blood collection to evaluate immune responses to anti-cancer therapy, for a more complete picture, comparison with the immune milieu in patient tumours could be enlightening. From this, we would be able to determine whether the peripheral blood immunome is truly a surrogate for that in the tumour or just part of the picture. Tumour tissue samples are of course harder to access, especially over the course of treatment, although in melanoma patients we initially investigated the practicalities of paired fresh tissue biopsies from skin metastases.

Similarly, we have been unable to fully interrogate the neutrophil population in either CRC or melanoma patients, due to the difficulties in working reliably with these cells. Towards the end of this project, early work into the investigation of neutrophil ectosomes was started which could be continued further. The hypothesis being that, as a high neutrophil count contributes to a high NLR, isolation of neutrophil ectosomes in patient plasma may reveal, as other data has suggested, the production of immunosuppressive cytokines such as IL-10 and TGF-β. This may go some way to explaining our findings in patients with a high NLR and also the contribution of high peripheral blood neutrophils to a poorer prognosis and OS in cancer.

Finally, in summary, these data show that we have met our initial objective of optimising and validating a panel of assays to interrogate immune responses in peripheral blood for use in batch analysis scenarios. In patients with CRC being treated with chemotherapy, these preliminary data suggest a role for chemotherapy in priming an immune response, particularly in patients with a high baseline NLR, which could potentially be exploited with the sequential use of immunotherapy. This is of particular interest given that until now these patients in general have not been shown to benefit from immunotherapy and thus has the potential to be practice changing. Going forward into clinical trials, this data would support the use of immune checkpoint inhibitors after an initial period of chemotherapy. Patients with stable disease after 12 weeks of chemotherapy (again stratified by baseline NLR) could, for example,

go onto receive an immune checkpoint inhibitor. This would allow for further collection of clinical samples, potentially with the addition of tumour biopsies for paired analysis. It would also be used to evaluate the benefit of immunotherapy after chemotherapy. Similarly, given that we saw some changes in immune function at 6 weeks, we could also look for early markers of disease progression in a larger group of patients.

In patients with melanoma being treated with checkpoint inhibitors, we have been able to reveal immune signatures, which may be used as initial data to investigate potential novel biomarkers in such patients. More particularly, these data may be more relevant in investigating biomarkers for toxicity and could have a role to play in patient selection and management of immune-mediated side effects in the future.

This work has expanded on our understanding of immune responses to anti-cancer therapy. It has revealed new opportunities and generated hypotheses for further investigation into the role of the immune system in cancer and anti-cancer therapy and may ultimately lead to better patient treatment and improved survival outcomes.

Chapter 7 References

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Chapter 8 Appendix

Table 8.1 *p* values for fresh and cryopreserved PBMCs either untreated or stimulated with 1pfu/cell reovirus at stated effector:target ratios (Figure 3.5).

	Fresh PBMCs			Cryopreserved PBMCs		
E:T Ratio	K562	SW620	Mel888	K562	SW620	Mel888
100:1	0.0282	0.0204	0.0048	0.305	0.0146	0.0176
50:1	0.0108	0.0063	0.0024	0.0091	0.0157	0.0047
25:1	0.0325	0.0125	0.002	0.0234	0.0182	0.0064
12.5:1	0.0289	0.0138	0.0009	0.0102	0.0176	0.0041
6.25:1	0.0399	0.0129	0.0002	0.0109	0.0048	0.0041
3.12:1	n/s	0.0043	0.0029	0.0130	0.0219	0.0051
1.56:1	n/s	0.0377	0.0016	0.0131	0.0025	n/s
0.78:1	n/s	n/s	0.0051	n/s	0.0377	0.0377

Table 8.2 *p* values for % CD69 expression on immune cell populations from 'same-day' and 'rested' PBMCs following treatment ± 1pfu/cell reovirus (Figure 3.10).

Population	'Same-day' PBMCs	'Rested' PBMCs
CD4+ T cells	0.0019	0.0005
CD8+ T cells	0.0018	0.0032
NK cells	0.0001	0.0045
NK cells (dim)	<0.0001	0.0054
NK cells (bright)	0.005	0.0081
NKT cells	0.0028	0.0007
γδT cells	0.0008	0.0012
Monocytes	0.0054	n/s
B cells	0.001	0.043

Table 8.3 Sample collection in patients with CRC on chemotherapy

All patient sample sets collected were complete, with the exception of the following (patients from NLR-23 onwards only had baseline and 6 week samples collected):

Patient	Baseline Sample	6 week Sample	12 week Sample
NLR-4	✓	×	×
NLR-6	✓	×	×
NLR-9	✓	×	×
NLR-14	×	×	×
NLR-17	✓	✓	×
NLR-29	✓	×	

Table 8.4 Activation markers on immune cell populations of CRC patients which did not differ between those with a high or low NLR at baseline

Activation Marker	Cell Population
CD69	CD4+ T cells, CD8+ T cells, NK cells, NKT cells, monocytes, B cells
PD-1/PD-L1	CD4+ T cells, CD8+ T cells, NK cells, NKT cells, monocytes, B cells, Tregs
HLA-DR	Monocytes, B cells
CCR7	CD4+ T cells, CD8+ T cells, NK cells, NKT cells

Table 8.5 Activation markers on immune cell populations of CRC patients which did not change over the first 6 weeks of chemotherapy

Activation Marker	Cell Population
CD69	CD4+ T cells, CD8+ T cells, NK cells, NKT cells, monocytes, B cells
PD-1	CD4+ T cells, CD8+ T cells, NKT cells, Tregs
PD-L1	CD4+ T cells, CD8+ T cells, NK cells, NKT cells, monocytes, B cells, Tregs
HLA-DR	Monocytes, B cells
CCR7	CD4+ T cells, CD8+ T cells, NK cells, NKT cells
CD62L	Tregs

Table 8.6 Cytokines/chemokines not detected by Luminex in CRC patient plasma

Cytokines/chemokines not detected/below standard curve					
IL-2	IL-1Rα	IL-12p40	IL-15	MCP-1	TNF-α
IL-1α	IL-5	IL-12p70	GM-CSF	M-CSF	TNF-β
IL-1β	IL-6	IL-13	LIF	B-NGF	VEGF

Table 8.7 Sample Collection in patients treated with ipilimumab

All patient sample sets collected were complete, with the exception of the following:

Patient	Baseline Sample	6 week Sample	12 week Sample
MelIpi-01	✓	×	×
MelIpi-02	✓	×	×
MelIpi-05	✓	✓	×
MelIpi-06	✓	×	×

Table 8.8 Sample Collection in patients treated with pembrolizumab

All patient sample sets collected were complete, with the exception of the following:

Patient	Baseline Sample	6 week Sample	12 week Sample
MelPem-02	✓	✓	×
MelPem-05	✓	×	×
MelPem-06	✓	✓	×
MelPem-07	✓	×	×
MelPem-08	✓	×	✓
MelPem-10	✓	✓	×
MelPem-17	✓	✓	×

Table 8.9 Activation markers on cryopreserved immune cell populations of patients treated with pembrolizumab which are unchanged over time

Activation Marker	Cell Population
CD69	CD4+ T cells, CD8+ T cells, NKT cells, monocytes, B cells
PD-1	NK cells, NKT cells, monocytes, B cells
PD-L1	CD4+ T cells, CD8+ T cells, NK cells, NKT cells, γδT cells, monocytes, Tregs
CD25 **CTLA-4**	CD4+ T cells, CD8+ T cells
HLA-DR **CD11c** **CD86**	Monocytes, B cells
CCR7	CD4+ T cells, CD8+ T cells, NK cells, NKT cells, γδT cells
CD16	NK cells, NKT cells, γδT cells

Table 8.10 Cytokines/chemokines not detected by Luminex in patients receiving pembrolizumab

Cytokines/chemokines not detected/below standard curve		
IL-1α	IL-10	GM-CSF
IL-2	IL-12p40	M-CSF
IL-6	IL-15	TNF-β

Table 8.11 Cytokines/chemokines with no change over time in patients receiving pembrolizumab

Cytokines/chemokines with no significant change over treatment period				
IFN-α2	IL-9	CTACK	MCP-1	RANTES
IL-1β	IL-12p70	Eotaxin	MCP-3	SDF-1α
IL-1Rα	IL-13	G-CSF	MIF	TNF-α
IL-2Rα	IL-17α	GRO-α	MIP-1α	TRAIL
IL-7	β-NGF	HGF	MIP-1β	VEGF
IL-8	Basic FGF	LIF	PDGF-BB	

Table 8.12 Cytokines/chemokines not detected by Luminex in patients receiving ipilimumab

Cytokines/chemokines not detected/below standard curve			
IL-1α	IL-10	IL-15	MCP-1
IL-2	IL-12p40	β-NGF	M-CSF
IL-6	IL-12p70	GM-CSF	TNF-β