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The effect of Macrophage Activation and Apoptosis in the Immune Response to Respiratory Pathogens

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Declaration

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Human monocyte derived macrophages were from peripheral blood obtained under ethical approval from South Sheffield regional ethics committee (07-Q2305/7). All animal studies were carried out in accordance with Animals (Scientific Procedures) Act 1986, with approval of the Sheffield Ethical Review Committee, Sheffield, United Kingdom and the GSK Policy on the Care, Welfare and Treatment of Animals.

Abstract

Macrophage activation is essential for macrophage function; however, the phenotypic profile of macrophages can become dysregulated during pulmonary disease. Two major causes of lower respiratory tract infections are *Streptococcus pneumoniae* (*S. pneumoniae*) and Non-Typeable *Haemophilus influenzae* (NTHi). These are problematic as current preventative treatments against these opportunistic pathogens are ineffective for those most vulnerable to infection. Macrophages can undergo apoptosis when infected with *S. pneumoniae* as part of the innate immune response. This apoptosis-associated bacterial clearance is reduced in alveolar macrophages (AMs) from COPD and HIV patient associated with increased expression of the anti-apoptotic protein Mcl-1.

I hypothesized that macrophages from Mcl-1 over-expressing transgenic mice would display an altered macrophage activation profile which would affect macrophage effector functions after bacterial challenge. Given the prominence of NTHi infection in diseases with defective macrophage function I also predicted NTHi challenge would cause macrophage apoptosis associated killing, which is inhibited by over expression of Mcl-1, similar to previous observations for *S. pneumoniae*.

Induction of classical and alternative macrophage activation was analysed between wild-type and transgenic Bone Marrow Derived Macrophages (BMDM) and determined in human Monocyte Derived Macrophages (MDM). The effect of macrophage activation on killing of *S. pneumoniae*, microbicidal production and apoptotic activity was then assessed in both BMDM and MDM models. A transcriptomics study was conducted to understand changes in gene expression between wild-type and transgenic AMs at 16 hours, a critical time point of *S. pneumoniae* challenge, at the onset of macrophage apoptosis associated killing. Macrophage killing and microbicidal production in MDMs after NTHi challenge were observed and apoptosis and Mcl-1 protein levels were assessed over a 72-hour time course. The effects of macrophage activation on NTHi challenge was also measured.

I found that Mcl-1 did not alter macrophage activation but activation of BMDM and MDM with IFN-γ (M(IFN-γ)) caused enhanced clearance of intracellular bacteria at early and late time points of *S. pneumoniae* challenge. Furthermore, levels of apoptosis were increased in M(IFN-γ) at 16 hours of *S. pneumoniae* challenge. Transcriptomic analysis of wild-type and transgenic AMs after 16 hours of challenge revealed induction of a T-cell signature in transgenic samples. Challenge of MDMs with NTHi caused low levels of macrophage apoptosis at 20 hours of challenge which

increased at 48 and 72 hours. Levels of Mcl-1 remained high at 16-20 hours then decreased from peak at 20 hours onwards with increasing levels of macrophage apoptosis.

These results demonstrate the importance of IFN-γ production during *S. pneumoniae* challenge. They also highlight a potential role for T-cell involvement in transgenic macrophages when the apoptotic response is compromised. Finally, they give insights into an unexplored aspect of macrophage immunology after NTHi challenge, showing increased apoptosis at later timepoints of infection. These findings offer useful insights into host innate immunology and may provide groundwork for future therapeutic development.

Abbreviations

AIM2	Absent in Myeloma 2
AAM	Alternatively Activated Macrophages
Akt1	RAC-alpha serine threonine protein kinase
AM	Alveolar Macrophage
ANOVA	Analysis of variance
APC	Antigen Presenting Cell
Bak	Bcl-2 homologous antagonist killer
BAL	Bronchoalveolar Lavage
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma
BH	Bcl-2 Homology
BHI	Brain and Heart Infusion
BMDM	Bone Marrow Derived Macrophages
C3	Complement Component 3
CAM	Classically Activated Macrophages
CAP	Community Acquired Pneumonia
Caspases	Cysteine-ASPartic proteASES
CBA	Columbia Blood Agar
ChoP	Phosphorylcholine
CLP	Common Lymphoid Progenitor
CLR	C-type-Lectin Receptor
CMP	Common Myeloid Progenitor
COPD	Chronic Obstructive Pulmonary Disease
CR1	Complement Receptor 1
DAF-FM	4-Amino-5-methylamino-2',7'-difluorofluorescein
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DCF-DA	Dichlorofluorescin diacetate
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic
EEA-1	Early Endosome Antigen-1
EGTA	Egtazic acid
ELISA	Enzyme Linked Immunosorbant Assay

FAD	Flavin Adenine Dinucleotide
FADD	Fas-associated death domain protein
FasL	Fas Ligand
Fcγ	Fc gamma
FIZZ-1	Found in Inflammatory Zone-1
FMN	Flavin Mononucleotide
FMO	Flourescence Minus One
H_2O_2	Hydrogen peroxide
HIFBS	Heat Inactivated Foetal Bovine Serum
Hib	*Haemophilus influenzae* type B
HIV-1	Human Immunodeficiency Virus-1
HRP	Horse-Radish-Peroxidase
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA1	Immunoglobulin A subclass 1
IL-	Interleukin
ILC	Innate Lymphoid Cell
iNOS	inducible Nitrogen Oxide Synthase
IRAK	IL-1 Receptor Associated Kinase
IRF	Interferon regulatory factor
ITAM	Immunoreceptor Tyrosine Based Activation Motifs
ITIM	Immunoreceptor Tyrosine Based Inhibitory Motifs
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi- dazolylcarbocyanine iodide
LAMP	Lysosomal Associated Membrane Protein
LIMP	Lysosomal Integral Membrane Protein
LL-37	Catelicidin
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
Ly6C	Lymphocyte Antigen 6C
MAL	MyD88-Adaptor Like
MAPK	Mitogen-activated protein kinases
MARCO	Macrophage Receptor with Collagenous Structure
Mcl-1	Induced myeloid leukemia cell differentiation protein

MCP	Microbicidal Cationic Peptides
MDM	Monocyte Derived Macrophages
MHC I	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Proteins
MOI	Multiplicity of Infection
MOMP	Mitochondrial Outer Membrane Permebalisation
MSD	MesoScale Discovery
MTT	3- [4, 5-dimethylthiazol-2yl]-2, 5-diphenyl-tetrazolium bromide
MULE	Mcl-1 Ubiquitin E3 Ligase
MyD88	Myeloid Differentiation primary response gene 88
N_2O_3	Dinitrogen trioxide
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor kappa light chain enhancer of activated B-cells
NK Cell	Natural Killer Cell
NLR	Nod-Like-Receptor
NO	Nitric Oxide
NO2	Nitrogen dioxide
Nod	Nucleotide Oligomerisation Domain
NRAMP	Natural Resistance Associated Macrophage Protein
NTHi	Non-Typeable *Haemophilus Influenzae*
O.D.	Optical Density
$O_2^{\cdot -}$	Superoxide
OMP	Outer Membrane Proteins
OMV	Outer Membrane Vesicles
$ONOO^-$	Peroxynitrite
p38	p38 mitogen activated protein kinases
PAGE	Poly Acrylaminde Gel Electrophoresis
PAMP	Pattern Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3 Kinase
PLY	Pneumolysin
PRR	Pattern Recognition Receptor
PspA	Pneumococcal Surface Protein A

PspC	Pneumococcal Surface Protein C
RMA	Robust Multiarray Averaging
RNS	Reactive Nitrogen Species
RORγt	RAR-related Orphan Receptor gamma
ROS	Reactive Oxygen Species
qPCR	Real time quantitative Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
STAT	Signal Transducer and Activator of Transcription
TARC	Thymus and Activation Regulated Chemokine
T-bet	T box transcription factor
TBS	Tris-Buffered Saline
TCA	Trichloro-acetic Acid
TCR	T cell Receptor
TGF-β	Transforming Growth Factor Beta
Th	T helper cell
TIR	Toll/Interleukin Receptor
TLR	Toll like Receptor
TNF-α	Tumour Necrosis Factor alpha
TRIF	TIR domain containing adaptor protein inducing IFN-β

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1. Introduction

1.1. The Immune System

Lower respiratory infections, excluding tuberculosis, account for over 3 million deaths worldwide per annum and are now ranked 3rd most common cause of death globally (WHO, 2015). Aside from the barriers our bodies present to external world, such as our skin, mucous membranes and the acidic environment of the stomach, more complex mechanisms are required for protection against and eradication of pathogens and toxins. The immune system is divided into categories of highly specialised cells which are responsible for an abundance of multifaceted actives extending much further than just host defence. Here I will describe the importance of our immune system in bacterial infection with specific insight on the position of the macrophage in response to the respiratory pathogens *Streptococcus pneumoniae* (*S. pneumoniae*) and Non-Typeable *Haemophilus influenzae* (NTHi).

1.1.1. Division and Components

Multipotent haematopoietic stem cells which reside in the bone marrow give rise to all cells of the immune system. The two main lineages which mature here are known as Common Lymphoid Progenitors (CLPs), which give rise to the lymphoid lineage, encompassing B-cells, T-cells and Natural Killer (NK) cells and Common Myeloid Progenitors (CMPs) which account for the majority of the cellular components of blood, including megakaryocytes and erythrocytes. CMPs also give rise to polymorphonuclear leukocytes, circulating granulocytes; neutrophils, basophils and eosinophils. Mononuclear mast cells are derived from bone marrow progenitors but differentiate in the tissue. Mononuclear leukocytes, monocytes circulate the body and are precursors to macrophages, which differentiate in the tissue and are the professional phagocytes of the innate immune system. Alternatively, development of specific niches of tissue macrophages are being increasingly recognised as deriving from embryonic lineages such as the foetal liver or yolk sac (van de Laar et al., 2016). These macrophage populations have been shown to exist into adulthood and in many scenarios are capable of self-replenishment. Mast cells also differentiate in the tissues along with Dendritic Cells (DCs) which are also professional phagocytes and are an exception in that they can be derived from either CLPs or CMPs. Collectively the two distinct lineages of the immune system give rise to two different arms of immunity, the ancient, evolutionarily conserved innate immune response, crudely referred to as “non-specific” immunity and the “specific” adaptive immune response which is a more recently developed addition to our immune system, emerging in our jawed vertebrate ancestors (Cooper and Alder, 2006), primarily concerned with providing us with immunological memory.

However, increasingly we recognise that while CMP derived cells are all innate immune cells CLPs make major contributions to innate immunity with the identification of Innate Lymphoid Cell (ILC) populations (Artis and Spits, 2015).

1.1.2. Adaptive and Innate Immunity

Pathogen recognition occurs by employment of pattern recognition receptors (PRRs) located on the cell membrane which are highly responsive to Pathogen Associated Molecular Patterns (PAMPs), evolutionarily conserved sequences located on the pathogen surface which help phagocytes to identify molecules as "non-self". Several studies identified innate immune pathogen recognition in more detail. PRRs such as membrane bound toll-like-receptors (TLRs), C-type-lectin receptors and cytoplasmic PRRs such as Nucleotide Oligomerisation Domain (Nod)-Like-Receptors (NLRs) or RIG-1-like-receptors were shown to recognise PAMPs which include bacterial components such as Lipopolysaccharide (LPS), peptidoglycan, teichoic acid and bacterial DNA. Once triggered, PRRs initiate signalling cascades within innate immune cells to aid internalisation of pathogens and intracellular killing, (Doyle et al., 2004; Medzhitov et al., 1997). Furthermore, PRR activation triggers downstream upregulation of transcription factors such as Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) which is a crucial immunomodulator in effective escalation of the immune response (Lawrence, 2009). As well as providing an initial effector response to infection, many phagocytes of the innate immune system also act as Antigen Presenting Cells (APCs) whose role is to present molecular components of digested bacteria on their cell membrane, by association with Major-Histocompatibility-Complex (MHC) class II expression. This in turn activates cells of the adaptive immune response. The requirement for immune sensing cells as the activators of adaptive immunity was first postulated by Charles Janeway (Janeway, 1989).

1.1.2.1. Activation of lymphocytes by APCs

The role of the APC is to encounter B and T-cells and activate them according to the specific threat. B and T-cell development includes the recombination of DNA which encodes the variable portions of their membrane bound B-cell receptor antibody and T-cell receptor (TCR) antigen receptors, therefore the diverse population of B and T-cells in the body possess the capability of recognising an innumerate amount of pathogen epitopes. B and T-cells can also initially recognise self-antigens, however auto-reactive B and T-cells are negatively selected and silenced through mechanisms collectively known as central tolerance (Pelanda and Torres, 2012). Upon encounter of microbial sequences presented by APCs, the population of B and T cells which recognise the characteristic epitope of the specific pathogen are then capable of replicating

through a process known as 'clonal expansion' (Burnet, 1976). Clonal expansion results in 'effector' and 'memory' populations which either orchestrate the specific pathogen induced immune response or remain in the circulation until required to bring about a rapid immune response later due to a repeat infection with the same pathogen. Two main categories of T-cells exist; either 'helper' ($CD4^+$) or 'cytotoxic' ($CD8^+$). Cytotoxic T-cells recognise antigens from damaged, infected or abnormal cells such as tumour cells, presented by MHC class I expression, present on all nucleated cells. They also recognise antigens presented by MHC class I on the surface of APCs such as DCs which can endocytose antigens and subsequently present them (cross-presentation) (Heath and Carbone, 2001). Antigen recognition is then followed by cytotoxic T-cell maturation which results in elimination of target cells by induction of programmed cell death or cell lysis (Andersen, 2006; Janeway et al., 2001). T-helper cells on the other hand are activated by MHC class II antigen presentation and are instrumental in further activation of innate immune cells to bring about pathogen specific responses.

1.1.2.2. Professional Antigen Presenting Cells

DCs, macrophages and B-cells are all examples of 'professional' APCs, however antigen presentation is the primary role of the DC and mice depleted of DCs fail to stimulate lymphocytes demonstrating this cell type is crucial to the primary immune response (Steinman and Witmer, 1978). There are two main populations of DCs in the lung, $CD103^+$ which have superior MHC I loading machinery and cross presentation ability and are therefore mainly involved in antigen presentation to cytotoxic T cells and $CD11b^+$ DCs, primarily concerned with T helper cell antigen presentation (del Rio et al., 2007). Macrophages whist fulfilling the requirements of a professional APC, such as MHC class II expression, T-cell co-stimulatory molecule expression and cytokine release, are more-so involved in phagocytosis and microbicidal activity and they possess more acidified phagosomes than DCs (Delamarre et al., 2005; Nagl et al., 2002). Furthermore, DCs have been shown to localise to lymphoid organs where naïve lymphocytes reside, whereas the majority of macrophages remain in non-lymphoid tissues, supporting their role as important mediators of the secondary immune response (Itano and Jenkins, 2003). Interestingly, identification of the capacity for other haematopoietic cells to act as APCs is also emerging, including basophils, eosinophils, neutrophils, ILCs and $CD4^+$ T-cells and this role extends to non-haematopoietic cells such as endothelial and epithelial cells (reviewed in (Kambayashi and Laufer, 2014)).

1.1.2.3. T-helper cell polarisation

T-helper cells are naïve until activated by specific stimuli, which determines the type of T-helper cell they will become (Pennock et al., 2013). For example, signalling through the TCR induces a

transcriptional pattern which determines how the T-cell will be polarised. Furthermore, engagement of APC costimulatory (and co-inhibitory) molecules with T-cell costimulatory receptors (i.e. ligand B7-1 with CD28) is required as a second signal to direct T-cell proliferation and activation (Chen and Flies, 2013; Mueller et al., 1989). The 'second touch hypothesis' describes T-cells as only partially activated before migrating to the tissues where they become fully polarised by the specific cytokine environment of the inflamed tissues (Ley, 2014). Experiments with macrophage and T-cell co-cultures have shown differentially activated macrophages can then direct $CD4^+$ T-cell proliferation and phenotype depending on their proficiency as antigen presentors and their specific repertoire of cytokine release (Arnold et al., 2015). This polarisation state is then effective at inducing the correct mediators which modulate a specific immune response. Traditionally, T-helper cells have been categorised as Th1 or Th2 (Mosmann and Coffman, 1989). The former requires strong signalling through the T-cell receptor for activation and orchestrates responses which necessitate a pro-inflammatory element such as those stimulated towards bacterial or viral infections. Th1 cells execute a specific immune response through activation of their master-regulator transcription factor T-box transcription factor (T-bet) which controls production of the Th1 distinguishing marker, Interferon-γ (IFN-γ) a pro-inflammatory cytokine which subsequently activates further signalling and specific pro-inflammatory macrophage effector functions (macrophage classical activation) (Yamane and Paul, 2013). Weak signalling through TCR causes generation of Th2 cells which are responsive to parasitic infections and are regulated by the trans-acting T-cell-specific transcription factor 3 (GATA3) / signal transducer and activator of transcription 5 (STAT5) axis, inducing interleukin-4 (IL-4) production and causing macrophage alternative activation (Nakayama et al., 2017). Th17 cells were later described and have now been defined in detail (Harrington et al., 2005; Korn et al., 2009). They are effective in responding to bacterial infection and are activated by transforming growth factor beta (TGF-β) and IL-6 which activate master-regulator transcription factor RAR-related orphan receptor gamma (RORγt), causing production of the cytokine, IL-17 (Yamane and Paul, 2013). There are recent descriptions of emerging T-cell subsets, such as Th9 and Th22, which demonstrate the potential range of specific T-cell responses (Kara et al., 2014). These likely induce specific activation states in the innate effector cells, such as macrophages, to fine tune the immune response.

1.1.3. The Macrophage

Macrophages were first described as phagocytic cells by the Russian zoologist Elie Metchnikoff (Mechnikov, 1988), who observed their positive chemotaxis toward foreign bodies followed by engulfment and intracellular destruction. The name "macrophage" is derived from the Greek for

"big eater" due to their inherent propensity to efficiently recognise and devour pathogens, cell debris and toxic material. Although often referred to as "professional phagocytes" (Rabinovitch, 1995) macrophages possess diverse roles in tissue homeostasis, wound healing, tissue regeneration as well as the orchestration of the adaptive immune response (Wynn et al., 2013).

There is vast heterogeneity among myeloid cell types and various monocyte subsets have been identified (Geissmann et al., 2003). However, of all myeloid cells macrophages are possibly the most diverse. Many highly specialised groups patrol their anatomical departments, including Kuppffer cells (liver), osteoblasts (bone), histocytes (interstitial tissue) and of emphasis here, AMs which are present in the lung. Developmentally, macrophages have long been known to be able to be derived from blood monocytes, mononuclear leukocytes, which mature from a universal myeloid progenitor cell in the bone marrow, also known as the mononuclear phagocyte system hypothesis (van Furth et al., 1972). Once in the circulation, monocytes respond to environmental cues which direct their journey into the tissue where final differentiation takes place (Fogg et al., 2006). As well as Monocyte Derived Macrophages (MDM), we now understand that there are additional sources of macrophage precursors including yolk sac derived and foetal liver derived precursors which appear in waves earlier during development and exist through to adulthood in self-replicating niches (van de Laar et al., 2016). Furthermore, yolk-sac erythro-myeloid progenitors rather than hematopoietic stem cells have been identified as the common progenitor for most tissue resident macrophages (Gomez Perdiguero et al., 2015). The resident macrophages of the nervous system, microglia, derive from primitive macrophages which differentiate in the yolk sac and are maintained into adulthood independently of the circulation (Ginhoux et al., 2010). Foetal monocytes are the precursors for AMs which seed the lungs in the embryo and differentiate by exposure to granulocyte macrophage colony stimulating factor after birth (Guilliams et al., 2013). On the other hand, intestinal macrophages are distinct in that they are replenished by Lymphocyte antigen 6C (Ly6C)^{hi} circulating monocytes (Bogunovic et al., 2009), demonstrating circulating monocytes are still important for some niches, particularly those with shorter lifespans.

Macrophages possess various activation states which can be triggered by the chemical or physiological environment. This phenomenon is referred to as macrophage activation. Classically Activated Macrophages (CAM) and Alternatively Activated Macrophages (AAM) represent opposite extremities of the activation spectrum and exhibit pro-inflammatory and anti-inflammatory cytokine expression profiles respectively (Mills et al., 2000b). Although it is widely accepted that macrophages can exhibit specific activation profiles, the spectrum is a vast

continuum and categorising phenotypes is challenging and often inaccurate as macrophages are highly plastic cells with the ability to quickly shift activation profile when faced with altered external cues, a phenomenon referred to as functional adaptivity (Davis et al., 2013; Guiducci et al., 2005; Porcheray et al., 2005; Stout and Suttles, 2004).

Macrophages can shift their phenotype in response to pathogen recognition and subsequent innate immune responses are specifically tailored to the type of pathogen detected (Mosser, 1994). Macrophages express several families of PRRs on their cell surface and after pathogen recognition, macrophages engage in phagocytosis, a dynamic process which involves binding of the ligand coated pathogen to host cell surface receptors, followed by remodelling of the actin cytoskeleton to aid entry. Once engulfed, pathogens are contained within vesicles called phagosomes. Through a process of fusion and fission events, the phagosome evolves into a phagolysome, a competent intracellular killing compartment (Desjardins et al., 1994a). As the phagosome matures, the conditions within the pathogen containing vesicle become more hostile due to an increasingly acidic environment coupled with production of oxidative molecules e.g. reactive oxygen species (ROS) and acid hydrolases e.g. lysozyme, leading to the eventual break down and destruction of the organism (Flannagan et al., 2009). This is discussed in more detail later (section 1.3.3). In recent years shifts in macrophage activation have become an emerging theme in many diseases including chronic inflammatory diseases, cardiovascular diseases, auto-immune disorders, neurodegeneration and cancer (Wynn et al., 2013). It is therefore imperative that we continue to explore the mechanisms and consequences of skewed macrophage phenotypes in order to better understand pathophysiology and to guide development of more effective therapeutics.

1.2. Macrophage Activation

1.2.1. Discovery

Although significant advances have been made in recent decades, it is likely we have only just scratched the surface in our understanding of macrophage activation. Early findings discovered mononuclear phagocytes could become ‘activated’ enabling an increased ability to phagocytose microbial organisms and subsequently destroy them (Mackaness, 1962), utilising ROS and Nitric Oxide (NO) (Nathan et al., 1983). The unearthing of AAMs came about in the late 1980’s and early 90’s by groups who recognised a distinct expression profile could antagonise the pro-inflammatory effects of ‘activated’ macrophages (Stein et al., 1992). Mills suggested that the terms ‘M1’ and ‘M2’ be used to refer to CAM and AAM respectively, mimicking the T-helper cell nomenclature of Th1/Th2 cell activation (Mills et al., 2000a). However, owing to the plastic

nature of macrophages and the fact that the M1/M2 paradigm is a very broad concept and largely unrepresentative of the vast spectrum of macrophage activation, it was more recently suggested by a consortium of macrophage biologists that macrophage phenotypes be referred to by their activation stimuli to avoid confusion (Murray et al., 2014). Therefore, in this thesis macrophages will be referred to as either CAM or AAM when speaking in broader phenotypic terms or labelled as 'M(IFN-γ)', 'M(IL-4)' or 'M(IL-10)' when referring to activation states induced by a specific cytokine stimulus. It is important to note, most of the work published on macrophage activation has been conducted using murine macrophages, however, many activation markers are species specific and great differences in the degree of heterogeneity exists between the profile and functionality of human and murine models (Mestas and Hughes, 2004).

1.2.2. Macrophage Metabolism

1.2.2.1. Arginine Metabolism

Perhaps the most well-defined marker of macrophage activation in mice is the differential metabolism of L-Arginine. In 1988, Hibbs and colleagues showed that CAM synthesise NO from L-Arginine via inducible Nitric Oxide Synthase (iNOS) (Hibbs et al., 1988). Conversely, Corraliza demonstrated that Arginase-1 expression can be triggered by inhibition of iNOS by IL-4, IL-10 and prostaglandin E2 exposure (Corraliza et al., 1994). Furthermore, Gordon and colleagues discovered that upon exposure to IL-4, murine L-Arginine was metabolised by an opposite pathway to CAM, (Hesse et al., 2001; Mills et al., 2000b), and, Arginase-1 was found to be decreased upon addition of IL-4, IL-10 and IL-13 inhibitors (Munder et al., 1998a).

As suggested by Mills, it is widely believed that differential arginine metabolism is related to macrophage function (Shearer et al., 1997). Production of NO by iNOS allows generation of nitrogen species which aid rapid pathogen elimination. Shifting to the opposite Arginase-1 metabolic pathway allows polyamine production from urea and ornithine which can contribute to various functions characteristic of AAM, including wound healing and cell proliferation (Mills et al., 2000b), (Modolell et al., 1995). It is important to note however, although an effective marker of macrophage activation in mice, Arginine metabolism has limitations as a marker in human models (Geelhaar-Karsch et al., 2013).

Recent data shows the signalling pathways which instigate Arginase-1 expression in macrophages appear more complex than first thought. Murray and colleagues showed that Arginase-1 can be induced by *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) infection which signals through a TLR - Myeloid Differentiation primary response gene 88 (MyD88)

pathway to induce STAT3 rather than STAT6 (involved in Arginase -1 expression through IL-4 receptor engagement), which works to increase amounts of IL-10 and supress microbicidal macrophage activity, creating an environment which favours pathogen survival (El Kasmi et al., 2008), (Qualls et al., 2010). Furthermore, Arginase-1 expressing macrophages were found to supress Th2 cell proliferation and cytokine responses and depletion of Arginase-1 was shown to enhance fibrosis, challenging the supposed role for Arginase-1 mediated production of ornithine and polyamines in fibrotic disease (Pesce et al., 2009). These results indicate that in some instances the phenotypic profile of activated macrophages does not always relate to their specific function (Murray, 2017).

1.2.2.2. Mitochondrial Metabolism

Macrophages at the extremities of the activation spectrum possess alternative pathways of glucose metabolism closely related to their distinct functions. CAM prefer anaerobic glycolysis which favours their rapid energy requirements during infection and allows enhanced ROS production for increased microbicidal activity (Rodriguez-Prados et al., 2010). In contrast, oxidative glucose metabolism (fatty acid oxidation) and mitochondrial biogenesis, regulated by Peroxisome proliferator-activated receptor (PPAR) - co activator 1β, predominates in AAM (Vats et al., 2006). This slower, more consistent energy source is ideal for AMM when combatting chronic parasitic infection and implementing tissue repair and wound healing (Odegaard and Chawla, 2011).

1.2.3. Classically Activated Macrophages

CAM have popularly been described as pro-inflammatory cells which arise following priming by IFN-γ which is released by a host of haematopoietic cells involved in the immune response (Bach et al., 1997). The IFN-γ receptor located on the cell surface is activated upon binding and initiates the kinase activity of Janus Kinase and subsequently phosphorylation of STAT1. Activation of STAT1 causes transcriptional upregulation of several pro-inflammatory mediators such as IL-12 and IL-18, which induce autocrine IFN-γ production (Munder et al., 1998b). Along with STAT1, Interferon Regulatory Factor (IRF) 5 is also indispensable for IFN-γ stimulated classical activation and drives expression of classical activation markers such as tumour necrosis factor α (TNFα), IL-1β and IL-12 (Krausgruber et al., 2011). Furthermore, IRF5 expressing macrophages are capable of inducing upregulation of T-bet and RORγt in T cells and subsequently IFN-γ and IL-17 cytokine release (Krausgruber et al., 2011).

For complete classical activation, a second signal; TNFα or microbial products which provide a stimulus for TNFα are required in synergy with priming by IFN-γ (Mosser, 2003). TNFα is

produced by ligation of several TLRs e.g., TLR2 and TLR4 (Ozinsky et al., 2000). For example, TLR4 recognises LPS (Hoshino et al., 1999) which is released by several types of Gram-negative bacteria. TLR ligation yields intracellular production of TNFα, a response which is prevented in TRL4 -/- knock-out mice (Gallego et al., 2011). The signalling cascade initiated by TLR4 activation leads to the release of the transcription factor, NFκB which translocates to the nucleus and induces upregulation of pro-inflammatory genes (Cairo et al., 2011). Other PRRs which contribute to macrophage activation include NLRs which detect intra-cytoplasmic PAMPs and are involved in inflammasome formation. Activation of macrophages with IFN-γ has been shown to increase expression of Nod2 and inflammasome related genes including members of the IL-1 cytokine family and *CASP1*, encoding the gene for caspase-1 (Awad et al., 2017).

The bactericidal activities of CAM have been well documented. Upon microbial ingestion, significant increases in reactive oxygen intermediates, superoxide (O_2^-) and hydrogen peroxide (H_2O_2) contribute to an "oxidative burst" which is important for microbial killing (Murray et al., 1979). Increased enzymatic production of NO from L-Arginine also contributes to pathogen degradation (Pacelli et al., 1995). Enhanced phagocytosis by CAM is more controversial, several sources have reported complement and Fc gamma (Fcγ) mediated phagocytosis is abrogated upon macrophage activation with IFN-γ (Schlesinger and Horwitz, 1991a), (Frausto-Del-Río et al., 2012b). Others, however have demonstrated that complement-mediated-phagocytosis of *C. neoformans* is dependent upon inflammatory mediators associated with classical activation (Collins and Bancroft, 1992) and Fcγ receptors are also increased in response to IFN-γ (Beyer et al., 2012).

During pulmonary infection, recruitment of activated macrophages by specific chemokines is instigated by TNFα and believed to be involved in the containment and resolution of infection (Roach et al., 2002). However, in disease states where inflammation is unresolved, the continuous production of inflammatory mediators can cause permanent tissue damage which further potentiates the symptoms of chronic inflammatory diseases (Martinez et al., 2008). AAM macrophages were first described as mediators of the anti-inflammatory response. Induction of the AAM phenotype has been shown to counteract excessive inflammatory exposure and resolve inflammation (Geng and Hansson, 1992; Stein et al., 1992).

1.2.4. Alternatively Activated Macrophages

The concept of 'alternative' macrophage activation arose three decades ago when initial experiments suggested alterations in the cell surface expression of macrophage mannose receptor (CD206) in response to stimulation with macrophage activator, IFN-γ (Alan et al., 1984).

Later it was discovered that the Th2 cytokine, IL-4, operates antagonistically to IFN-γ, enhancing expression of CD206 (Stein et al., 1992) and in the same vein, members of the scavenger receptor families appeared to be more numerous after IL-4 treatment (Geng and Hansson, 1992; Högger et al., 1998). IL-13 is another Th2 associated cytokine which shares the same receptor as IL-4 and was also found to initiate the alternatively activated phenotype (Doyle et al., 1994; MacKenzie et al., 1998). Surface markers, cytokine and chemokine signatures can be used to distinguish AAM from CAM and altered transcriptional regulation has been defined for alternative activation (Lawrence and Natoli, 2011). Much like CAM, AAM, stimulated with IL-4 also require STAT activation, in this case, the phenotype is regulated by STAT6 (Gordon, 2003). Jumonji domain containing-3, a histone 3 Lys27 demethylase (Jmjd3), is also essential for alternative activation and has been shown to act in combination with IRF4 to co-ordinate alternative activation responses such as those involved in parasitic infection (Satoh et al., 2010). This study also opened up the potential role of epigenetics in macrophage activation which has since been further investigated and reviewed (Ivashkiv, 2013).

As mentioned above, Mills and colleagues, who first coined the terms 'M1, M2' due to parallels with the T helper cell Th1/Th2 paradigm, suggested that the shift in L-Arginine metabolism aids polyamine production which has implicated AAM in wound healing, (Shearer et al., 1997), (Mills et al., 2000a). Other genes which are upregulated during alternative activation give clues for AAM functionality. For example; increased mannose and scavenger receptor surface expression indicate enhanced endocytic activity (Mantovani et al., 2002). In mice, upregulation of Found in Inflammatory Zone 1 (FIZZ-1) participates in resolution of inflammation and protection against Helminth infection and increased expression of chi-lectins Ym1 and Ym2 play a role in resolution of parasitic infection and allergic reaction (Raes et al., 2002). However, contribution to fibrosis and epithelial thickening by FIZZ-1 in response to the fungal allergen Alternaria, has been recorded; suggesting AAM may also contribute to airway pathology (Doherty et al., 2012). Other markers of AAM are chemokines, such as thymus and activation regulated chemokine (TARC) which has been shown to prevent generation of CAM from unstimulated macrophages exposed to a pro-inflammatory stimulus (Katakura et al., 2004) and also CCL22 and CCL24 (Mantovani et al., 2004).

1.2.5. Other Macrophage Activation States

David Mosser and colleagues unearthed a macrophage subset distinct from AAM as they had been originally described but which still drew some parallels with the alternative phenotype. He named this subset 'type II activated macrophages' or M2b macrophages (Anderson and Mosser,

2002a). The M2b phenotype is instigated by Fcγ receptor ligation and TLR receptor activation and shares some traits with CAM. Although both CAM and M2b are activated by immunogenic substances, distinct categorisation closer to an alternative phenotype is necessary due to M2b amplified anti-inflammatory IL-10 and decreased pro-inflammatory IL-12 production (Gerber and Mosser, 2001). Furthermore, M2b macrophages induce IL-4 production which facilitates the Th2 response (Anderson and Mosser, 2002b). However, in contrast to M2a, AAM stimulated by IL-4/IL-13, M2b macrophages fail to upregulate Arginase-1 expression (Mosser, 2003) suggesting they do not play a role in wound healing and regeneration processes but rather take part in immunoregulation as suggested by Mantovani (Mantovani et al., 2004). M2b macrophages are therefore distinct from M2a in terms of functionality as well as activation and expression profile.

M2c or M(IL-10) macrophages are a third sibling of the alternatively activated subset. They are induced by IL-10, TGFβ, glucocorticoids and like IL-4/IL-13 stimulated macrophages, they use the Arginase-1 pathway of L-Arginine metabolism (Corraliza et al., 1995) and upregulate IL-4 receptor α which further increases Arginase-1 expression upon IL-4 binding (Gordon, 2003). Due to stimulation by and high output of anti-inflammatory IL-10, M(IL-10) are implicated in immune regulation and suppression of inflammation (Mantovani et al., 2004). Murray and colleagues, demonstrated that IL-10 blocks transcription of pro-inflammatory cytokines indirectly via activation of STAT3 (Murray, 2005) in agreement with earlier publications which report TNFα and oxidative intermediate blockade by IL-10 (Bogdan et al., 1991). Bogdan *et al* illustrated the ability of M(IL-10) macrophages to down regulate the pro-inflammatory response on two levels, firstly; degradation of TNFα and IL-1 mRNA by IL-10 release 3 hours after macrophage activation, coupled with delayed inhibition of pro-inflammatory cytokine expression at 12-16 hours by TGFβ, operating at the level of translation (Bogdan et al., 1992). These findings cement a role for M(IL-10) in inflammation resolution as a protective mechanism to prevent local tissue damage.

1.2.6. Emerging Activation States

Demonstrative of the diversity of macrophage activation –several less well-defined macrophage subsets have been recorded. Tumour associated macrophages (TAM) are identified as a unique subset, which share characteristics with both CAM and AAM. They have been shown to be instrumental in angiogenesis, tissue remodelling and immune cell infiltration of the tumour site (Sica et al., 2006). Another subset has been coined “M2d” and appears to be functionally similar to TAM in the ability to suppress certain immune responses such as inhibition of T cell proliferation (Duluc et al., 2007). Additional documentation includes *in vitro* mesenchymal stem

cell activated macrophages which have a suggested role in tissue repair (Kim and Hematti, 2009) and a macrophage subset which participates in suppression of T-cell mitogenesis as well as promoting the Th17 immune response, preliminarily coined “M17” macrophages (Tatano et al., 2014). More obscure subsets include $CD169^{+}$ macrophages located in the spleen which activate invariant natural killer T (iNKT) cells and are important in the adaptive immune response against viral pathogens (Chávez-Galán et al., 2015). TCR^{+} macrophages display high levels of phagocytosis and have proposed roles in atherosclerosis and bacterial challenge (Chávez-Galán et al., 2015). These emerging macrophage activation states represent a shift in the dogma from the historical, linear model of macrophage activation where classical and alternative macrophages reside at the polar ends, to a spherical model which is multi-dimensional in that activation can be both extrinsic and intrinsic, potent and mild and that in each tissue setting and scenario it is likely that the specific macrophage activation profile will be unique (Sudan et al., 2015), (Murray, 2017). Established and emerging macrophage phenotypes are outlined in Figure 1-1.

1.2.7. Macrophage Activation in Disease

As mentioned, macrophage activation is involved in a multitude of disease states and dysregulation of macrophage phenotype has been identified in atherosclerosis, cancer, metabolic disorders and pulmonary diseases to name just a few. In some cancers, TAM are recruited to the tumour site and activated into a specific polarisation state by IL-4, IL-10 and immunoglobulin, produced by local immune cells, which encourages further proliferation, tumour angiogenesis and metastasis (Yang and Zhang, 2017). In atherosclerosis, which has been associated with Th1 type responses, macrophage phenotypes extend beyond classically and alternatively activated and new phenotypes such as Mox which are activated by oxidised phospholipids, M(Hb) activated by haemoglobin / haptoglobin complexes and Mhem, polarised by haem, have emerged, all which serve different functions in plaque formation and stability (Chinetti-Gbaguidi et al., 2015). In obese individuals, CAM are predominant in adipose tissue and pro-inflammatory cytokine release contributes to insulin resistance whereas AMM are associated with insulin sensitivity and predominate in lean, healthy individuals (Castoldi et al., 2015). Th2-type responses of both T-cells and macrophages have been shown to be protective against helminth infection in both removal of the pathogen and in control of Th1 induced inflammation (Anthony et al., 2007). Therefore, furthering our understanding of macrophage activation and the switches which control macrophage phenotype is crucial to multiple areas of medical research. Dysregulation of AM activation has also been implicated in many pulmonary

diseases which has ramifications for both lung tissue integrity and the handling of respiratory pathogens.

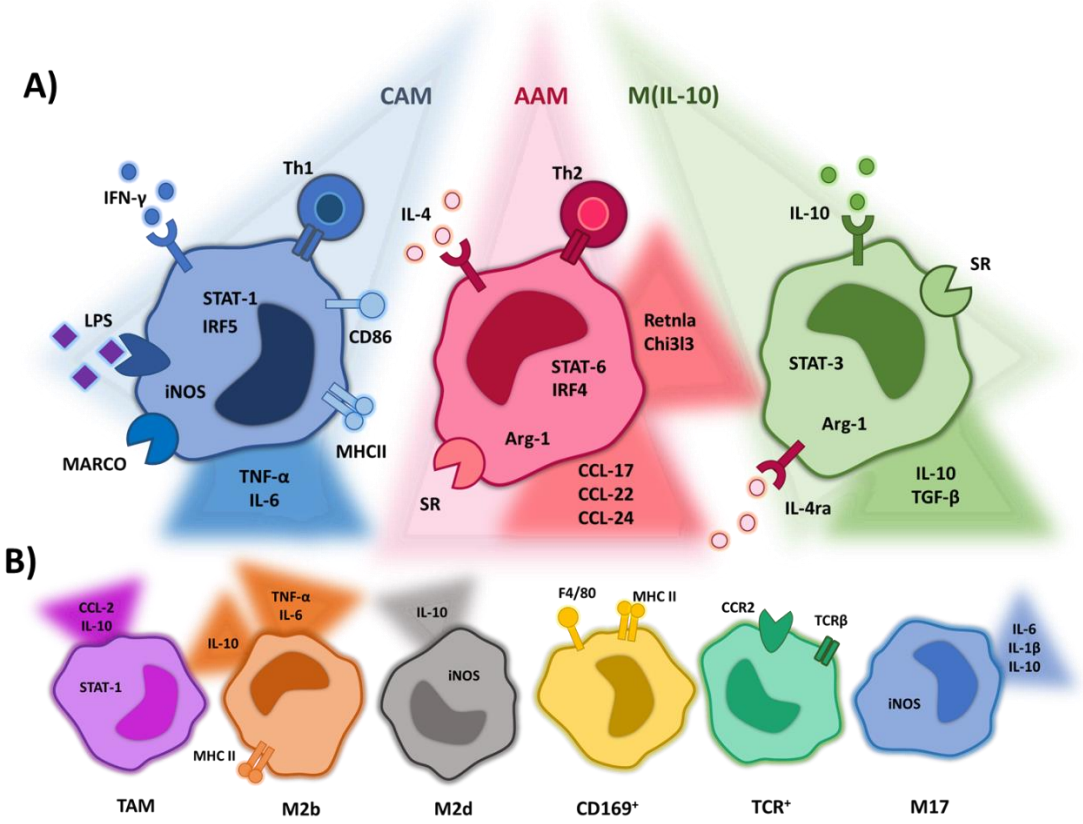


Figure 1-1: Defined subsets of macrophage polarisation in response to differential external stimuli

Macrophages can be activated into specific activation states *in vitro* which theoretically mimic those induced by Th1 / Th2 T-cell subsets or other external stimuli *in vivo*. While it is now recognised that this method of analysing macrophage activation is limited as it does not consider the myriad of activating stimuli macrophages are physiologically exposed to, it is in many ways the best methodology we have for gaining insight of macrophage activation in health and disease. Activation stimuli and upregulation of phenotype specific markers are included in this diagram. **(A)** The phenotypes depicted in this figure are the archetypal murine CAM, AAM and IL-10 stimulated macrophages M(IL-10) which are of importance to this thesis. Note: this diagram includes markers of activation expressed in murine models which differ to human activation markers **(B)** Other defined and emerging subsets of macrophage activation which have been shown to play a role in immunoregulation or are perturbed during certain diseases.

1.2.7.1. Dysregulation of macrophage activation in pulmonary diseases

Asthma is chronic inflammation of the airways associated with bronchial hyper-responsiveness, airflow obstruction and inflammation. Symptoms recur and are often reversible, occurring

towards a wide range of stimulants such as allergens, environmental toxins, viruses and bacteria (Program, 2007). A plethora of immune cells are thought to be involved in the pathogenesis of asthma, including myeloid cells and lymphocytes and Th2 responses have long been thought to be critical in disease development. Indeed many of the symptoms of the asthmatic lung can be linked to Th2 cell responses, such as tissue remodelling and collagen deposition (Elias et al., 1999). In 2003, Rothenberg and colleagues demonstrated that Arginase-1 and 2 are highly expressed in two murine models of experimental asthma induced by IL-4 and IL-13 production which is increased in the asthmatic lung. This paper also showed that Arginase expression is induced in human asthma, implicating a possible role for AAM in disease propagation (Zimmermann et al., 2003). In keeping with an alternatively activated profile, asthmatics are also seen to produce greater levels of immunosuppressive IL-10 (Balhara and Gounni, 2012). Furthermore, data suggests asthmatics are more susceptible to invasive pneumococcal disease amongst other microbial infections, indicating perturbed or supressed functions of CAM (Hansel et al., 2013). On the other hand, macrophages which produce pro-inflammatory IL-17, have been implicated in causation of severe inflammation seen in allergic asthma and IFN-γ has been suggested to have a role in development of inflammation in the asthmatic lung, (Hayashi et al., 2007; Song et al., 2008). Therefore, the skewed phenotypes of both CAM and AMM appear to be crucial to the pathophysiology of asthma.

Chronic Obstructive Pulmonary Disease (COPD) is widely accepted as a disease caused by the inhalation of harmful substances such as those present in cigarette smoke or biomass fuels (Coggon and Taylor, 1998). As a result, patients are subject to lung inflammation and increasing airflow limitation resulting in chronic bronchitis and emphysema (Boorsma et al., 2013). Due to the complex and shifting micro-environment in the lung and given macrophages are highly plastic cells, it is likely heterogeneous subsets of AMs play various roles in the pathophysiology of obstructive lung diseases. It is believed alveolar destruction or emphysema in COPD may be attributable to the migration of inflammatory cells into the airways, instructed by chemo-attractants such as Macrophage Inflammatory Protein 1 beta (MIP1β) (Bless et al., 2000; Demedts et al., 2007; Song et al., 2008). Chemokines which influence cell migration are likely to be produced in response to tissue injury caused by noxious substances (Boorsma et al., 2013). Therefore, the type of immune cell present in the lung and the secretory products of these cells are of paramount importance in the aetiology of COPD.

Although numerous publications have speculated in recent years, the exact contribution of distinct AM subsets to COPD is controversial and largely unknown. CAM have been implicated

to play a significant part in generating severe lung inflammation as cigarette smoke contains several activators of CAM cell surface receptors, including the bacterial endotoxin, LPS (Hasday et al., 1999; Stedman, 1968). In addition, various markers of CAM activation have been found in AMs from COPD patients, including iNOS (Maestrelli et al., 2003), and TNFα (Demirjian et al., 2006). However, the COPD lung is subject to acute exacerbations caused by bacteria, primarily; NTHi and *S. pneumoniae* (Murphy et al., 2004; Sethi et al., 2002), and increased bacterial colonisation suggests defective macrophage phagocytosis and microbial killing possibly due to dysregulated classical activation (Berenson et al., 2006a; Taylor et al., 2010).

In the same vein, others have described a down regulation of CAM markers in AMs from COPD patients and healthy smokers (Shaykhiev et al., 2009a). Shaykheiv and colleagues demonstrated AAM genes involved in tissue remodelling and immunoregulation were increased with progression of the disease whilst expression of CAM genes was suppressed. A recent study discovered that alternative activation markers, CD163, CD204 (macrophage scavenger receptor I) and CD206 are over expressed on cells from ex-smokers with severe to very severe COPD (Kaku et al., 2014). Furthermore, reports have found TLR signalling (an archetypal CAM characteristic) is impeded by upregulation of CD204 (Ohnishi et al., 2011) in line with previous findings demonstrating downregulation of TLR2 and TLR4 receptors in smokers and COPD patients (Droemann et al., 2005).

Taken together these contrasting reports on the activation of AMs might indicate that macrophages exhibit various activation states at different stages during the development of disease or, given their plastic behaviour, are capable of rapidly reprogramming their activation state in response to the altered lung milieu. Evidence favouring the latter has recently emerged in a series of non-invasive experiments which demonstrate CAM and AAM AMs co-existing as a mixed population in the COPD lung (Al Faraj et al., 2014).

1.3. Macrophage Response to Bacterial Infection

1.3.1. Pathogen recognition

As mentioned earlier, macrophages recognise invading pathogens by recognition of evolutionarily conserved bacterial PAMPs which bind to PRRs expressed on the cell surface. PRRs are highly expressed on macrophages, given their role as innate immune sensors, however, they are also abundant on other APCs of the immune system including DCs and B-cells and are expressed less abundantly on other cells involved in innate immunity such as epithelial cells. Of the four main classes of PRRs, TLRs were the first class to be discovered and have been most extensively studied.

1.3.1.1. Toll like Receptors

Out of the total of 10 TLRs, TLR2 and TLR4 are most commonly associated with bacterial infection, given their necessity for detection of Gram-positive cell wall products such as lipoproteins and lipopeptides (TLR2) and Gram-negative LPS (TLR4) (Takeuchi et al., 1999).

TLR9 is located on intracellular vacuoles such as phagosomes and lysosomes. It is specialised to recognise unmethylated CpG DNA repeats which are released upon bacterial digestion inside phagosomes (Wolf et al., 2011). A study by Albiger *et al*., highlights the importance of TLR9 in pneumococcal infection as TLR9 knockout mice are unable to clear invasive disease as efficiently as other TLR family mutants. Furthermore, although removal of TLR9 does not affect nasopharyngeal colonisation, it does result in decreased survival of animals and increased levels of bacteraemia (Albiger et al., 2007). These results demonstrate the importance of receptor location; cell surface TLRs are key in the initial recognition and control of bacterial species, however a more specialised immune response for a more dangerous intracellular location is reliant on further microbial sensing which can only transpire after initial processing.

Once bound, TLRs can heterodimerise with other TLRs or homodimerize (Ozinsky et al., 2000) to allow the propagation of a specialised signal in response to specific ligands. TLRs contain Toll/Interleukin-1 receptor (TIR) domains which interact with adaptor proteins also containing TIR domains. TLR signalling is dependent on either TIR domain containing MyD88 or TIR-domain-containing adaptor protein inducing IFN-β (TRIF) (De Nardo, 2015). There are a total of 5 TIR adaptor protein family members (reviewed in (O'Neill and Bowie, 2007)). MyD88 is the most critical adaptor protein in TLR signalling as downstream TLR2, TLR4, TLR5, TLR7 and TLR9 signalling is lost in MyD88 deficient mice (Kawai et al., 1999; Takeuchi et al., 2000). In the place of MyD88, TRIF is required for TLR3 signalling and is also required for TLR4 signalling along with the bridging protein TRIF-related adaptor molecule (TRAM), which promotes TRIF recruitment to TLR4 (Yamamoto et al., 2003a), (Yamamoto et al., 2003b) and TRIF deficient mice are incapable of type 1 Interferon transcription by TLR3 and TLR4 ligation, whereas TLR4 mediated NFκB signalling remains intact through the MyD88 pathway. MyD88-adaptor-like (MAL) is instrumental in recruiting MyD88 during TLR2 and TLR4 signalling and functions a similar role as a bridging adaptor protein (Fitzgerald et al., 2001). The final TIR domain adaptor protein; sterile α-armadillo motif works as an inhibitor, causing blockade of TIRF-dependant Toll signalling (Carty et al., 2006) by negative regulation of NFκB. TLR signalling is also negatively regulated by IRAK-M (IL-1 Receptor Associated Kinase) which prevents phosphorylation of MyD88 downstream effectors, c-Jun N-terminal Kinase, p38 mitogen-activated protein kinases (p38) and Extracellular Signal-Related Kinases 1/2 (Kobayashi et al., 2002).

After TLR binding, MyD88 forms a complex termed the "Myddosome" which requires the recruitment of IRAKs, firstly IRAK4 followed by IRAK1, to the MyD88 death domain (Motshwene et al., 2009). The subsequent signalling cascade ends with the release of the NF-κB dimer from inhibitor of κB (IκB) and pro-inflammatory gene transcription.

1.3.1.2. Nod Like Receptors

Another class of intracellular PRRs are the NLRs which are located in the host cytosol. Nod1 and Nod2 are effective in recognising components of both Gram-negative and Gram-positive bacteria (Girardin et al., 2003a; Girardin et al., 2003b), including peptidoglycan fragments. Upon activation, they result in NFκB activation and Mitogen-Activated Protein Kinases (MAPK) signalling, similar to TLR activation and can therefore initiate cytokine and chemokine production as well as microbicidal gene upregulation (Caruso et al., 2014). This shows us NLRs are essential for amplification of the innate immune response after TLR activation. Furthermore, in a TLR tolerance model, NLRs were shown to be key in responding to intracellular bacterial infection, demonstrating their importance in areas of the body where TLR receptors are likely to be tolerised such as the gut and the colonised nasopharynx (Kim et al., 2008). NLRs also play a key role in inflammasome formation. The inflammasome is a protein complex composed of a NLR protein, adaptor proteins and caspase-1 which upon cleavage, activates pro-inflammatory cytokines from the IL-1β family and induces pyroptosis, a pro-inflammatory form of cell death (Fink and Cookson, 2005). Unlike apoptosis, pyroptosis is a more rapid process, causing the leakage of cytosolic contents, however despite the chaotic nature, pyroptosis serves an important purpose in eradication of intracellular pathogens (Bergsbaken and Cookson, 2009; Fink and Cookson, 2007).

1.3.1.3. Scavenger Receptors

As well as PRRs, scavenger receptors such as CD163 have also been shown to bind Gram-positive and Gram-negative bacteria and upon ligand binding, they too are capable of triggering a downstream cytokine response (Fabriek et al., 2009). Macrophage Receptor with Collagenous Structure (MARCO) is a class A scavenger receptor with variable tissue expression. It's expression is induced in response to microbial stimuli on highly phagocytic macrophages (van der Laan et al., 1997). Another scavenger receptor important in pathogen recognition is CD36 and CD36 deficient mice show impaired *S. pneumoniae* phagocytosis and bacterial clearance (Sharif et al., 2013). These reports demonstrate the importance of scavenger receptors as immune sensors and phagocytic receptors.

1.3.1.4. Opsonisation

As well as direct receptor interaction with pathogens, macrophages can also recognise targets which have been tagged by complement proteins and Immunoglobulin G (IgG) antibodies. Recognition by complement deposition is initiated by soluble proteins released from hepatocytes in response to pro-inflammatory cytokines. These proteins initiate a signalling cascade once bound to the pathogen surface via three different complement pathways, the classical, alternative and lectin pathway, which each culminate with cleavage of complement Component 3 (C3) to C3b by C3 convertase at the pathogen surface and subsequent coating of the target cell in C3b molecules resulting in opsonisation. C3b can then be further cleaved to inhibitory iC3b, C3c or C3dg which reveal different receptor binding sites for recognition by different complement receptors, allowing for a pathogen specific response (van Lookeren Campagne et al., 2007). Complement receptors present on the phagocyte surface can then bind to complement deposits which triggers phagocytosis. These receptors include Complement Receptor 1 (CR1), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) which recognise targets coated with various complement opsonins including C3b and also C1q and mannose binding lectin. Similarly, immunoglobulins can act as opsonins by coating pathogens via epitope recognition involving the Fab localised antigen binding site and their Fc region which tags the pathogen for recognition by specific Fc receptors present on phagocytes (Daëron, 1997). Macrophages possess a range of receptors which identify various immunoglobulin isotypes and bind with different affinities thereby providing another mechanism of infection specific response (Bruhns et al., 2009). Fcγ receptors either contain Immunoreceptor Tyrosine Based Activation Motifs (ITAM) or Immunoreceptor Tyrosine Based Inhibitory Motifs (ITIM) which activate or inhibit phagocytosis respectively (Barrow and Trowsdale, 2006). Furthermore, cross talk between Fc and complement mediated phagocytic signalling has been shown to enhance pathogen internalisation, emphasising the requirement for receptor collaboration in pathogen clearance (Underhill and Ozinsky, 2002).

1.3.2. Phagocytosis

Numerous receptors are involved in phagocytosis and often completion of internalisation requires crosstalk between different receptor families for activation of the appropriate phagocytic response. The most well described model of phagocytosis is Fc mediated, so I will focus on describing this pathway in more detail, however complement receptors, scavenger receptors and lectin receptors are also known to be ‘phagocytic entry receptors’ and Toll-like receptors, although not directly phagocytic, can contribute to induction by co-operating with phagocytic receptors (Gordon, 2016). Different characteristics in the engulfment of particles

have been described for each of these pathways (Kaplan, 1977). It is important to note however, that there is not one overarching model of phagocytosis even within receptor families and internalisation appears to be tailored, to some extent, to the specific particulate in the specific situation.

Pathogens opsonised with immunoglobulins bind to Fc receptors on the cell surface as described above. Once bound to Fc receptors, phagocytosis has been described to proceed in a 'zipper' like fashion whereby the plasma membrane is shaped around the pathogen surface by engagement of multiple Fc receptors with opsonins at multiple sites (Griffin et al., 1975). Formation of the phagocytic cup has been shown to be driven by phosphorylation of tyrosinse residues on the ITAM of Fc receptors by the Src family of tyrosine kinases. This event occurs upon engagement of Fc receptors and initiates further recruitment of Fc receptors to the plasma membrane for receptor clustering and efficient binding to immunoglobulins (Sobota et al., 2005). Zippering is also guided by actin polymerisation which drives the formation of the phagocytic cup up until closure. The microtubule associated GTPase, Dynamin-2 has been shown to be recruited to early phagosomes and is involved in pseudopod extension and scission of the advancing phagosome at the membrane edge (Gold et al., 1999; Marie-Anaïs et al., 2016). Other important modulators include Phosphoinositide 3 Kinase (PI3K), phospholipase C and members of the Ras superfamily of small GTPases, blockade of which terminates phagocytosis at various steps (Swanson, 2008).

The process of internalisation allows the macrophage to sample the molecule which it is engulfing. Underhill and Goodridge suggest we can think about this process as the cell first 'tasting' its target, to understand the chemical composition which initiates the right kind of phagocytosis. It then moves on to 'feeling' how large the molecule is and exploring the cell surface which provides information on how it should be processed once it has been 'swallowed'. This model highlights the key role of 'tasting' and 'feeling' in initiating the correct cytokine response and pro-inflammatory gene transcription (Rettig et al., 2010; Underhill and Goodridge, 2012).

Once fully ingested, the 'nascent' phagosome membrane is mostly composed of the elements of the plasma membrane and the phagosomal environment is similar to that of the extracellular milieu (Canton, 2014). The phagosome must then acquire the materials it needs to correctly handle the ingested material, this occurs through a process of phagosomal maturation which is, in brief, a sequence of fission and fusion events with other intracellular vacuolar compartments (Desjardins et al., 1994b; Flannagan et al., 2009). The first stage, the formation of the early

phagosome, is thought to be similar to that of early endosome formation which is mediated by the GTPase Rab5, activated by its nucleotide-exchange factor (GEF) Rabex-5 and the effector Rabaptin-5 which increases Rabex-5 activity, providing continuous activation of Rab5 (Horiuchi et al., 1997; Lippé et al., 2001). Rab5 recruits human vacuolar sorting protein 34, a class III phosphatidylinositol 3-kinase, which generates phosphatidylinositol 3 phosphate and together with Rab5 recruits and retains Early Endosome Antigen-1 (EEA-1), an important marker of early phagosomes and a key player in docking and fusion of compartments (Christoforidis et al., 1999). Late phagosomes are identified by inactivation of Rab5 and Rab7 recruitment (Vieira et al., 2003) which is required for fusion with lysosomal compartments. The tethering of late phagosomes to lysosomes involves SNAP (Soluble NSF Attachment Protein) receptors (SNAREs), such as vesicle associated membrane protein (VAMP) 7 and 8. In addition to vesicle associated VAMP 7 and 8 (Antonin et al., 2000), late phagosomes also contain Lysosomal Associated Membrane Protein-1 (LAMP1) and LAMP2 (Desjardins et al., 1994b). Subsequent fusion with lysosomes results in recruitment of Lysosomal Integral Membrane Protein-II (LIMPII), cathepsin content and low luminal pH, (Harrison et al., 2003; Jović et al., 2012).

Interestingly, macrophage phagocyte maturation has been shown to vary amongst macrophage activation phenotypes and the kinetics of maturation vary within a macrophage population (Canton, 2014; Podinovskaia et al., 2013). This is likely reflective of the different functions certain populations of macrophages are required for, for example, classical activation provides a slower maturation, likely to benefit the processes of antigen presentation and intrerphagosomal killing by ROS whereas phagosomes in AAM progress much quicker through the endosomal pathway for rapid clearance of apoptotic bodies (Canton, 2014). It is the phagolysosomal phase when the majority target degradation occurs, the mechanisms of macrophage killing will be discussed next.

1.3.3. Microbicidal Mechanisms

1.3.3.1. Acidification of the vacuole

Phagocytic maturation is associated with increasing acidity of the phagosome lumen to pH 4.5-5.5 and this acidification is achieved by employment of V-type H^+-ATPases which pump protons into the phagosomal compartment and by direct delivery of H^+ from lysosomes (Futai et al., 2000). Increases in H^+ are key to pathogen degradation as they create a hostile environment for microbe survival and activate enzymes such as hydrolases which work at optimum in low pH environments and indirectly play key roles in bacterial killing by enhancing the release of microbial components that stimulate microbicidals via PRR or induce apoptosis associated killing

respectively (Bewley et al., 2011a; Ip et al., 2010). H^+ availability also encourages formation of various ROS species in the presence of oxygen generated by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) (Flannagan et al., 2009; Vatansever et al., 2013). Certain intracellular bacteria species have evolved ways to inhibit phagosomal acidification by preventing recruitment of v-ATPases, as evidenced by *Mycobacterium avium-intercellulare* (Sturgill-Koszycki et al., 1994).

1.3.3.2. Reactive Oxygen Species (ROS)

ROS are key effectors in phagocytic control of bacterial infection. In phagocytes, ROS is generated by NOX2, NADPH oxidase system which is comprised of two membrane subunits: β subunit: $gp91^{phox}$ and α subunit: $gp22^{phox}$ which together form "flavocytochrome b" (Segal and Jones, 1978) and three cytoplasmic subunits: $p47^{phox}$, $p40^{phox}$ and $p67^{phox}$ which upon phosphorylation associate with NOX2 (Panday et al., 2015). To generate ROS, electrons are passed from NADPH to Flavin Adenine Dinucleotide (FAD) which are then be passed to heme molecules and finally to oxygen which becomes reduced to $O_2^{\cdot-}$ (Bedard and Krause, 2007). $O_2^{\cdot-}$ is then capable of forming several species of ROS including H_2O_2 mediated by superoxide dismutase (Bray et al., 1974), hydroxyl radicals and singlet oxygen (Hoidal et al., 1979). The methods by which ROS causes bacterial damage have not been well described however documented methods of microbicidal activity include damage to bacterial DNA, lipids and proteins (Imlay and Linn, 1988; Kohanski et al., 2007).

1.3.3.3. Reactive Nitrogen Species (RNS)

In phagocytes, RNS are generated by inducible nitric oxide synthase (NOS2 or iNOS). Unlike endothelial NOS (eNOS) or neuronal NOS (nNOS) which are constitutively active and require calcium for activation, iNOS is inducible and controlled by increases in transcription of iNOS enzyme induced by pro-inflammatory cytokine production (Stuehr et al., 1991). The NOS enzyme exists as a dimer, containing an oxygenase domain with heme and L-arginine binding regions and a reductase domain containing Flavin Mononucleotide (FMN), FAD and NADPH binding sites. Electrons are transferred from NADPH to FAD to FMN to heme which produces NO and citrulline from L-Arginine and oxygen (Feng et al., 2014; Stuehr, 1999). Once produced, NO forms intermediates including; nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$) by reaction with $O_2^{\cdot-}$, and dinitrogen trioxide (N_2O_3) all which can damage bacterial membranes, enzymes and DNA (Darrah et al., 2000; Hurst and Lymar, 1997). These include inhibition of bacterial respiration, oxidative injury and damage to iron clusters, used by bacteria for electron transport and are potentiated when combined with H_2O_2 (Pacelli et al., 1995; Stevanin et al., 2000).

1.3.3.4. Antimicrobial molecules

Phagosomal antimicrobial proteins and peptides are alternative, non-oxidative forms of host defence which are effective in limiting bacterial nutrients, inducing bactericidal activity and hydrolysis of key bacterial lipids, proteins and carbohydrates (reviewed in (Flannagan et al., 2009)). In macrophages, Natural Resistance-Associated Macrophage Protein (NRAMP) 1 has been shown to cause metal efflux from the phagosome, restricting the key bacterial nutrients, iron, manganese and cobalt, the presence of NRAMP1 is therefore thought to be particularly damaging to intracellular pathogens (Cellier et al., 2007). Other antimicrobial molecules include β-defensins which are small cationic polypeptides that can lyse bacterial cell walls, resulting in the release of bacterial DNA, and subsequent DNA damage by oxidative stress (McGlasson et al., 2017). Defensins have also been shown to prevent intracellular pathogen replication by blocking phagosomal escape of *Listeria monocytogenes* (Arnett et al., 2011). Calthelicidins are another family of small proteins which are mostly abundant in neutrophil granules however calthelicidin (LL-37) is also active in AMs and is induced by TLR2, TLR4 and TLR9 activity in response to *Mycobacterium tuberculosis* (Rivas-Santiago et al., 2008) this protein is also implicated in eradication of *Salmonella typhimurium* and has a role in promoting bacterial phagocytosis (Rosenberger et al., 2004; Wan et al., 2014). Cathepsins are associated with the acidic environment of the phagolysosome, they are proteases which can contribute to bacterial hydrolysis and degradation of some bacterial species. Protective roles for a variety of cathepsins including, cathepsin E, cathepsin B, cathepsin L and cathepsin D against Gram-positive and Gram-negative bacteria have all previously been described (Bewley et al., 2011a; Qi et al., 2016; Tsukuba et al., 2006; Xu et al., 2013). These actions may, however, be indirect rather than directly microbicidal. Of particular interest to this report is cathepsin D which plays a key role in macrophage control of *S. pneumoniae* infection by modulation of apoptosis associated killing, described in more detail later. As well as cathepsins, other macrophage antimicrobial endopeptidases exisit including asparagine endopeptidase which is activated by phagosome acidification and helps to kill the bacteria *Pseudomonas aeruginosa* (Descamps et al., 2012).

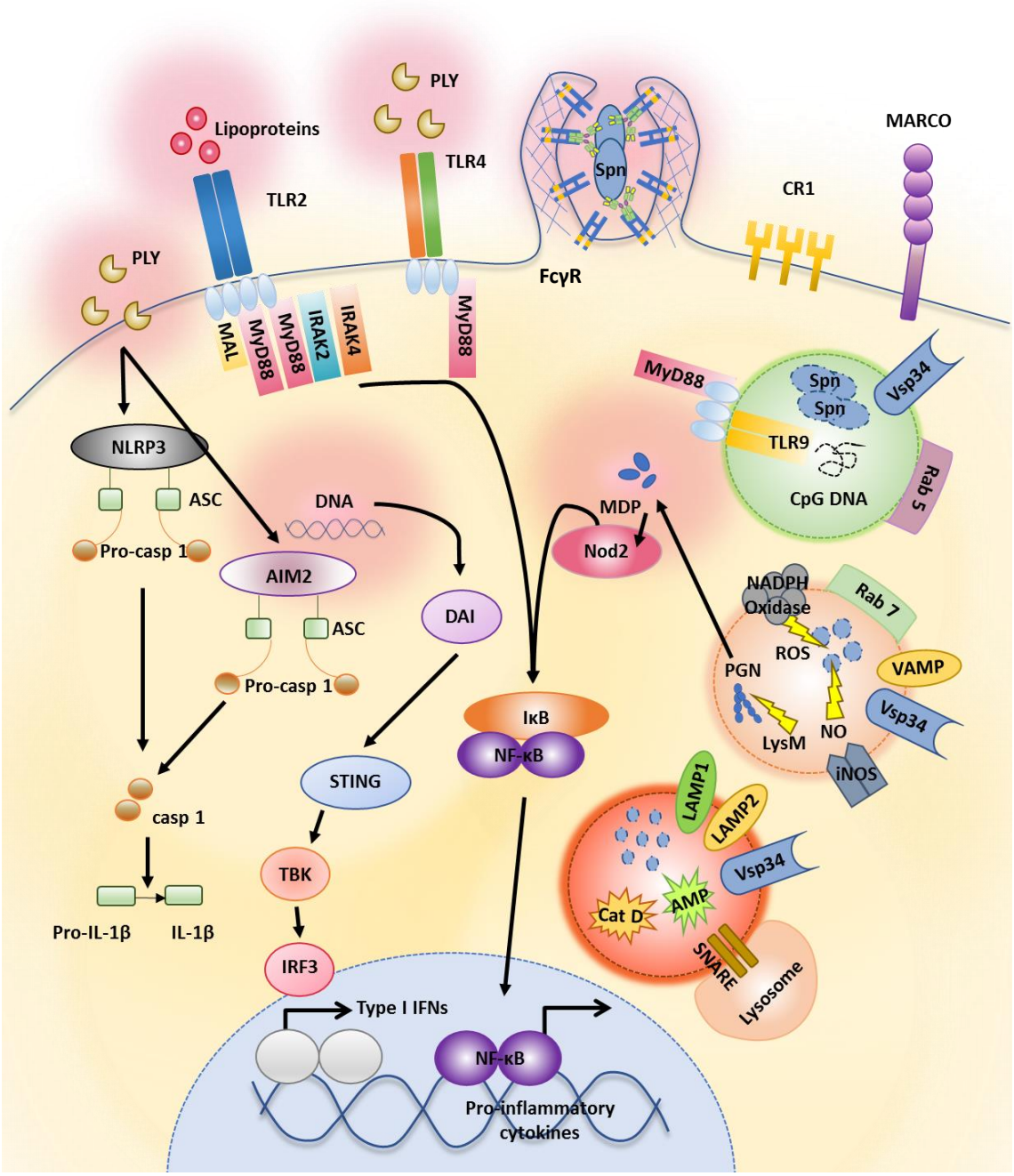


Figure 1-2 Overview of the components involved in the macrophage response to bacterial infection

Signalling pathways in the response of macrophages to *S. pneumoniae* infection are depicted, however many of these responses are common for the handling of numerous bacterial pathogens. Virulence components of *S. pneumoniae* such as pneumolysin (PLY) and pneumococcal lipoproteins are capable of activating Toll-Like Receptors (TLRs), primarily TLR2 and TLR4. Activation of TLRs leads to MyD88 signalling and myddosome formation causing release of Inhibitor of kappa B (IκB) and translocation of the Nuclear Factor Kappa B (NF-κB) dimer to the nucleus for initiation of pro-inflammatory gene transcription. PLY has also been shown to activate apoptosis associated speck like protein containing a CARD (ASC) domain containing inflammasomes including NLR Family Pyrin Domain Containing 3 (NLRP3) and Absent In

Melanoma (AIM2), activation which leads to cleavage of pro-caspase-1 and activation of Interleukin-1β (IL-1β). AIM2 has also been found to be activated by cytosolic double stranded DNA which can be sensed by DNA dependant activator of IRFs (DAI) leading to signalling via Stimulator of Interferon Genes (STING), Tank Binding Kinase 1 (TBK1) and Interferon Regulatory Factor 3 (IRF3) and transcription of type I Interferons (IFNs). Internalisation of *S. pneumoniae* can occur by a variety of cell surface receptors including opsonic uptake by Fc gamma receptors (Fcγ) and complement receptors (CR1) and non-opsonic uptake by Macrophage Receptor with Collagenous Structure (MARCO). Once phagocytosed, bacteria are contained within the intracellular vacuole; the phagosome, which matures through the endosomal pathway and acquires various associated proteins which contribute to the development of an increasingly hostile intraphagosomal environment. Early phagosomes are associated with Rab5 which is required in the recruitment of the V-ATPase, Vsp34 instrumental in lowering the pH of the phagosome. TLR9 is another member of the Toll family which associates with phagosomes and recognises CpG DNA repeats, a common component of microbial DNA. As the phagosome matures, Rab 5 is exchanged for Rab 7 and phagosomes acquire Soluble NSF Attachment Protein (SNARE)s such as Vesicle Membrane Associated Protein (VAMP) 7 which is needed for lysosome fusion. With lysosome fusion, intracellular bacterial killing intensifies with the employment of Nicotinamide Adenosine Dinucleotide Phosphate (NADPH) derived Reactive Oxygen Species (ROS) and Nitric Oxide (NO) produced by inducible Nitric Oxide Synthase (iNOS) which can combine to form damaging Reactive Nitrogen Species (RNS). Macrophages also utilise Lysozyme M (Lys M) which degrades bacterial peptidoglycan (PGN) to muramyldipeptide (MDP) which is then recognised by the cytosolic Pattern Recognition Receptor (PRR) Nucleotide-binding oligomerization domain-containing protein 2 (NOD-2). As the phagosome matures to become a phagolysosome, it acquires expression of Lysosomal Associated Membrane (LAMP) 1 and 2 and Cathepsins, including Cathepsin D (Cat D) of significance in pneumococcal infection and other Anti-Microbial Peptides (AMPs) which are optimally active at a low pH are therefore employed in the final stages of intraphagasomal bacterial killing.

1.4. *Streptococcus pneumoniae*

1.4.1. Pathogenesis

The Gram-positive, alpha haemolytic bacteria, *S. pneumoniae*, was first identified in independent studies by pioneering bacteriologists, Louis Pasteur (Pasteur, 1881) and George Sternberg (Sternberg, 1881) during the late 19th century. Referred to at first as the diplococcus due to its rounded shape and tendency to form pairs, it was later established as the pneumococcus, descriptive of its prominent involvement in pulmonary disease (Frankel, 1886). Today, *S. pneumoniae* is the most common cause of Community Acquired Pneumonia (CAP). Infection is most frequent in children under 5 years of age, causing clinical syndromes in the upper respiratory tract such as otitis media, sinusitis and bronchitis in addition to pneumonia

(WHO, 2017). The elderly (>65 years) and patients who are immunocompromised or exhibit co-morbidities (van der Poll and Opal, 2009) such as COPD are at increased susceptibility. Furthermore, invasive pneumococcal infection is responsible for a significant number of bacterial meningitis and septicaemia cases per annum (Kadioglu and Andrew, 2004). At the turn of the century, almost 1 million deaths in children under 5 years old were estimated to be attributable to pneumococcal infection (O'Brien et al., 2009). Although use of antibiotics and more recently vaccinations, have proven effective in resolution and prevention of infection (Musher et al., 1993), there are now over 90 serotypes of *S. pneumoniae* (Weinberger et al., 2010) and environmental pressures are aiding the evolution of drug resistant strains (Campbell and Silberman, 1998), particularly in the post vaccine era (Miller et al., 2011). In this section, I will discuss the factors which make *S. pneumoniae* such a successful pathogen and how our immune systems are equipped to combat pneumococci.

1.4.2. Virulence Factors

S. pneumoniae is capable of colonising the human nasopharynx using several virulence factors which have evolved to facilitate colonisation and tissue invasion. The transformable nature of the pneumococcus (Johnsborg and Havarstein, 2009) aids its ability to incorporate new methods of host evasion and drug resistance. Many microbial characteristics of *S. pneumoniae* are immunogenic, including the pneumococcal cell wall which causes activation of immune cells and release of inflammatory mediators such as cytokines, chemokines and the intracellular production of NO (Orman et al., 1998). The most important virulence factor for invasion of *S. pneumoniae* is the polysaccharide capsule as without it virulence is significantly reduced (Morona et al., 2004).

1.4.2.1. Capsule

The polysaccharide capsule forms the outermost layer of the bacterium and is genetically distinct in each serotype, this variation renders some strains more virulent than others (Briles et al., 1992). The capsule functions protectively by concealing the cell surface and preventing complement mediated phagocytosis (Jonsson et al., 1985b). Through use of un-encapsulated mutants, Hyams *et al.,* found that the pneumococcal capsule functions to evade aspects of both the classical and alternative complement pathways. The group then went on to determine that some capsular serotypes of *S. pneumoniae* are more susceptible to complement–mediated phagocytosis than others which may influence the potential invasiveness of pneumococcal strains (Hyams et al., 2010). The capsule has also been suggested to aid colonisation of the human nasopharynx by avoiding mucosal transport out of the respiratory tract, mutants which

lack a polysaccharide capsule are less able to colonise epithelia due to an increased tendency to adhere to luminal mucous (Nelson et al., 2007).

1.4.2.2. Pneumolysin

The cholesterol dependent cytolysin, Pneumolysin (PLY), is a pneumococcal toxin which upon release is capable of creating large holes in cholesterol containing cell walls which compromises the integrity of host cells and worsens tissue injury. The presence of PLY is also instrumental in activating aspects of the host inflammatory response including the complement cascade, (Rubins et al., 1996), enhanced activation of pro-inflammatory cytokines; TNFα, IL-1β, IL-6, bactericidal molecules such as ROS and NO that can further enhance tissue injury (Braun et al., 1999; Shoma et al., 2008) and inflammasome formation, which contributes to host protection against *S. pneumoniae* (McNeela et al., 2010). Although PLY is a prominent virulence factor for *S. pneumoniae* (Berry et al., 1989; Kadioglu et al., 2000), studies have noted that PLY is not essential for successful colonisation (Rubins et al., 1998). Furthermore, PLY mutants which do not have either complement activating or haemolytic function display increased virulence compared to PLY negative strains, however, this result is only modest, implying other factors account for the effect of PLY on virulence (Benton et al., 1997). The presence of PLY however, has been shown to trigger T-cell responses and promote clearance of pneumococci, suggesting the host response may require the presence of PLY for effective pneumococcal recognition and clearance (van Rossum et al., 2005).

Early reports suggested recognition of PLY is mediated by TLR4 (Malley et al., 2003). Evidence suggested activation of TLR4 signalling by PLY may play a role in host mediated macrophage apoptosis (Srivastava et al., 2005), a mechanism implemented by the host for effective infection resolution. Additionally, infection with PLY deficient mutants is associated with less macrophage apoptosis in the respiratory tract (Marriott et al., 2004) providing further evidence that the presence of PLY may actually facilitate host defence. On the other hand, as noted by Marriott, 2008, PLY induced macrophage apoptosis could potentially cause unnecessary cell death and become detrimental to the host (Marriott et al., 2008). More recently NLRs such as NLR family pyrin domain containing 3 and Absent in Melanoma 2 (AIM2) have emerged as the major PRR involved in PLY recognition and some authorities now question the role of TLR4 and speculate whether these results might reflect contamination of PLY with LPS or other TLR4 ligands (Fang et al., 2011; McNeela et al., 2010; Witzenrath et al., 2011).

1.4.2.3. Other Virulence Factors

Other defined virulence factors of *S. pneumoniae* include pneumococcal surface protein A (PspA) and C (PspC) which are located on the pneumococcal cell wall (McDaniel et al., 1984). PspA and C enhance pneumococcal survival by interference with the complement cascade (Cheng et al., 2000; Tu et al., 1999). A number of pneumococcal serotypes also express pili, which aid pneumococcal colonisation of the respiratory tract by promoting adherence to epithelial cells (Barocchi et al., 2006). In addition, LPTXG-anchored surface proteins such as neuraminidase and hyluronidase are involved in adherence and enhancement of the inflammatory response (Brittan et al., 2012). Although it has been recorded that pneumococcal virulence is reduced without the autolysin, LytA, (Hirst et al., 2008) a role in virulence for cell wall hydrolases is less well defined, one review suggests autolysis of pneumococci might enable release of intracellular toxins such as PLY but PLY is also capable of release independent of autolysis (Balachandran et al., 2001; Mitchell et al., 1997).

1.4.3. Macrophage Response to *S. pneumoniae*

1.4.3.1.Macrophage Recognition of S. pneumoniae

As part of the innate immune response, well equipped to recognise and kill pathogens even before the evolution of adaptive immunity, macrophages are capable of effective recognition of *S. pneumoniae* via TLRs. TLR2, TLR4 and TLR9 have well defined roles in recognition of *S. pneumoniae* (Figure 1-2). TLR2 has been shown to recognise components of the pneumococcal cell wall, including lipoteichoic acid and lipopetides (Schwandner et al., 1999). TLR4 knockout mice display increased vulnerability to lethal doses of *S. pneumoniae* due to lack of interaction with PLY (Malley et al., 2003). TLR9 is an endosomal PRR which is capable of recognising pneumococcal DNA (Albiger et al., 2007). In a study by Lee and colleagues, mice lacking either TLR2 and TLR4 or TLR2 and TLR9, demonstrated decreased secretion of pro-inflammatory cytokines and chemokines compared to controls, suggesting these receptors work synergistically to activate downstream effectors in response to detection of *S. pneumoniae* (Hemmi et al., 2000). Other PRRs known to be involved in pneumococcal recognition include Nod2 which recognises pneumococcal peptidoglycan (Opitz et al., 2004), NLRs (including NLRP3 and AIM2 which recognise PLY (Fang et al., 2011; McNeela et al., 2010; Witzenrath et al., 2011) and DNA recognition receptors such as DNA-dependent activator of IFN-regulatory factors (Parker et al., 2011). Deposition of complement components on the bacterial cell surface also play a key role in host recognition and mediation of the immune response to *S. pneumoniae* (Winkelstein, 1981).

1.4.3.2. Macrophage intracellular trafficking and killing of S. pneumoniae

Macrophages interact, bind and phagocytose *S. pneumoniae* forming an intracellular membrane bound vesicle in the cytoplasm known as a phagosome. As described in section 1.3, the *S. pneumoniae* containing phagosome matures to a highly acidic killing compartment co-localising with lysosomes and containing a range of late phagosoamal membrane markers, including LAMP-1, and and lysosomal markers, including cathepsin D (Bewley et al., 2011a; Gordon and Read, 2002; Huynh et al., 2007), where early killing of pneumocci occurs by employment of ROS and RNS. Pneumococcal cell wall and PLY release from intact bacteria stimulate NO production (Braun et al., 1999; Orman et al., 1998), which decreases viability of *S. pneumoniae* in the airways. When iNOS was depleted in mice, viable pneumococci recovered from Bronchoalveolar Lavage (BAL) fluid 24 – 36 hours post infection were significantly increased compared to control mice (Kerr et al., 2004).

Non-oxidative mechanisms of bacterial killing are also employed by macrophages. Activation of the defensins Microbicidal Cationic Peptides (MCP-1 and MCP-2) by AMs has been shown to permeabilise the outer cell membrane of Gram-positive bacteria causing leakage of intracellular contents and access of macrophage microbiocidal molecules to the cell interior, possibly causing DNA damage (Lehrer et al., 1993). In addition, matrix metalloprotease has also been shown to disrupt cell membrane integrity of ingested bacteria (Houghton et al., 2009). Weiser and collagues showed that phagosomal lysozyme digests *S. pneumoniae* peptidyglycan which is then sensed by Nod2, which activates mediators of the pro-inflammatory response (Davis et al., 2011). Furthemore, the host utilises the iron sequestering molecule lactoferrin to restrict the growth of *S. pneumoniae* and one report demonstrates concominant expression of lysozyme and lactoferrin contributes to pneumococcal clearance (André et al., 2015).

However, evidence suggets that canonical microbiocidal killing mechanisms employed inside the phagosome are less important for AMs than they are for other phagocytic cells such as neutrophils and intraphagosomal killing of pneumococci can become exhausted (reviewed by (Aberdein et al., 2013)). However, apoptosis associated killing, regulated by the anti-apoptotic protein, Mcl-1 (induced myeloid leukaemia cell differentiation protein-1), has been shown to enable re-engagement of pneumococcal killing in AMs. It is a defect in this pathway which is thought to cause failure of AMs from COPD patients to clear pneumococcal infection effectively (Bewley et al., 2017), (discussed in section 1.5.2).

1.4.4. T-cell Response to *S. pneumoniae*

T-cell mediated immunity is key to host defence against *S. pneumoniae* infection (Chen and Kolls, 2013). MHC class II deficient mice, which lack $CD4^+$ T-cell populations, have impaired bacterial clearance from lung and blood and have a lower survival rate than wild-type mice (Kadioglu et al., 2004). This study showed firstly that $CD4^+$ T-cells were important in the early phases of pneumococcal infection, secondly, migration of T-cells towards pneumococci is reliant on PLY expression and finally, that $CD4^+$ T-cells were activated (Kadioglu et al., 2004). Th1 cells which release IFN-γ have been shown to be involved in pneumococcal host defence, however, many reports also suggest the involvement of Th17 cells (Olliver et al., 2011; Zhang et al., 2009). Infant mice which are more susceptible to pneumococcal infection than adult mice show delayed production of IL-17A in response to pneumococci (Bogaert et al., 2009). Furthermore, Th17 cells were demonstrated to be the most abundant type of activated T-cell in the lung following pneumococcal challenge and showed even greater levels of recruitment in mice with pneumococcal immune memory, indicating Th17 cell responses are key to initial pneumococcal infection and confer significant protection against reinfection (Wang et al., 2017). It is however important to note that in this study Th1 cell populations were also present, albeit in low numbers, demonstrating the requirement for a range of T-cell populations in protection against pneumococcal infection. IFN-γ producing NK cells also have a clear role in pneumococcal clearance at early stages of infection, as do $CD8^+$ regulatory T-cells which repress $CD4^+$ T-cell responses. Whether the role of all T-cells is beneficial for pneumococcal clearance, since roles in clearance may be associated with increased production of pro-inflammatory factors that might promote tissue injury, is yet to be determined (Mertens et al., 2009) however the immunomodulatory role of regulatory T-cells has been shown to be essential for balancing pathogen clearance with pro-inflammatory responses (Neill et al., 2012). In the same vein, T-cell responses can be dampened by monocyte induced apoptosis in response to *S. pneumoniae* as opposed to T-cell necrosis which occurs in the absence of monocytes, highlighting the importance for regulation of the immune response and in the cross talk between cell types during pneumococcal infection (Daigneault et al., 2012).

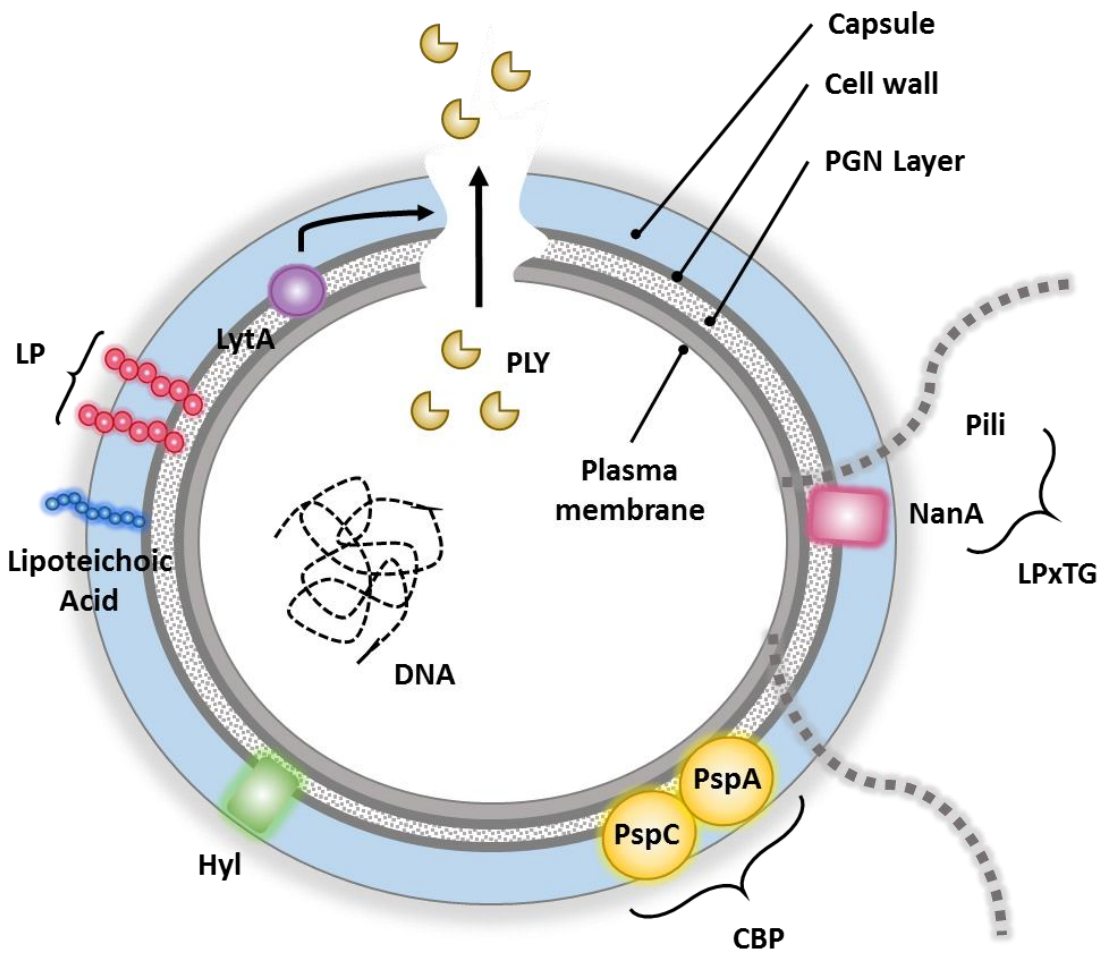


Figure 1-3 *S. pneumoniae* structure and virulence factors

The structure of *S. pneumoniae* includes an outer polysaccharide capsule which is unique to each serotype. The cell wall sits below the capsule and is composed of both inner and outer membranes and a peptidoglycan layer situated in between. Several varieties of immunogenic proteins are located on the outer membrane of the pneumococcal cell wall including Choline Binding Proteins (CBP), Pneumococcal surface protein A (PspA) and C (PspC) and also included in this category is LytA, a major pneumococcal autolysin. The second class of cell surface proteins are those anchored by the LPxTG motif and this group includes the neuraminidase (NanA) and pili proteins, present on some pneumococcal serotypes. The final class of cell wall proteins is the pneumococcal lipoproteins (LP) which are anchored by a lipoprotein domain. Other virulence components of *S. pneumoniae* which enable host recognition of the bacteria are Pneumolysin (PLY) the choline-dependant cytolysin, which is released upon lysis of the pneumococcal cell wall, the pneumococcal DNA which contains recognisable CpG methylated domains and Lipoteichoic Acid a cell wall glycolipid.

1.5. Apoptosis

Apoptosis, a type of regulated cell death is, in general, a compacted, neat form of cell deletion which occurs rapidly and causes little impact on close by cells and tissues. It is biochemically distinct from other forms of cell death such as necrosis, autophagy and necroptosis (reviewed by (Galluzzi et al., 2015)). Apoptosis is an essential process in many different aspects of biology, it has a prominent role during development in that it is required for the sculpting of organs and anatomical structures and the refinement of cell numbers. It is also important for the discrete removal of damaged cells and for maintaining the correct balance of cell numbers throughout life (reviewed by (Alberts, 2002)). Apoptosis is associated with distinct morphological features including nuclear and cytoplasmic condensation and fragmentation which forms apoptotic bodies of various sizes, containing nuclear chromatin and cytoplasmic contents (Kerr et al., 1972). Changes to the cell surface during apoptosis allow for rapid ingestion of apoptotic bodies by phagocytes (efferocytosis) and intracellular processing which results in degradation or recycling of phagosomal contents (Kerr et al., 1972). There are two main pathways of apoptosis, the extrinsic and intrinsic pathway both executed by the family of cysteine-aspartic proteases known as caspases. Briefly, the extrinsic pathway is initiated by extracellular signalling and involves the binding of a death ligands such as Fas Ligand to its cell surface receptor, e.g. death receptor Fas, which recruits Fas-associated death domain (FADD) protein and cleaves initiator caspases 8/10 which subsequently cleave effector caspases 3/7 (reviewed in (Fulda and Debatin, 2006)). The intrinsic pathway converges with the extrinsic pathway at the point of caspase 3/7 cleavage, however the prior events are centered around the mitochondria and the release of mitochondrial proteins after Mitochondrial Outer Membrane Permebalisation (MOMP), the reason why this pathway is also referred to as the mitochondrial pathway. I have described this pathway in more detail below and it is further reviewed in (Tait and Green, 2010)).

1.5.1. The Mitochondrial Pathway of Apoptosis

The mitochondrial pathway of apoptosis is primarily modulated by the B cell lymphoma 2 (Bcl-2) family of mitochondrial proteins. This family is comprised of members which share the Bcl-2 Homology (BH) domains. Anti-apoptotic members including Bcl-2, Mcl-1, Bcl-extra-large and A1 share BH domains 1-4 and resist apoptosis by maintaining mitochondrial membrane integrity (Vaux et al., 1988; Youle and Strasser, 2008). The pro-apoptotic members Bcl-2 associated X protein (Bax) (mostly cytosolic) and Bcl-2 homologous antagonist killer (Bak) (tethered to the mitochondria outer membrane) contain BH domains 1-3 and the presence of either is essential for the execution of apoptosis (Wei et al., 2001). Upon commitment to apoptosis, Bax and Bak translocate from the cytosol to the mitochondria and form homodimers to trigger apoptosis

(Dewson et al., 2008; Hsu et al., 1997). The second group of Bcl-2 pro-apoptotic proteins contain only BH3 domains (BH3 only proteins) and act as 'sensitisers' to apoptosis by inhibiting anti-apoptotic proteins or stimulating pro-apoptotic family members (Youle and Strasser, 2008).

The mitochondrial pathway of apoptosis can be triggered in response to DNA damage, cytokine deprivation, oncogene activation and growth cycle abnormalities via activation of tumour suppressor gene p53 which can then initiate the transcription of BH3 only proteins and also directly interact with Bax and Bak to promote apoptosis or supress anti-apoptotic Bcl-2 family members (Elkholi et al., 2014). Bax and Bak form pores in the mitochondrial outer membrane leading to MOMP (Peixoto et al., 2009). Subsequently, MOMP allows for the release of mitochondrial proteins from the mitochondrial intermembrane space such as cytochrome c which then can bind to Apoptotic Protease Activating Factor 1 leading to apoptosome formation and activation of initiator caspase-9 (Sanchis et al., 2003). The apoptosome is then capable of cleaving executioner caspases 3/7 (Srinivasula et al., 1998). Caspases 3/7 induce cell death by proteolysis of hundreds of proteins key to cellular integrity, including cytoskeletal components, the destruction of which contribute to rounding of the cell and membrane blebbing, hallmark characteristics of apoptosis (Lüthi and Martin, 2007). The mitochondrial pathway of cell death is the pathway used by macrophages when undergoing apoptosis associated killing of *S. pneumoniae* (Bewley et al., 2011a; Marriott et al., 2005).

1.5.2. Macrophage Apoptosis Associated Killing

It is well documented that many pathogens have evolved mechanisms of evading the host response by interfering with apoptosis. In some cases, cell death is induced by the pathogen to prevent phagocytosis and bacterial killing, in others the cell cycle is prolonged to aid intracellular microbial replication (DeLeo, 2004). However, in recent years, a role for host mediated apoptosis as a mechanism for bacterial clearance has emerged. Firstly, phagocyte apoptosis was shown to be beneficial to the host during *Bacillus Calmette-Guérin* infection by causing a reduction in the number of viable bacteria (Molloy et al., 1994). In the early 2000's, Dockrell and colleagues discovered that during pneumococcal infection AMs are capable of undergoing apoptosis during the resolution stages of infection (Dockrell et al., 2001a; Dockrell et al., 2003). Infection resolution was abrogated upon depletion of AMs and prevention of apoptosis by caspase inhibition, indicating a novel role for AM induced apoptosis in bacterial clearance. At the same time, the group observed that opsonisation increases *S. pneumoniae* induced apoptosis and that it is the intracellular burden of pneumococci which dictates the induction of apoptosis rather than cell surface receptor interactions (Ali et al., 2003). This study also noted the potential role

of the PI3K pathway in macrophage survival as blockade of PI3K activation partially inhibited pneumococcal associated apoptosis. This finding is in line with earlier work which highlighted a role Mcl-1, which belongs to the Bcl-2 family, in macrophage survival. Liu and colleagues discovered that upon inhibition of PI3K, ras-related C3 botulinum toxin substrate (RAC)-alpha serine threonine-protein kinase (Akt1) is no longer able to promote Mcl-1 upregulation, resulting in cell death (Liu et al., 2001). During pneumococcal infection enhanced iNOS levels were observed leading to an increased yield of NO, contributing to bactericidal activity (Marriott et al., 2004). Interestingly, higher levels of NO lead to MOMP, an important marker of apoptosis. Inhibition of iNOS was found to upregulate Mcl-1 levels and inhibit apoptosis, but instead of cell survival macrophages underwent necrosis. This evidence again supports a role for Mcl-1 (and NO) in the instigation of pneumococcal associated cell death.

1.5.2.1.Mcl-1

Mcl-1 was originally identified by Craig and colleagues as an early induction, pro-survival gene, responsible for maturation of the ML-1 human myeloid leukaemia cell line which under certain conditions can differentiate along the monocyte/macrophage pathway (Kozopas et al., 1993). This report also found Mcl-1 to have high sequence similarity to Bcl-2 and suggested the emergence of a Bcl-2 family of apoptosis regulating proteins. Later findings by the Craig laboratory demonstrated that Mcl-1 is capable of delaying cell death after exposure to a range of apoptosis inducing stimuli including growth factor deprivation and UV radiation and it is also able to interact with the pro-apoptotic proteins Bax and Bak through which it can block apoptosis (Thomas et al., 2010; Zhou et al., 1997). However, unlike Bcl-2 which is a stable protein with prolonged survival effect, Mcl-1 is subject to rapid turnover due to additional PEST (Prolene, Glutamic acid, Serine and Threonine) sequences in its n-terminus which make it an ideal candidate for responses requiring swift generation and degradation, for example, in promoting the short-term viability of the cell (Thomas et al., 2010; Yang et al., 1995). As well as offering protection from cytotoxic agents, Craig and colleagues went on to discover Mcl-1 mRNA is increased during differentiation of monocytes and macrophages (Yang et al., 1996) and it was later shown by Opferman and colleagues that Mcl-1 is required for the survival of haematopoietic stem cells (Opferman et al., 2005) and development of T and B lymphocytes (Opferman et al., 2003). Furthermore, mice, heterozygous for Mcl-1 deletion were unable to produce Mcl-1 homozygous null pups as full deletion of Mcl-1 results in peri-implantation and embryonic lethality (Rinkenberger et al., 2000) demonstrating its absolute requirement in development.

Supporting its role as a rapid response gene with multiple functions (Craig, 2002), Mcl-1 is subject to multiple post-transcriptional modifications, one of which is alternative splicing. A naturally occurring isoform of Mcl-1 was found, Mcl-1 short (Mcl-1_s) which is a BH3-only splice variant and therefore has pro-apoptotic, rather than anti-apoptotic functionality (Bae et al., 2000). Furthermore, another splice variant, Mcl-1 extra short (Mcl-1_{es}) was shown to localise to the mitochondria and is capable of associating with full length Mcl-1 and promoting cell death by the mitochondrial pathway, terminating in cytochrome c release (Kim et al., 2009). Further exploration into three mitochondrial isoforms of Mcl-1 showed differential mitochondrial location, while anti-apoptotic 40kDa and 38kDa isoforms locate to the mitochondrial outer membrane and sequester pro-apoptotic Bcl-2 family members, 36kDa Mcl-1 localises to the mitochondrial matrix and is involved in the maintenance of mitochondrial structure and fusion and normal mitochondrial bioenergetics (Perciavalle et al., 2012). More recently, it was shown that the ratio of full-length Mcl-1 and Mcl-1_s is important for maintenance of mitochondrial physiology, with a higher ratio of Mcl-1_s resulting in mitochondrial hyper-fusion which is thought to support cell death (Morciano et al., 2016). These studies show the importance of Mcl-1 as an apoptotic switch and as a constituent of the mitochondria where it exerts its role as both a modulator of cell death and a modulator of mitochondrial respiration.

1.5.2.2. Mcl-1 acts as the switch for apoptosis associated killing

The Bcl-2 family of pro-apoptotic and anti-apoptotic proteins regulate cell death by preserving mitochondrial integrity until induction of MOMP which leads to cytochrome c release and subsequent caspase activation, reviewed by Corey *et al* (Cory and Adams, 2002). As discussed, Mcl-1 has a short half-life in comparison to other Bcl-2 proteins therefore its high turnover renders it an ideal candidate for an apoptotic switch. Marriott and colleagues conducted a series of experiments which lead to confirmation that Mcl-1 is a key regulator of delayed apoptosis in pneumococcal infection. Firstly, they found that Mcl-1 protein levels remained elevated up to 14 hours after infection, after which there is a gradual decline in expression (Marriott et al., 2005). By using transgenic mice which over expresses Mcl-1 and therefore significantly delays the onset of pneumococcal associated apoptosis, the group were able to determine that the inhibition of apoptosis extended the survival of intracellular pneumococci, which caused bacteraemia at a lower dose than is required in wild type mice. Additionally, the group discovered the expression of an alternative Mcl-1 isoform which appeared 12 hours after infection and exhibited pro-apoptotic activity, furthering the notion of Mcl-1 as the apoptotic switch. Furthermore, this study revealed that upon proteasome inhibition levels of Mcl-1 increased at 20 hours post infection, indicating proteasomal degradation plays a role in Mcl-1

turnover. Doug Green and colleagues have since established MOMP can be induced by glycogen synthase kinase 3 β which is negatively regulated by Akt and phosphorylates Mcl-1 causing ubiquitination by Mcl-1 ubiquitin ligase E3 (MULE) leading to subsequent degradation via the proteasome (Maurer et al., 2006b). More recently, cathepsin D, a protein known to be involved in the initiation of apoptosis through cleavage of Bcl-2 family members (Blomgran et al., 2007), has been shown to enhance interaction of Mcl-1 with MULE (Bewley et al., 2011a). The ability of cathepsin D to downregulate Mcl-1 is set in place after lysosomal membrane permeabilisation has been triggered by the internalisation of pneumococci. The regulation of Mcl-1 in apoptosis associated killing is summarised in Figure 1-2.

1.5.2.3. The CD68 Mcl-1 transgenic mouse

The Craig laboratory constructed a transgenic mouse which over expressed Mcl-1 in all tissues studied (Zhou et al., 1998). This mouse showed enhanced viability of lymphoid and myeloid cells and when transgenic cells were explanted into tissue culture, they were capable of inducing an immortalised myeloid cell line (Zhou et al., 1998). With the Mcl-1 transgenic mouse, the Dockrell group were able to determine the importance of Mcl-1 as the switch for apoptosis associated killing, described above (Marriott et al., 2005). To ensure that the effects of Mcl-1 were macrophage specific, the group in collaboration with Steve Schapiro, University of Pittsburgh, Ruth Craig Dartmouth Medical School and David Greaves, University of Oxford, expressed a construct containing the human Mcl-1 cDNA used by Zhou and colleagues in the Craig laboratory, on a CD68 promoter, using a method previously described by Greaves and colleagues (Gough et al., 2001). The CD68 Mcl-1 mouse lacks transgenic expression of human Mcl-1 in neutrophils and lymphocytes, mice exhibit normal survival and lack any gross developmental phenotype (Preston et al., unpublished data), therefore, with this model, the group was able to determine the role of Mcl-1 in resolution of infection in a macrophage specific context. CD68 Mcl-1 transgenic mice displayed decreased macrophage apoptosis after pneumococcal infection and failed to clear bacteria as effectively as wild-type mice at late time points (Bewley et al., 2017, Preston et al., unpublished data). Furthermore, it was shown that generation of mitochondrial ROS is required for apoptosis associated killing and that Mcl-1 transgenic mice display increased oxygen consumption rates and increased levels of mitochondrial ROS at baseline, however, the clearance defect in these cells is thought to be due to an inability of transgenic macrophages to increase caspase dependant mitochondrial ROS after *S. pneumoniae* challenge, mimicking a defect which is also seen in AMs from COPD patients (Bewley et al., 2017). In vivo, CD68 Mcl-1 transgenic mice fail to clear low dose pneumococcal infection (10^4 cfu/ml) and bacteraemia ensues, in contrast to wild-type mice which are capable of clearance

without neutrophil recruitment. With an intermediate dose of 10^5 cfu/ml transgenic mice were found to have increased cfu in the blood compared to wild-type macrophages and at a high dose of 10^7 cfu/ml both wild-type and transgenic mice became overwhelmed. This data indicates the deficit which transgenic mice exhibit is most important when AM are the major cell type involved in bacterial clearance. The potential of restoring apoptotic responses in this model is currently being explored with the use of BH3 mimetics and lysomotrophic agents which are inductive of lysosomal membrane permebalisation leading to activation of the apoptotic cascade (Preston et al., unpublished data).

1.5.3. Significance in Disease

Previously, it has been recorded that patients with COPD exhibit decreased neutrophil apoptosis coupled with decreased mRNA levels of the pro-apoptotic protein, Bak and increased levels of Mcl-1 mRNA (Zhang et al., 2012). In the same vein, AMs from patients with COPD display less nuclear fragmentation (an indicator of apoptosis) and enhanced bacterial survival (Bewley et al., 2017). Furthermore, higher expression of Mcl-1 is apparent in in both uninfected and *S. pneumoniae* exposed AMs from COPD patients, which correlates with a decrease in apoptosis after pneumococcal challenge. Likewise, COPD AMs display higher levels of baseline mitochondrial ROS which fails to increase upon challenge with *S. pneumoniae* in line with decreased bacterial killing. This observation is also associated with a reduced ratio of mitochondrial ROS to superoxide dismutase (SOD), an antioxidant which is responsible for the dismutation of $O_2^{\cdot-}$. These observations indicate that Mcl-1 upregulation is a key event in the pathogenesis of COPD and contributes to *S. pneumoniae* induced acute exacerbations.

HIV patients have increased susceptibility to invasive pneumococcal disease and macrophage apoptosis associated killing in response to *S. pneumoniae* infection has also been shown to be perturbed in HIV positive individuals on fully suppressive anti-retroviral therapy. AMs from these patients show increased levels of Mcl-1, altered mitochondrial ROS generation and decreased levels of apoptosis. These effects can be stimulated with the HIV envelope glycoprotein gp120 (Collini *et.al*, in submission). These results indicate defective apoptosis associated killing in HIV positive individuals may cause an increased risk to invasive pneumococcal disease, a phenotype which is again modulated by increased levels of Mcl-1.

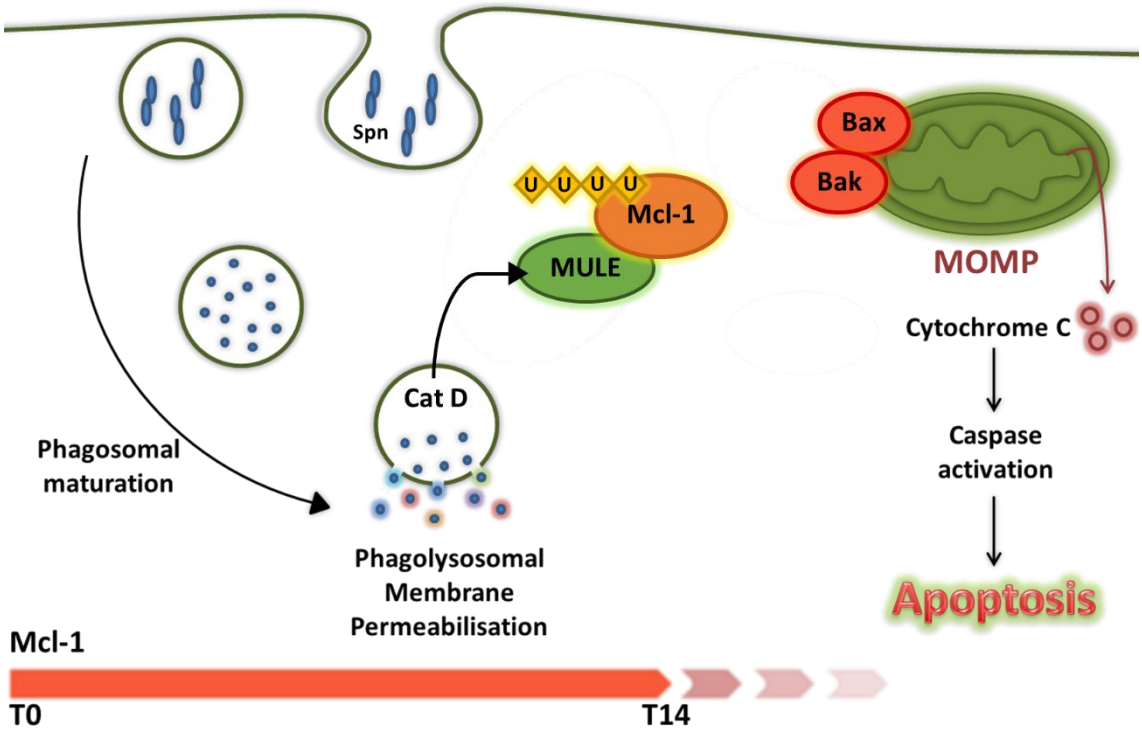


Figure 1-4: Overview of macrophage apoptosis associated killing

S. pneumoniae (Spn) is internalised by the macrophage into the phagosome which undergoes phagosomal maturation until it becomes an effective phagolysosomal killing compartment. Early phase killing of *S. pneumoniae* then ensues by the employment of ROS and NO which is eventually overwhelmed by *S. pneumoniae* resistance mechanisms. Late phase killing is triggered by phagolysosomal membrane permeabilisation and the lysosomal protease cathepsin D (Cat D), activation of which triggers MULE activity. MULE tags Mcl-1 for proteasomal degradation which is followed by activation of Bax and Bak which activate MOMP and cytochrome C release leading to caspase 3/7 activation and apoptosis. Apoptosis is associated with increased release of mitochondrial ROS and late phase killing of *S. pneumoniae*.

1.6. Non-Typeable *Haemophilus Influenzae*

1.6.1. Pathogenesis

Non-typeable *Haemophilus influenzae* is an un-encapsulated, small, Gram-negative bacterium. It is a facultative anaerobe and a common coloniser of the nasopharynx, existing in around 75% of the population at any one time, however upon invasion of the lower respiratory tract and other sterile sites, NTHi is a common cause of infection, particularly in the immunocompromised such as those at the extremities of age and those with lung disease (Bandi et al., 2001; Murphy, 2003; Sethi and Murphy, 2008). Furthermore, 90% of *Haemophilus* strains isolated from the middle ear during otitis media are attributable to NTHi and NTHi accounts for 50% of all COPD

exacerbations (Murphy and Apicella, 1987). The success of this pathogen is likely attributable to its affinity for phase variation and high strain turnover allowing for effective evasion of the immune response. Additionally, NTHi has been shown capable of intracellular persistence despite its identification as an extracellular pathogen, which could lead to dysregulation of immune responses and enhanced tissue damage by sustained activation of the inflammatory response (Craig et al., 2001; Craig et al., 2002; Morey et al., 2011). Although primarily interacting with and stimulating bronchiole epithelial cells residing on the surface of lung tissue epithelial cells are quick to recruit macrophages and other leukocytes to execute an effective immune response which is severely impaired during lung diseases such as emphysema and COPD (Clementi and Murphy, 2011).

1.6.2. Virulence Factors

1.6.2.1. Outer Membrane Proteins and Outer Membrane Vesicles

There are around 36 Outer Membrane Proteins (OMPs) expressed on the surface of *H. influenzae* either categorised as major or minor based on their relative expression (Foxwell et al., 1998c). Major OMPs include Proteins P1, P2, P4, P5 and P6 and high molecular weight (HMW) proteins 1 and 2 which are conserved across almost all *H. influenzae* strains. OMP expression contributes to the high amount of strain to strain variation seen between NTHi subtypes which likely participates in evasion of the host immune response (Foxwell et al., 1998c). P2 (porin), P5 (Protein D) and HMW proteins have documented importance in bacterial adhesion to mucin and epithelial cells (Reddy et al., 1996; St Geme et al., 1993) and the presence of P5 has been linked to decreased complement deposition via binding of the negative regulator of alternative pathway complement deposition, factor H, and decreased Immunoglobulin M (IgM) binding, two important mechanisms for host internalisation of invading NTHi (Rosadini et al., 2014). However, protein D (P5) is required for effective NTHi adherence and internalisation to monocytic cells via macrophage β-Glucan receptors (Ahrén et al., 2001). Furthermore, OMP are potent inducers of the host pro-inflammatory response; P6 has been shown to enhance TNFα and IL-8 cytokine release from human macrophages (Berenson et al., 2005). Although this provides protective functions in healthy subjects, it likely contributes to tissue damage and propagation of infection through development of excessive inflammatory responses, as seen in immunocompromised individuals such as those with COPD.

An emerging role for Outer Membrane Vesicles (OMVs), suggested to be regulated by OMPs, in the virulence of *H. influenzae* species is being increasingly documented. OMVs are transporters of bacterial DNA, virulence factors and signalling molecules with indicated roles for biofilm

formation (Kaparakis-Liaskos and Ferrero, 2015). They are released from all Gram-negative bacteria and contribute to activation of the immune response in epithelial cells and macrophages. OMVs are enriched in certain OMPs from the outer membrane of NTHi strains including P2, P5 and P6 and less frequently P1 and P4, therefore making OMVs a valid contender for vaccine development (Roier et al., 2015). Certain strains of NTHi have also been shown to release more OMVs than others which might confer increased virulence of selected NTHi isolates (Roier et al., 2015; Roier et al., 2016).

1.6.2.2. Lipooligosaccharide

The lipooligosaccharide (LOS) located on the outer membrane of *H. influenzae* shares many similarities to LPS of other Gram-negative bacteria but has a lower molecular weight as it lacks O-polysaccharide side chains (Murphy and Apicella, 1987). The LOS is composed of 3 parts; a membrane anchored portion, Lipid A, an inner core and an outer core. Lipid A is also accountable for most of endotoxicity of LOS and mutants which lack the *htrB* gene, responsible for Lipid A acylation reactions, show attenuated pro-inflammatory responses, less effective airway cell activation and airway colonisation compared to parent strains (Nichols et al., 1997; Swords et al., 2002).

Phosphorylcholine (ChoP) is a host derived epitope found on the cell surface components of respiratory bacteria including *H. influenzae* and *S. pneumoniae*. ChoP expression on the LOS is controlled by NTHi phosphocholine kinase encoded by the *lic-1* locus and the first gene in the *lic-1* locus, *lic-A*, allows for phase variation of ChoP expression (Weiser et al., 1989; Weiser et al., 1997). *Lic-1* together with three other chromosomal loci induce phase variation of ChoP and other LOS structures which allows NTHi to adapt to alternative host environments, persist in colonisation of the airways and evade host immune responses leading to development of disease (Tong et al., 2000; Weiser et al., 1998).

Conversely, ChoP can be bound by C-Reactive Protein which subsequently activates the complement system it is therefore potentially beneficial for NTHi pathogenicity if the amount of ChoP expression can be decreased by phase variation (Weiser and Pan, 1998; Weiser et al., 1998). Conversely, presence of *igtC, lex2* and *losA* genes, encoding structures which decorate the LOS all confer complement resistance and when removed, complement deposition is increased (Hallström and Riesbeck, 2010). More recently, variation in NTHi *VacJ* and *Yrb* transporter genes, also contributing to OMV production, have been shown to alter IgM antibody binding to the LOS (Roier et al., 2016). Strains with increased *VacJ* / *Yrb* expression, have been shown to have enhanced serum resistance, in isolates collected from the lower respiratory tract

of patients with COPD (Nakamura et al., 2011). Modifications in LOS structure have also been shown to protect NTHi against host IgM in otitis media models (Langereis et al., 2012). It is therefore clear that the phase variation of the LOS on NTHi is essential to colonisation of the airways and propagation of disease.

1.6.2.3. IgA Proteases

IgA proteases are highly conserved across all strains of NTHi and were originally implicated in cleavage of human immunoglobulin A, subclass 1 (IgA1) Fab domains from Fc domains, which are then expressed on the bacterial cell surface, preventing the function of IgA1 antibody in promoting opsonophagocytosis and masking the bacteria by expression of host molecules (Mistry and Stockley, 2006; Murphy et al., 2015). They have suggested roles in NTHi colonisation of the airways (Poole et al., 2013) and are also implicated in propagation of the inflammatory response as they have been shown to increase levels of pro-inflammatory cytokines including TNF-α and IL-6 (Mistry and Stockley, 2006). More recently an alternative IgA protease gene, known as *IgaB* was discovered in around 40% of NTHi strains and shown to be more frequently expressed in clinical isolates (Fernaays et al., 2006a; Fernaays et al., 2006b). Further investigation of the IgaB protease interestingly revealed a role in cleavage of LAMP-1 which helps the bacterium withstand lysosome mediated killing and aids persistence of NTHi in host immune cells (Clementi et al., 2014).

1.6.2.4. SapA

The SapA transporter protein is conserved across Gram-negative bacteria and involved in the transport of heme and other essential nutrients across the bacterial inner membrane (Mason et al., 2011). SapA has been shown to modulate NTHi resistance against host derived antimicrobial peptides by binding and subsequently targeting these for intracellular degradation (Shelton et al., 2011). Mason and colleagues showed that SapA was also essential for heme transport, a compound which NTHi are incapable of producing and therefore must acquire from their local environment. Furthermore, they revealed that that antimicrobial peptides compete with heme for SapA binding, providing evidence of multiple roles for SapA in influencing NTHi survival (Mason et al., 2011; Stojiljkovic and Perkins-Balding, 2002).

1.6.3. The Macrophage Response to non-typeable *Haemophilus Influenzae*

AM are essential for the ingestion and clearance of NTHi from the respiratory tract and are supplemented in the airway by recruited macrophages in response to acute airway challenge (Foxwell et al., 1998a). As the resident immune cells of the lung which respond to NTHi colonisation and infection, AMs are responsible for recognition, ingestion and killing of NTHi

following phagolysosomal trafficking and these functions can be complemented by similar activities in recruited inflammatory airway macrophages (Clementi and Murphy, 2011; Foxwell et al., 1998a; Martí-Lliteras et al., 2009). Macrophages are also critical for neutrophil recruitment and subsequent T-cell activation to bring about the second phase of bacterial killing (Foxwell et al, 2008).

NTHi is readily ingested by macrophages perhaps owing to its lack of capsule (Noel et al., 1992). Several pathways of NTHi uptake have been suggested including opsonic phagocytosis by complement deposition, which can be activated by several NTHi virulence factors, and non-opsonic uptake by the β-Glucan receptor and scavenger receptors (Ahrén et al., 2001; Hallström and Riesbeck, 2010; Provost et al., 2015). The transcription factor nuclear erythroid–related factor 2 increases NTHi phagocytosis and clearance by upregulation of the scavenger receptor MARCO (Harvey et al., 2011). Studies have also shown that lipid rafts are important for effective NTHi internalisation in both macrophages and bronchiole epithelial cells, as cholesterol depletion impairs phagocytosis (Martí-Lliteras et al., 2009; Morey et al., 2011). Furthermore, cytochalasin D treatment of AMs reduces NTHi uptake indicating F-actin dependent cytoskeletal rearrangement also contributes to effective internalisation.

Internalisation of NTHi and signalling by PRRs such as TLR2 (involved in recognition of OMP P6) and TLR4 (involved in recognition of LOS) induces PI3K and Akt signalling which has many downstream effects on phagocytosis, cytoskeleton rearrangement and endosomal maturation and trafficking (Berenson et al., 2014; Gillooly et al., 2001; Tamura et al., 2009; Wieland et al., 2005). Signalling via TLRs is also effective in generating pro-inflammatory responses through activation of NFκB. NTHi in conjunction with TNFα can activate NFκB via the p38/MAPK and PI3K/Akt signalling pathways to promote the inflammatory response by release of cytokines such as TNFα, IL-1β, IL-6 and IL-8 (Watanabe et al., 2004). TLR2 and 4 mediated cytokine responses are diminished in COPD AMs leading to impaired NTHi clearance (Berenson et al., 2014). This does not necessarily result in diminuation of the overall inflammatory response though. Interestingly, activation of the NLRP3 inflammasome by NLRs and subsequent activation of caspase-1 and production of IL-1β has been implicated as a source of enhanced acute inflammatory responses in the NTHi infected lung, which may also contribute to the potent inflammatory nature of NTHi infection (Rotta Detto Loria et al., 2013).

Once internalised, phagosomes containing NTHi have been shown to undergo phagosomal maturation, first co-localising with markers of early phagosomes such as EEA-1 then acquiring late phagosomal markers such as LAMP-1 and LAMP-2 which is also associated with subsequent

recruitment of lysosomes and development of low pH (Martí-Lliteras et al., 2009). In bronchiole epithelial cells, NTHi have been shown to utilise phagosomes as protected intracellular niches where they can reside without proliferation for extended periods. These phagosomes do not fuse with lysosomes and only very low numbers associate with cathepsin D, which compromises intracellular killing (Morey et al., 2011). As discussed earlier, the NTHi IgaB protease may play an essential role in bacterial survival inside the phagosome by cleavage of late phagosomal markers, however, this protease does not appear to inhibit phagosomal acidification and functions more effectively at a neutral pH so the exact role of LAMP1 cleavage in this model on intracellular killing is incompletely defined (Clementi et al., 2014). It is therefore conceivable that other virulence factors may be employed in conjunction with the IgaB protease to aid NTHi survival at a lower pH. However, it is not known if similar effects occur in macrophages since these observations are all in epithelial cells. In view of the importance of these pathways in macrophages it is theoretically possible that in macrophages, that if they occur, the reduction in lysosomal fusion and cathepsin D activation could prevent the onset of macrophage apoptosis associated killing as a host mechanism of NTHi clearance.

Recent studies have opened the door for micro RNA signalling in macrophages and neutrophils in airway disease. Antagonism of miR-328 was recently found to increase NTHi phagocytosis, cathepsin D phagosome co-localisation, ROS production and ultimately bacterial clearance (Tay et al., 2015). Macrophages are known to increase ROS production in order to kill NTHi (Jyotika Prasad 2013). NTHi have also shown susceptibility to anti-microbial peptides such β-defensins (Starner et al., 2002). The presence of the SapA transporter defence mechanism, by which NTHi degrade anti-microbial peptides released from immune cells, further implicates these molecules as effective mediators of NTHi killing (Shelton et al., 2011). Macrophage production of the antimicrobial peptide LL-37 has however been positively correlated with COPD patients colonised with NTHi and with markers of inflammation (Persson et al., 2017), raising questions about how effective this response is in clearing the bacteria. Other studies have reported low levels of lactoferrin and lysozyme in response to NTHi infection (Parameswaran et al., 2011).

1.6.3.1. NTHi in COPD Exacerbation

During COPD, damage to the lung prevents normal ingestion and clearance of NTHi by macrophages (Berenson et al., 2006b; Martí-Lliteras et al., 2009). This creates a lung milieu where bacteria can thrive and evade the usual immune response. Evidence suggests the altered macrophage response in COPD pathogenesis might be due to dysregulated macrophage activation which contributes to an ever-more damaging lung environment (Kunz et al., 2011; Shaykhiev et al., 2009b), and promotes the “vicious circle” of events central to disease

progression, characterised by persistent bacterial colonisation and inflammatory tissue injury (Murphy and Sethi, 1992).

1.7. Aims of the thesis

Macrophage activation is important in both health and disease however the role macrophage activation in *S. pneumoniae* infection and more specifically the effect of macrophage activation on apoptosis associated killing, has not been explored in detail before. Further, a role for macrophage apoptosis associated killing has previously been outlined in response to *S. pneumoniae* and *Escherichia coli* and shown to be evaded by *Staphylococcus aureus* and *Neisseria meningitidis* yet it has not been established whether apoptosis associated killing is activated in response to NTHi infection.

Broadly, I hypothesise that since apoptosis associated killing is critical to pathogen clearance, when host or pathogen factors alter it there will be consequence to the clearance of bacteria. More specifically, I hypothesise that:

1. Mcl-1 over-expression alters macrophage activation which in turn alters macrophage response to *S. pneumoniae* challenge
2. When capacity for apoptosis associated bacterial clearance is overwhelmed additional layers of the host response must be activated
3. Macrophage apoptosis associated killing is activated in response to NTHi challenge

The main aims of my thesis are:

1. Analyse if there is any difference in macrophage activation between wild-type and CD68 Mcl-1 transgenic Bone Marrow Derived Macrophages (BMDMs) and characterise activation in BMDMs and MDMs.
2. Determine the effect of macrophage activation on wild-type and transgenic BMDM and MDM macrophage effector functions after *S. pneumoniae* challenge
3. Understand how the CD68 Mcl-1 transgene alters the transcriptional response of AMs to *S. pneumoniae* at 16 hours of challenge and validate these findings using multi-colour flow cytometry
4. Analyse macrophage effector functions including macrophage apoptosis and bacterial killing in response to NTHi challenge

2. Materials and Methods

2.1. Cell culture and differentiation

2.1.1. Human monocyte-derived macrophages

Blood isolation was carried out in the OPHAT room, E floor, Royal Hallamshire Hospital. 300ml of blood was collected in blood bags (Fresenius Kabi Ltd) with the anticoagulant, Citrate Phosphate Dextrose Adenine (CPDA) solution, from consenting donors by a trained member of staff. All donors were given an information sheet and signed both a consent and a screening form which were checked and filed in the Infectious Diseases Department (E floor). Monocyte isolation from human blood was conducted by a member of the departmental technical team with ethical approval from South Sheffield Regional Ethics Committee (07-Q2305/7). Blood was decanted into T75ml tissue culture flasks and 25ml of blood from each donor was layered onto 12.5ml Ficoll-Paque (GE Healthcare) in 50ml Falcon tubes (Starstedt). Blood was then centrifuged at 1500 rpm for 23 minutes with the brake off. Cell layers were isolated and added to fresh 50ml tubes and centrifuged at 1000 rpm for 13 minutes. Supernatants were discarded and pellets resuspended in 10ml macrophage media (RPMI (Lonza), 10% Heat Inactivated Foetal Bovine Serum (HIFBS) with low LPS (Gibco), 1% L-Glutamine (Lonza)). Peripheral blood mononuclear cells were then counted using the haemocytometer method and further diluted in macrophage media to a concentration of 2 x 10^6 /ml and plated out.

2.1.2. Murine Bone Marrow Derived Macrophages

Mice were culled by cervical dislocation. Legs were removed and placed in Dulbecco's Modified Eagle Medium (DMEM), (Lonza). Bones were cleaned of remaining tissue and bone marrow cells isolated from mouse femurs and tibias. Cells were flushed from bones using a 0.5x16mm needle and 1ml syringe. Cells were counted using a haemocytometer and resuspended in medium (DMEM plus 10% HIFBS and 10% L929 conditioned medium plus antibiotics; 100µg/ml Streptomycin with 100U/ml penicillin) at a concentration of 2x10^5 cells/ml. Cells were incubated in medium at 37^{o}C for 5 days after which non-adherent cells were removed and fresh media added. Medium was then replaced every other day with DMEM plus 10% HIFCS and 10% L929 conditioned medium until complete macrophage differentiation at 14 days. Antibiotics were excluded from the medium after 7 days of culture. As BMDMs proliferate in cell culture, a cell count was performed on day 14. Cells were washed in 1ml of Phosphate Buffered Saline (PBS) and incubated with 200µl Accutase® (Biolegend) for 15 minutes. They were then gently scraped and counted using a haemocytometer (Section 2.1.3). For extended storage of BMDMs, cells were flushed and centrifuged at 200 x g for 10 minutes. Cells were then resuspended in HIFBS +

10% DMSO and added to cryovials. To achieve a slow rate of cooling which is essential for cell preservation, BMDMs were transferred to a Mr. Frosty™ freezing container (Thermo Scientific) containing isopropyl alcohol. They were first cooled to -80°C before transfer to the biorepository liquid nitrogen storage (C floor, Royal Hallamshire Hospital). To thaw, cells were kept on dry ice until transfer to a water bath set to 37°C. Once defrosted, cells were quickly diluted in 50ml of media to avoid the toxic effects of DMSO.

2.1.3. Quantifying the number of cells

Bone marrow cells were diluted in 50ml complete medium and 10µl of the suspension was added to the haemocytometer by capillary action. Cells were counted on the Olympus CK2 microscope, 10x objective. A cell count was obtained from all four 4 x 4 grids and divided by 4 to give the average cell count per grid. The average cell number was then multiplied by the 10,000 to give cells/ml. The calculated number of cells /ml was divided by the desired number of cells/ml to give the dilution factor. The dilution factor was multiplied by total volume of the cell suspension (50ml) to estimate the total volume needed to achieve the desired number of cells/ml.

2.1.4. Macrophage activation

Macrophages were activated with either human or murine IL-4, IL-10, IFN-γ or IFN-γ + ultra-pure LPS (Enzo). All cytokines were purchased from PreproTech. Upon arrival cytokines were reconstituted first in distilled H_2O then further diluted in PBS containing 0.1% bovine serum albumin (Sigma Aldrich) to a concentration of 20µg/ml. To avoid freeze – thaw cycles, vials were aliquoted and stored at -20°C until use. At 14 days of culture, macrophages were stimulated with 20ng/ml cytokine and 100ng/ml LPS for a duration of 24 hours unless otherwise stated.

2.1.5. Macrophage viability test

TRIVIGEN® TACS MTT Cell Proliferation Assay Kit was purchased from R&D Systems. A volume of the tetrazolium salt 3- [4, 5-dimethylthiazol-2yl]-2, 5-diphenyl-tetrazolium bromide (MTT) was added to each well. After 4 hours of incubation at 37°C, an equal volume of detergent reagent containing Sodium dodecyl sulfate (SDS) was added to the cells and 37°C incubation was resumed for a further 4 hours. The plate was read at a wavelength of 562nm (Labsystems Multiscan Ascent). Wells containing media alone (without cells) were used as a blank. To create a standard curve, cells were seeded at increasing densities in a 96-well plate. To analyse the effect of macrophage activation on cell viability, cells cultured for 14 days were seeded at $2x10^5$ cells/ml and left to adhere to the plate overnight. Cells were then activated with cytokines or left unstimulated. Viability was assessed at either 24 or 48 hours' post activation.

2.2. Bacterial culture

2.2.1. Streptococcus pneumoniae

2.2.1.1. Stock preparation

Experiments were carried out using serotype 1 *S. pneumoniae* WHO reference laboratory strain SSISP; (Statens Serum Institut). *S. pneumoniae* were plated on Columbia Blood Agar (CBA) plates and incubated overnight at 37°C, 5% CO_2. Colonies were collected and grown in growth media (25ml Brain and Heart Infusion (BHI) broth (Oxoid) supplemented with 20% HIFBS) incubated at 37°C + 5% CO_2 with agitation. The Optical Density (O.D.) was measured at hourly time points at a wavelength of 600nm by spectrophotometry (Jenway). Once the O.D. reached 0.6 (mid-log) the broth was aliquoted and stored at -80°C. At each time point colony forming units were calculated using the Miles Misra technique (section 2.2.3.). *S. pneumoniae* colonies were identified by α-hemolysis of blood agar and optochin sensitivity.

2.2.1.2. Opsonisation and Macrophage infection

Human serum was obtained from healthy volunteers previously vaccinated with the 23-valent polysaccharide pneumococcal vaccine (Pneumovax®23). Effectiveness of opsonisation treatment was assessed by flow cytometry. Binding of fluorescein isothiocyanate (FITC) conjugated goat human IgG Fc (Dako) to immune serum treated and untreated *S. pneumoniae* was compared. Opsonisation with human immune serum caused a positive shift in FITC expression compared to untreated bacteria.

Murine serum was pooled from mice vaccinated with Pneumovax®23. Serum samples were collected initially from a tail vein bleed and antibody levels checked by Enzyme Linked Immunosorbant Assay (ELISA). 6 weeks post-vaccination was sufficient for animals to raise an antibody response.

S. pneumoniae serotype 1 from frozen stock was thawed, pelleted (9000xg, 3 minutes) and washed 3 times in 1ml sterile PBS. For opsonisation, the pellet was then re-suspended in serum free DMEM + 10% mouse immune serum for infection of murine BMDMs or RPMI + 10% human immune serum for infection of human MDMs and incubated for 30 minutes at 37°C with agitation. Post incubation, bacteria were pelleted (3400xg, 3 minutes) and washed twice with 1ml sterile PBS. The pellet was then re-suspended in 950µl of appropriate media + 10% HIFBS and added to cells at the correct Multiplicity Of Infection (MOI). Miles Misra surface viability counts were conducted on the re-suspended bacteria grown overnight on CBA plates to confirm the concentration of *S. pneumoniae* per aliquot.

2.2.2. Non-Typeable *Haemophilus influenza*

2.2.2.1. Stock preparation

NTHi requires agar plates which have been "chocolated" (heated) to break up erythrocytes enabling release of Hemin destruction of NADases which cause digestion of Nicotinamide Adenine Dinucleotide (NAD) released by red blood cells (Krumwiede and Kuttner, 1938), (Musher, 1996). Freeze-dried NTHi (strain number: ATCC49247) was reconstituted in BHI broth supplemented with factors required for *Haemophilus influenza* growth; 2µg/ml NAD (Sigma Aldrich) + 10µg/ml Hemin (Sigma Aldrich) dissolved in 20mM NaOH, bead stocks were created and stored at -80°C. Individual beads were collected from the stock and plated on chocolate blood agar (EO Labs). After overnight incubation, colonies were collected and added to 25ml growth media which was incubated at 37°C plus 5% CO_2 with agitation and measured hourly by spectrophotometry at a wavelength of 600nm until the O.D. reached 0.6. 20% HIFBS was then added and the volume divided into 1ml working aliquots (Kirkham et al., 2013). Aliquots were stored at -80°C. Glycerol stocks of the clinical isolate obtained from GSK (NTHi strain number 1479) was also grown and stored using the same method.

2.2.2.2. Macrophage infection with NTHi

NTHi, stored at -80°C, was thawed and pelleted (9000xg, 3 minutes) and washed 3 times in 1ml sterile PBS. The pellet was then re-suspended in 950µl of media + 10% HIFBS and added to cells at the correct MOI. 10-fold serial dilutions were conducted on the re-suspended bacteria and grown overnight on chocolate agar plates to confirm the concentration of NTHi per 1ml aliquot.

2.2.3. Miles Misra viable bacterial count

In order to quantify the amount of bacteria in a suspension, the Miles Misra technique for estimating concentration of viable bacteria was used (Miles et al., 1938). Bacteria was re-suspended in infection media and vortexed. 6 x 10-fold serial dilutions were performed in sterile PBS and plated in 3 x 10µl drops per quadrant on blood agar plates. Plates were incubated overnight at 37°C with 5% CO_2. The following day colonies were counted on the first quadrant which displayed visible individual colony forming units. The number of colonies was then multiplied by the dilution factor, divided by 30 to give cfu/µl and multiplied by 1000 to give cfu/ml.

2.2.4. Preparation of cells for infection

Before infection, cell number was quantified using a hemocytometer for accurate calculation of the MOI. Media was discarded and replaced with fresh media containing only 10% HIFBS

(500µl/well). If appropriate, macrophages were activated with cytokines 24 hours prior to infection and washed once in PBS before addition of fresh media.

2.3. Measurement of protein Expression by Western Blot

2.3.1. Protein Extraction

Cells were lysed at room temperature by the trichloro-acetic acid (TCA) protein extraction method (Wang et al., 1996). Non-adherent cells were first removed by washing in PBS. Cells were then washed in Tris buffered saline -Ethylenediaminetetraacetic acid (TBS-EDTA) (2% 1M Tris pH7.4, 15% 1M NaCl, 1% 0.5M EDTA, 1% 0.5M egtazic acid (EGTA), H_2O) for removal of metal ions and other unwanted media constituents. Cells were lysed using TBS-EDTA-SDS lysis buffer (0.8% 1M Tris pH7.4, 6% 1M NaCl, 1% 0.5M EDTA, 1% 0.5M EGTA, 2% SDS (20%) H_2O), containing complete protease inhibitor (Sigma) at a ratio of 1:25. DNA was then aggregated and proteins precipitated using 100% TCA (Sigma). Protein pellets were washed once in 1ml 2.5% TCA and dissolved in ~40µl 3M Tris base (Severn Biotech Ltd.) which was diluted to 1.5M Tris base with distilled H_2O after solubilisation. All protein samples were stored at -20^{o}C.

2.3.2. Protein quantification

Protein content was quantified using the colorimetric DC Protein Assay kit (Biorad). BSA protein standards dissolved in lysis buffer at concentrations ranging from 10mg – 0.5mg/ml were used to construct a standard curve. 5µl of protein standards and samples were added to a 96-well plate with kit reagents: alkaline copper tartrate solution and dilute Folin. The plate was incubated for 15 minutes which is the time taken for maximal colour change reaction (proteins react with the copper and Folin is reduced by the copper treated proteins). The plate was then read at a wavelength of 630nm (Labsystems Multiscan Ascent). Protein concentrations were calculated from interpolation of the standard curve using GraphPad Prism v7.02.

2.3.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE). To denature, equal amounts of protein were added to Lammeli buffer (4% SDS, 20% Glycerol, 0.004% Bromophenol Blue, 10% 2-mercaptoethanol, 0.125M Tris HCl) at a ratio of 1:1 and boiled at 95^{o}C for 5 minutes. Proteins were then loaded into lanes of a 1.5mm thick 12% or 10% polyacrylamide gel (Table 2-1) in running buffer (0.025M Tris-Base, 0.19M glycine and 0.1% SDS). The electric current was then applied at 100 V as proteins travel through the stacking gel and 140 V through the resolving gel. Molecular weight of the protein was determined by addition of 5µl Blue Wide Range Protein ladder 10 – 245 KDa (Geneflow).

Table 2-1: Resolving and Stacking Gel Ingredients

	Resolving Gel (µl) 12%	Resolving Gel (µl) 10%	Stacking Gel (µl)
H_2O	6400	7150	3000
40% 29:1 Acrylamide	4500	3750	620
1.5M Tris, pH 8.8	3800		
0.6M Tris, pH 0.8			1260
20% SDS	75		25
20% Ammonium Persulphate	150		50
T-Med	6		5

2.3.4. Semi-dry transfer

The resolving gel was then separated from the stacking gel and placed on top of nitrocellulose paper membrane (GE Healthcare) with filter paper on either side, soaked in transfer buffer (0.047M Tris-Base, 0.038 Glycine, 0.04% SDS, 10% Methanol, H_2O). The gel sandwich was then transferred to the semi-dry transfer blotter (Trans-Blot SD transfer cell, BioRad) and rolled flat to prevent bubbles on the membrane. Electric current was passed through the blotter at 15 V for 45 minutes.

2.3.5. Chemiluminescence

After transfer, the nitrocellulose membrane was washed in H_2O and transfer of protein was determined by Poncheau S staining. Poncheau S (Sigma) was made up 0.1% weight:volume in 5% Acetic Acid (Fisher Bioreagents). Membranes were incubated in Poncheau S for 2 minutes then staining was removed by washing in TBS (0.1M Tris-HCl, 0.16M NaCl, H_2O) with agitation. The membrane was then blocked for 1 hour in blocking buffer (TBS + 5% skimmed milk powder). Membranes were incubated overnight at 4°C or for 1 hour at room temperature in TBS-Tween (Fisher Bioreagents) (TBS + 0.05% Tween-20) containing 5% skim milk powder (Sigma) + 1:1000 dilution of the primary antibody. The following antibodies were used:

Table 2-2: Primary Antibody List

Antibody	**Clone**	**Company**	**Concentration**
Murine Mcl-1	Anti-rabbit	Rockland	1:1000
Human Mcl-1	Anti-rabbit	Santa Cruz	1:1000

Murine iNOS	Anti-mouse	BD Pharmingen	1:1000
Murine Arginase-1	Anti-mouse	BD Pharmingen	1:1000
Poly-clonal pSTAT1 (Tyr701)	Anti-rabbit	Thermofisher	1:1000
Poly-clonal pSTAT6 (Tyr641)	Anti-rabbit	Cell Signaling (CST)	1:1000
Poly-clonal pSTAT3 (Tyr705)	Anti-rabbit	Cell Signaling (CST)	1:1000
Poly-clonal SOCS3	Anti-rabbit	Cell Signaling (CST)	1:1000
Poly-clonal α-tubulin	Anti-mouse	Sigma	1:2000

Following primary incubation, membranes were washed 3 x 10 minutes in TBS-Tween then incubated with Horse-Radish-Peroxidase (HRP) conjugated polyclonal Goat anti-Mouse or anti-Rabbit secondary antibody (Dako) for 1 hour at room temperature. Membranes were washed 3 x 10 minutes in TBS-Tween and incubated in Bio-Rad Clarity™ ECL substrate for 5 minutes at room temperature. Membranes were then sealed with film and analysed using ChemiDoc™ XRS+ System (BioRad). Membranes were then stripped with 0.2M NaOH and H_2O and reprobed with α-tubulin antibody as a loading control.

2.3.6. Densitometry

Chemiluminescent images generated using the ChemiDoc™ system were merged with colorimetric image or the ladder and converted to TIFF files. The optical density of the protein band was then analysed using Fiji biological image processing software (Schindelin et al., 2012). The optical density of each antibody band was compared to that of the loading control – α-tubulin to correct for differential protein loading. When comparing infection conditions, fold change from the control condition (mock infected) was calculated.

2.4. Measurement of gene expression by qPCR

2.4.1. Sample preparation

Macrophages were activated with cytokines or left unstimulated for 24 hours. RNA was isolated based on a protocol by Chomczynski and colleagues (Chomczynski and Sacchi, 1987). The monolayer culture was washed once in sterile PBS before addition of 200µl of TRI reagent (guanidine thiocyanate and phenol solution) (Sigma) per $2x10^5$ cells. Lysates were homogenised by gentle pipetting, then transferred to 1.5ml eppendorfs and stored at -80^oC.

2.4.2. RNA isolation

For phase separation, samples were thawed on ice and 200µl of chloroform (BHR Pharmaceuticals Ltd.) per 1ml of TRI reagent was added to each sample. Samples were then incubated at room temperature for 15 minutes followed by centrifugation for 15 minutes at

12,000xg, 4°C. The aqueous phase was removed from the sample and added to a fresh tube. Samples were incubated at room temperature for 10 minutes with 500µl of 2-Propanol (Fisher Bioreagents) per 1ml of TRI reagent, followed by centrifugation at 12,000xg, 4°C, for a duration of 10 minutes. The supernatants were discarded and 1ml of 75% ethanol (Fisher Bioreagents) per 1ml of TRI reagent was added to each sample before further centrifugation at 7,500xg, 4°C for 5 minutes. Supernatants were then discarded and pellets air-dried before resuspension in 35µl of sterile water. RNA samples were stored at -80°C.

2.4.3. Genomic DNA removal

DNA-*free*™ kit (Ambion, Life Technologies) was used per the manufacturer's instructions for digestion of contaminating DNA and removal of DNase and divalent cations. All samples were incubated with DNase digestion reagents (3.5µl DNase I Buffer + 1µl rDNase I) for 30 minutes at 37°C. For the removal of divalent cations, which can catalyse RNA degradation, samples were incubated with 3.5µl DNase Inactivation reagent for 2 minutes at room temperature. Samples were then centrifuged at 10,000xg for 1.5 minutes to pellet the DNA and RNA was transferred to a fresh tube.

2.4.4. Quantification of total RNA

Total RNA content was quantified by nanodrop spectrophotometry model no. ND-1000 (LABtech international) using ND1000 v.3.2.1. software.

2.4.5. cDNA generation

cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 2x Master Mix (10x RT buffer, 25x dNTP Mix, 10x RT Random Primers, Multiscribe™ Reverse transcriptase, RNase Inhibitor and Nuclease free H_2O) was prepared as per the manufacturer's instructions. 10µl of RNA sample was added to an equal volume of 2x Master Mix in 0.5ml Eppendorf tubes and mixed by gentle pipetting. Tubes were then sealed, centrifuged and placed in the thermal cycler (MJ research PTC-200) programmed to the following conditions:

Table 2-3: Thermal cycler settings for cDNA generation

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	Hold

2.4.6. Real time Quantitative PCR (qPCR)

Real-time PCR was performed using specific primers for iNOS, Arginase-1 and β-actin and Taqman Gene expression probes (Invitrogen) (see below). Per well: 600mM of forward / reverse primer and 350mM of probe, 5µl PrecisionPLUS master mix and 3µl H_2O were loaded to a PCR plate (Bio-Rad) along with 400ng cDNA. Plates were centrifuged at 1500rpm for 10 minutes before running on a Bio-Rad i-Cycler PCR machine (CFX384 Real Time System), conditions (Table 2-4) Steps 2-4 were repeated x40. All assays were performed in triplicate and are normalised to levels of β-Actin housekeeping gene. Fold changes were calculated compared to the unstimulated control using the δδCt method.

iNOS: Forward:5'AAGAGGCAAAAATAGAGGA, Reverse:5'TGGTAGGTTCCTGTTGTTTC, Probe:CTGTCAGTGTGAGCATC

Arginase-1: Forward:5'CGGAGACCACCACAGTTTGGC, Reverse:5'TGGTTGTCAGTGGAGTGTTG, Probe:TCCTTTGTTACAGCTTC

β-actin: Forward: 5'-GGATGCAGAAGGAGATCACTG, Reverse: 5'-CGATCCACACGGAGTACTTG, Probe 5'-CCCTGGCACCCAGCACAATG

Table 2-4: Temperatures for qPCR reaction

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	95°C	95°C	60°C	60°C
Time	2 min	15 seconds	1 min	5s

2.5. Measurement of cytokine content

2.5.1. ELISA

Supernatants were collected from unstimulated controls and cells stimulated with cytokines for 24 hours or 48 hours or as otherwise stated. Supernatants were also collected from mock infected cells and cells infected with *S. pneumoniae* serotype 1 or NTHi for 4 – 24 hours or as otherwise stated. Ready-Set-Go!® IL-6 and TNFα ELISA kits were purchased from eBioscience. MIP-2 ELISA kit was purchased from R and D Systems. Assays were performed per the manufacturer's instructions. High binding plates were coated in 100µl capture antibody (pre-titrated purified antibody) diluted in coating buffer and incubated at 4°C overnight. Plates were then washed in sterile PBS containing 0.05% Tween and blocked for 1 hour in 200µl 1x Assay

Diluent (PBS with HIFBS) (eBioscience) at room temperature. Standards and samples were defrosted and diluted in 1x Assay Diluent and added to the plate in duplicate in a volume of 100µl/well. Top standards (Recombinant cytokine or chemokine) were diluted in 1x Assay Diluent and 1:2 serial dilutions conducted for construction of the standard curve. Plates incubated at 4^{o}C overnight then washed and detection antibody added for 1 hour at room temperature. Plates were washed again and Avidin-HRP added for 30 minutes at room temperature. Plates were washed and substrate solution 1xTMB added for up to 15 minutes at room temperature. Reaction was then stopped with 2M Sulphuric Acid and plate read at 450nm (Thermo Multiscan Ex). Data was analysed using GraphPad Prism software. Unknowns were interpolated from the standard curve using a non-linear regression sigmoidal dose-response equation.

2.5.2. MesoScale Discovery Analysis

All MesoScale Discovery (MSD) analysis was conducted at Glaxo Smith Kline (GSK), Bioscience Hub, Stevenage. Supernatants were collected from unstimulated controls and cells stimulated with cytokines for 24 hours. Reagents were prepared as per the manufacturers guidelines and samples diluted 5 or 10-fold with diluent provided. 50µl of each sample was added to the MSD plate, sealed and incubated for 2 hours room temperature with agitation. Plate was washed 3x in 150µl of wash buffer and 25µl of 1X detection antibody solution was added to each well. Incubation was resumed for a further 2 hours at room temperature with agitation. Plates were then washed 3x in wash buffer and 150µl 2x Read Buffer T added to each well. The plate was then immediately read on an MSD instrument (Model 1201, Meso Sector S 600). Lyophilised calibrator for construction of the standard curve was reconstituted in 1000µl of Diluent 41 and 7 x 4-fold serial dilutions were performed. Diluent 41 was used as the zero calibrator. For analysis, the MSD WORKBENCH® analysis software was used. Standard curves were constructed from the calibrators using a sigmoidal dose-response curve. Sample concentrations were then back-fitted from the standard curve.

Table 2-5: MSD Sensitivity Ranges:

Pro-inflammatory Panel 1 (mouse)	**Range (pg/ml)**	**Pro-inflammatory Panel 1 (human)**	**Range (pg/ml)**	**Chemokine Panel 1 (human)**	**Range (pg/ml)**
IFN-γ	0.40 – 570	IFN-γ	7.47 - 938	Eotaxin	0.36-1500
IL-1β	0.72 – 1030	IL-1β	2.14 – 375	MIP-1β	0.24-1010

IL-2	1.03 - 1570	IL-2	0.89 – 938	Eotaxin-3	1.21-4970
IL-4	2.58 - 1060	IL-4	0.45 – 158	TARC	0.36-1500
IL-5	1.60 – 590	IL-5	1.58 – 488	IP-10	0.58-2400
IL-6	7.61 – 3140	IL-6	1.13 – 375	MIP-1α	0.23-942
KC/GRO	3.29 - 1230	KC/GRO	0.68 – 233	IL-8	18.5-75900
IL-10	19.8 - 2030	IL-10	1.22 – 315	MCP-1	0.10-448
IL-12p70	179 – 20600	IL-12p70	4.21 – 353	MDC	2.59-10600
TNF-α	0.97 - 403	TNF-α	0.69 - 248	MCP-4	0.16-602

2.6. Macrophage functional assays

2.6.1. Macrophage phagocytosis assay

Latex beads (2µm) carboxylate-modified polystyrene, fluorescent yellow-green (Excitation: ~470 nm; Emission: ~505 nm) were purchased from Sigma. Beads were diluted in sterile PBS (1:1000) and centrifuged at 9000xg for 3 minutes. Supernatant was discarded and beads were resuspended in RPMI containing 10% immune serum and then incubated at 37°C for 30 minutes to opsonise beads (see section 2.2.1.2). Beads were then centrifuged at 3400xg for 3 minutes, supernatant discarded and beads resuspended in PBS to wash. A 100µl solution of beads, diluted to ensure an MOI of 10, was added to macrophages previously activated for 24 hours with cytokines. Macrophages were left on ice for 1 hour then incubated for 3 hours at 37°C. Cells were then washed 3x with 1ml PBS and fixed with 2% paraformaldehyde. Coverslips were mounted on slides with VECTASHEILD™ containing 4',6-diamidino-2-phenylindole (DAPI) and sealed.

2.6.2. Gentamicin protection assay

2.6.2.1. Internalisation of bacteria

To determine the number of intracellular bacteria, macrophages were infected with *S. pneumoniae* or NTHi until the desired time point. At the time point, Miles Misra dilutions were performed on the supernatant of each well to assess the number of bacteria in the extracellular medium. To eliminate extracellular bacteria, cells were washed 3 times in sterile PBS ahead of a 30 minute incubation at 37°C with 1ml medium containing antibiotics (20µg/ml Gentamicin (Sigma) and 40uM Penicillin (Sigma)). Wells were washed 3 times with 2ml sterile PBS, on the final wash supernatants were collected and 5-fold Miles-Misra dilutions plated out to determine complete removal of extracellular bacteria. To lyse, cells were incubated at 37°C for 15 minutes with 250µl of 0.02% Saponin (Sigma) dissolved in H_2O. 750µl of PBS was then added to wells to make the total volume up to 1ml. Cells were scraped and vigorously pipetted to ensure lysis.

Supernatant samples were taken from each well and 5-fold Miles-Misra dilutions plated. Colonies of *S. pneumoniae* or NTHi were counted the following day and cfu/ml calculated.

2.6.2.2. Killing assay

If intracellular killing of bacteria between time points was to be determined, infected wells were maintained in low dose antibiotics, after the initial pulse of antibiotic treatment to prevent regrowth of extracellular bacteria. 0.75µg/ml vancomycin (*S. pneumoniae*) or 2µg/ml gentamicin (NTHi) was added until the desired time point. At each time point, cells were washed 3 times in 2ml sterile PBS and lysed as described above. 5-fold Miles Misra dilutions were plated and grown on blood agar overnight. Colonies were counted the next day and cfu/ml calculated.

2.6.3. Detection of Nitric Oxide

Diaminofluoresceins are cell permeable due to their diacetate (DA) group and non-fluorescent. They produce fluorescent triazole derivatives (Benzotriazoles) upon interaction with NO (Kojima and Ikeda, 1998). This reaction is NO specific and does not occur in the presence of other reactive nitrogen species (RNS) or reactive oxygen species (ROS). 4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM), originally described by Kojima and colleagues, is an improvement on a previous Diaminofluorescein used for the live detection of NO, DAF-2, which could only be applied in a limited pH range (Itoh et al., 2000).

MDMs and BMDMs were infected with *S. pneumoniae* at an MOI of 10. Cells washed at 4 hours post infection in PBS, media was replaced and incubation resumed until desired time point. At time point, cells were washed 3 times in PBS and media replaced with fresh media containing 5µM of DAF-FM DA (Sigma) (Excitation: ~495 nm; Emission: ~515 nm) in phenol-red free RPMI, cells were incubated at 37^{o}C / 5% CO_2 for 30 minutes. Macrophages were then washed 3 times in sterile PBS to remove excess dye and then incubated for a further 30 minutes in fresh phenol-red free medium without dye. Macrophages were again washed 3 times in PBS and scraped in 300µl of PBS before analysis on the FACs Calibur flow cytometer (BD Biosciences) using the FL-1 channel.

2.6.4. Detection of cellular ROS

As with DAF-FM, Dichlorofluorescin diacetate (DCF-DA) is a compound capable of passive diffusion across the cell membrane. Upon entry to the cell, DCF-DA is hydrolysed by esterases to a non-fluorescent compound and upon oxidisation by certain species of ROS it becomes highly fluorescent. DCF-DA was originally described as a method of measuring H_2O_2 (Brandt & Keston, 1965) however it has since been described as successfully detecting a variety of ROS and RNS.

The complex interactions between ROS and RNS mean there is a variety of reactions which can lead to the oxidation of DCF-DA therefore it is difficult to determine the exact species which are being quantified in each experiment. Furthermore, there are conflicting reports on the ability of some species to generate a signal via DCF meaning this dye may not be a reliable method of quantifying ROS in general (Myhre et al., 2003). To this end, it is important to also target specific ROS species to gain a more accurate understanding of their contribution to the oxidative state of the cell.

2',7'- (DCF-DA) (Excitation: ~504 nm; Emission: ~524 nm) was purchased from Sigma. Culture media was replaced with fresh media (RPMI / DMEM + HIFBS) containing 20µM of DCF-DA, cells were incubated at 37°C / 5% CO_2 for 45 minutes. MDMs and BMDMs were then washed 3x PBS and infected with *S. pneumoniae* or NTHi. At desired time point, cells were washed 3 times in PBS and scraped in 300µl of PBS before analysis on the FACs Calibur flow cytometer (BD Biosciences), channel: FL-1.

2.6.5. Measurement of mitochondrial membrane potential

Impaired mitochondrial function and loss of mitochondrial transmembrane potential is one of the early indicators of apoptosis induction (Zamzami et al., 1995). The fluorescent cyanine dye JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolocarbocyanine iodide) enters the mitochondria and forms either monomers or at higher mitochondrial membrane potentials, J-aggregates. The formation of J-aggregates causes a bathochromic shift (a shift in the absorbance maxima to a higher wavelength) from an emission wavelength of 520nm for green fluorescing monomers to 585nm emission of red fluorescence (Smiley et al., 1991). To identify apoptosis in macrophages, the loss of red fluorescence and/or the gain of green fluorescence can be quantified.

One vial of JC-1 (Excitation: ~520 nm; Emission: ~596 nm) lyophilized powder (Sigma) was dissolved in DSMO for a final concentration of 10mM. JC-1 solution was stored in working aliquots at -20°C. JC-1 stock solution was diluted 1:1000, in media without serum, to give a concentration of 10µM. 300µl of staining solution was added to adherent cells seeded at $2x10^5$/ml. Macrophages were incubated with JC-1 for 30 minutes at 37°C / 5% CO_2. They were then washed 3x and scraped in 300µl of sterile PBS. Healthy samples from mock infected controls were first analysed on the FACs Calibur flow cytometer (FL2 Channel). Voltages were selected which ensured the fluorescent spectra were towards the right of the histogram display so shifts to the left indicating loss of fluorescence could be observed when *S. pneumoniae* exposed samples were analysed.

2.7. Measurement of Cell Death

2.7.1. DAPI Staining

Macrophages seeded on coverslips were challenged with *S. pneumoniae* following pneumococcal infection. At 4 hours, all cells were washed 3 times with 1ml sterile PBS. At the desired time point, macrophages were washed again 3 times with 1ml sterile PBS and fixed in 500µl 2% paraformaldehyde and stored at 4^{o}C. Upon staining, macrophages were washed 3 times in 1ml sterile PBS and coverslips were carefully removed and mounted on slides in DAPI containing mounting medium (Vectorshield®) purchased from Vector Laboratories. Coverslips were sealed using nail varnish and cell density and percentage of apoptotic cells was quantified by fluorescent microscopy.

2.7.2. NucView Staining

'Effector' caspases 3 and 7 recognise and cleave substrates containing the DEVD amino acid sequences to mediate the execution phase of apoptosis. The mitochondrial events which occur during apoptosis are regulated by caspases 3 and 7 and MOMP, a cellular event associated with apoptosis, is prevented in caspase 3 and 7 double knock-out transgenic mice (Lakhani et al., 2006). NucView™ comprises a DEVD motif containing substrate conjugated to a DNA binding fluorogenic dye. This construct can pass through the cell membrane and locate in the cytoplasm in live cells. Upon caspase 3/7 activity, the DEVD motif is recognised and the substrate cleaved which releases the fluorogenic dye allowing binding to DNA, resulting in nuclear fluorescence in apoptotic cells (Biotium).

NucView™ 530 (Excitation: ~528 nm; Emission: ~563 nm) Caspase-3 Substrate, 1mM in DMSO was purchased from Biotium. Macrophages were challenged with *S. pneumoniae* serotype 1 until desired time point. Media was removed and replaced with NucView 530 substrate in infection media at a concentration of 1µM. 200µl of solution was added to each well and incubated at 37^{o}C for 30 minutes. Macrophages were then washed twice in sterile PBS, fixed in 2% paraformaldehyde and stored at 4^{o}C. Macrophages were incubated for 4 hours with 250ng/ml of Staurosporine (Sigma) per 1ml of cells (2x10^{5}) as a positive control. Cells were mounted in Vectorsheild™ mounting medium containing DAPI, used as a counterstain. Slides were then analysed by fluorescent microscopy.

2.8. Transcriptomics Study

2.8.1. Mice

Generation of the CD68 Mcl-1 transgenic mouse was carried out by previous group member Julie Preston in collaboration with Steven Shapiro, University of Pittsburgh, USA. The 1.5 kb cDNA sequence for human Mcl-1 was cloned into a 2.9 kb plasmid containing the CD68 promoter, a gene which encodes a glycoprotein specific to monocytes and macrophages, with the first intron being IVS-1 which acts as a macrophage specific enhancer (Gough et al., 2001). After restriction enzyme digestion and gel purification, the transgene was microinjected into C57Bl/6J oocytes (Washington University School of Medicine, St Louis, USA). Founder and progeny genotype was identified by tail or ear biopsy and subsequent PCR amplification using the forward: 5'-ACCATCTCCTCTCTGCCAAA-3' and reverse: 5'-GGGCTTCCATCTCCTCAA-3' primers. The CD68 Mcl-1 transgene was found to be present only in cells of the macrophage lineage and absent from neutrophils and lymphocytes. Transgenic mice were not associated with any developmental phenotype nor did the transgene effect fertility, survival, leukocyte subset cell count or show any abnormalities in the lungs or lymphoidal tissue after histological analysis, (Preston et al, unpublished data), (Bewley et al., 2017).

2.8.2. Ethics

All animal experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986, authorized under a UK Home Office License 40/3726 with approval of the Sheffield Ethical Review Committee, Sheffield, United Kingdom.

2.8.3. *In vivo* infection and tissue collection

CD68 – human Mcl-1 and wild-type littermate mice were anaesthetized with Ketamine and ACP and instilled with 20µl of PBS or 20µl of $1x10^4$ cfu/ml serotype 1 *S. pneumoniae*, via tracheostomy, for mock infected and infected conditions respectively. Mice were culled by overdose with sodium pentobarbitone at 16 hours of infection. Blood was immediately collected into a heparinized syringe by exsanguination via the inferior vena cava and stored on ice. Lungs were then exposed and lavaged with ice cold saline via a cannula inserted into the trachea. Lungs were removed and kept on ice with BAL until processing. Mice were age and sex matched.

2.8.4. Collection of RNA

The volume of BAL collected from each mouse was measured and cells counted using a hemocytometer. 150µl of BAL was used for bacterial counts using the Miles Misra technique. The remainder of the BAL was centrifuged at 1000 x g, 4^{o}C and supernatants stored at -80^{o}C for later analysis of cytokine content. Half of each cell pellet was lysed in TRI Reagent; 1ml TRI

Reagent was used per $5x10^6$ cells and samples stored at -80°C. The residual pellet was re-suspended in 50µl of PBS, 40µl of which was used to conduct cytospins. Samples containing more than 10% neutrophils were excluded from the study.

2.8.5. Analysis of viable bacteria in cell pellets, lungs, blood and BAL

10µl of the remaining cell suspension was pooled and lysed in 2% saponin, incubated at 37°C for 12 minutes. Samples were then diluted in PBS and vigorously pipetted to ensure lysis. The lysate was then diluted for Miles Misra surface viable counting and plated on CBA plates. Lungs were added to 1.5ml screw top micro-centrifuge tubes with 6x3.2mm stainless steel beads and 300µl sterile PBS and homogenized for a duration of 5 minutes using a Bullet Blender (Next Advance). 200µl was then added, samples were vortexed and viable bacteria determined using the Miles Misra technique. Colony forming units contained in both the BAL and the blood were also calculated using the same technique.

2.8.6. Microarray

Cell pellets in TRI Reagent were sent to GSK Stevenage and RNA was isolated, quantified and analysed on Affymetrix GeneChip Mouse Genome 430 2.0 Array. Data was normalised with the Robust Multiarray Averaging (RMA) method and raw p-values calculated with Array server.

2.8.7. Analysis

Data was compared as 4 separate experiments: -

Experiment 1: non-infected transgenic → non-infected wild-type

Experiment 2: *S. pneumoniae* transgenic → *S. pneumoniae* wild-type

Experiment 3: *S. pneumoniae* wild-type → non-infected wild-type

Experiment 4: *S. pneumoniae* transgenic → non-infected transgenic

Data was analysed using Array Studio (Omicsoft, QIAGEN). The 45,100 probes probes with an absolute fold change of >1.5 were considered potentially interesting. False discovery rate (FDR) values were then calculated for these probes and p-values with <5% significance selected. Probe sets were then loaded into NextBio online database (illumina), Metabase and SPIA (Bioconductor) for further pathway analysis.

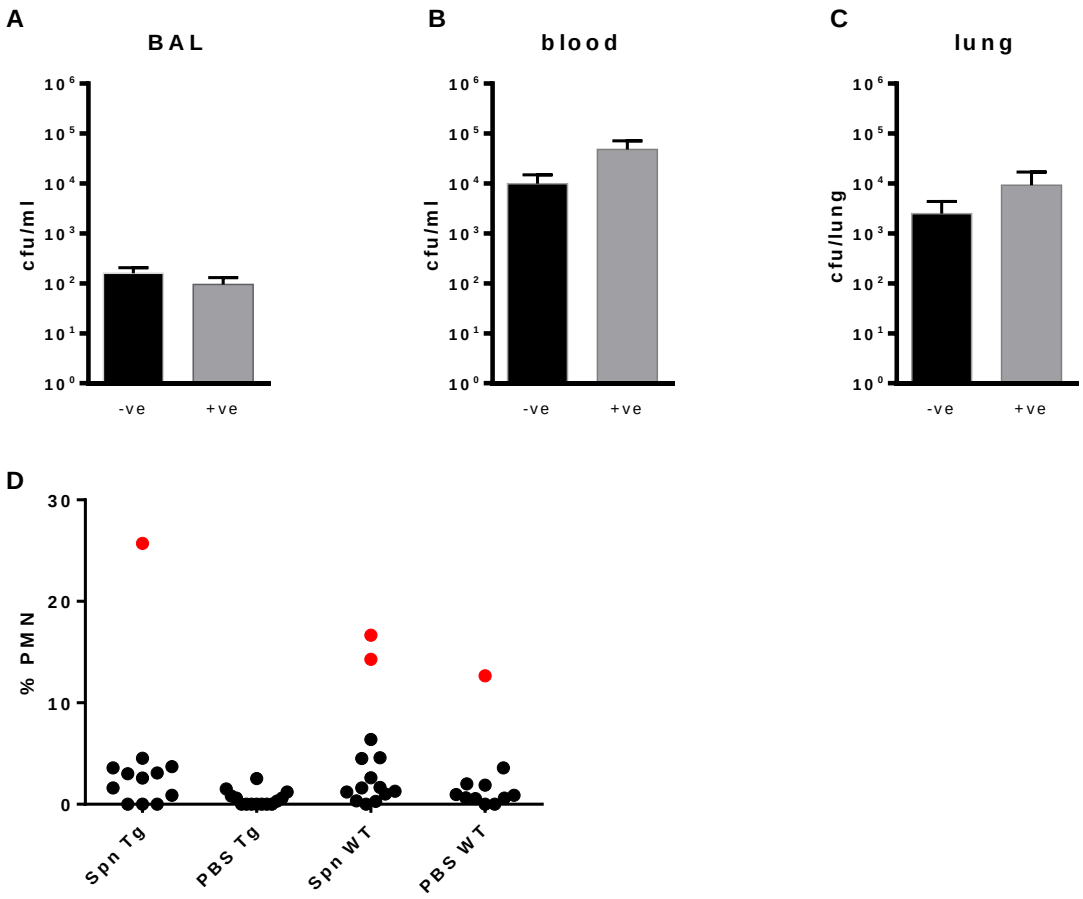


Figure 2-1: Bacterial counts and neutrophil percentages in samples

Wild-type and transgenic mice were challenged with *S. pneumoniae* serotype 1 for a duration of 16 hours**.** At the timepoint, broncho alveolar lavage (BAL) and blood were collected and lungs extracted and homogenised **(A-C)** Bacterial cfu/ml determined my Miles Misra counts in BAL fluid, blood and lungs (n=15), mean ± SEM. **(D)** Percentage neutrophils in BAL were confirmed by cytospin, samples containing more than 10% were discarded (red), transgenic *S. pneumoniae* (n=12), transgenic PBS (n=13), wild-type *S. pneumoniae* (n=14), wild-type PBS (n=11). Missing values are due to insufficient cells on cytospins for accurate differentials. Experiments conducted by Dr Helen Marriott.

2.9. Flow Cytometry

2.9.1. Flow Cytometry

All flow cytometry experiments were completed in the University of Sheffield Medical School Core Flow Cytometry Facility using a 4 laser 13 colour LSRII™ (BD Biosciences) and BD FACSDiva version (?) software. Results were analysed using FlowJo™ software version 10.3 (Tree Star Inc.)

2.9.2. *In vivo* infection

CD68-human Mcl-1 and wild-type littermate mice were anaesthetized with isoflurane and instilled with 50µl of PBS or 50µl of $1x10^4$ cfu/ml serotype 1 *S. pneumoniae*, via intranasal installation. Mice were culled by overdose with sodium pentobarbitone at 16 hours after infection. Blood lung and BAL were collected as in section 2.8.3.

2.9.3. Bronchiole Alveolar Lavage preparation

BAL volumes were measured, and centrifuged at 1500xg for 5 minutes. Supernatants were stored at -80°C for future analysis. The pellets were resuspended in PBS, cell count was obtained before proceeding.

2.9.4. Lung preparation

Lungs were harvested, rinsed in saline and kept at 4°C until use. They were then digested using GentleMACS lung dissociation kit and MACS tissue dissociator (Miltenyi Biotec). Lungs were added to gentleMACs C tubes and dissociated using the provided enzyme mix, using the gentleMACs dissociator program: m_lung_01. Suspensions were then incubated with agitation for 30 minutes at 37°C before further dissociation using program: m_lung_02. Sample was then centrifuged and resuspended and material strained using a 70µm cell strainer and rinsing with provided buffer. Samples were then centrifuged at 300xg for 10 minutes, washed twice and resuspended in PEB buffer (PBS, 0.5% BSA, 2mM EDTA). A cell count was then obtained before samples were stained.

2.9.5. Spleen Preparation

Spleens were isolated from the animal and stored on ice until use. Spleens were placed in gentleMACs C Tubes containing 3ml (1-2 Spleens) of PEB Buffer. Spleens were then dissociated using the gentleMACS Dissociator program: m_spleen_01. Dissociated spleens were then applied to a 30µm cell strainer placed over a 50ml centrifuge tube and washed through with 5ml of PEB Buffer. Cell suspension was then centrifuged at room temperature for 10 minutes at 300g. The supernatant was discarded and pellet re-suspended.

2.9.6. Antibody Staining

BAL and Lung tissue were lysed using Iotest 3 (Beckman Coulter). Samples were then centrifuged at 1500g for 5 minutes and washed twice in FACS Buffer (PBS containing 1% HIFBS). CD16/CD32 Mouse FC Block (BD Pharmingen) was added to all samples except the unstained control which was resuspended in FACs Buffer. Fluorescence Minus One (FMO) controls were set up for successful gating of each fluorophore in each panel. Single stained samples and compensation

beads (invitrogen) were also used to compensate for each fluorophore. All control samples used cells from lung tissue due to higher abundance than BAL fluid. Antibodies for staining samples were diluted to the correct concentration in FACS Buffer and a cocktail containing all antibodies, was added to each BAL and lung experimental sample. FMOs were stained with every antibody except the one of interest and single stained samples were stained with just the antibody of interest. All samples and controls were mixed and incubated for 30 minutes at 4°C protected from light. Samples were then centrifuged at 1500xg and washed 3 times in FACs Buffer then resuspended in 200µl of FACs Buffer. 1 drop of negative and 1 drop of positive compensation beads were added to tubes containing the antibody cocktail, tubes were vortexed and incubated in the dark at 4°C for 10 minutes. Beads were washed and resuspended in 200µl of FACs Buffer. Below are the details of the antibody panels used in this experiment.

Table 2-6: Myeloid Panel

Marker	Fluorochrome	Company Details	Host Species and Isotype
CD45	BV510	BD Biosciences (563891)	Rat IgG2b K
CD11b	FITC	Biolegend (101206)	Rat IgG2b k
CD64	PE-Cy7	Biolegend (139314)	Mouse IgG1, κ
CD24	APC-Cy7	EBioScience (47-0242)	Rat IgG2b κ
MHC class II	PerCp-Cy5.5	(Biolegend) 107626	Rat IgG2b, κ
CD11c	APC	(Biolegend) 117310	AR Hamster IgG
LY6g	BV421 (pacific blue)	(Biolegend) 127628	Rat IgG2a k
Siglec F	PE	(Miltenyi biotech) 130-102-274	Rat IgG1

Table 2-7: Th1 Th17 and NK + B cell Panel

Marker	Fluorochrome	Order Details	Host Species and Isotype
CD45	BV510	BD Biosciences (563891)	Rat IgG2b k
CD4	APC/FIRE™	Biolegend (100460)	Rat IgG2b k
TCRβ	VioBlue	Miltenyi (130-104-815)	Recombinant human IgG
CD49b (DX5)	PE-Cy7	BioLegend (108922)	Rat IgM k
CCR6 (CD196)	ALEXA-647	BD Biosciences (557976)	Rat IgG2a k

CXCR3 (CD183)	PE	BD Biosciences (562152)	Hamster IgG1 k
CD19	PerCPCy 5.5	Biolegend (115534)	Rat IgG2a, κ

2.10. Statistics

All statistical analysis was performed using GraphPad Prism v.7.02 (GraphPad Inc.). All data represented as Mean ± Standard Error of the Mean (SEM) unless otherwise indicated. For comparison between two unmatched groups, an unpaired t-test was used (parametric data) or a Mann-Whitney test (non-parametric data). For comparison of more than two groups a one-way ANOVA (parametric) with Tukey's post- test was used, non-parametric data was analysed using Kruskal-Wallis test with Dunn's post-test. Grouped data (two or more variables) was analysed using two-way ANOVA with Sidak's post-test. For flow cytometry analysis median fluorescence intensity and geometric mean flourescence intensity was genetated using Flowjo software version 10.3. D'Agostino & Pearson normality test was used to decide on use of parametric or non-parametric statistical tests. Group sizes were determined based on previous publications by our group, where possible, *in vitro* data is based on 3 separate experiments from 3 individual donors. *In vivo* data is based on at least 3 donors from 3 independent experiments, unless otherwise stated (Bewley et al., 2017; Marriott et al., 2005). Where n numbers are stated, n refers to the number of individual human or murine donors.

3. Characterisation of macrophage activation in wild-type and CD68 Mcl-1 transgenic macrophages

3.1. Introduction

Macrophage activation is essential for macrophage function in both health and disease. Macrophage activation profile has also been shown to be reversible which allows macrophages to quickly adapt to changes in the microenvironment (Porcheray et al., 2005). This same plasticity can lead to the skewing of macrophage subsets by diseases which manipulate the local milieu (Ruffell et al., 2012; Shaykhiev et al., 2009b). For example, during bacterial infection macrophages typically switch to classical activation to resolve infection however if the increasing pool of CAM is not tightly regulated, CAMs can trigger lasting tissue damage through excessive inflammation (Benoit et al., 2008b).

In response to *S. pneumoniae* infection, macrophages avoid harmful inflammation by undergoing apoptosis as a mechanism of pathogen clearance (Dockrell et al., 2001a). This phenomenon is associated with the mitochondria and mediated by the anti-apoptotic protein Mcl-1 (Marriott et al., 2005) which is tightly linked to mitochondrial activity and integrity (Perciavalle et al., 2012). Reports highlight the importance of metabolism and therefore, by implication, mitochondrial activity in orchestrating macrophage phenotype by switching between either glycolysis (classical activation) or oxidative phosphorylation (alternative activation), reviewed by (Galván-Peña and O'Neill, 2014). However, the relationship between over-expression of Mcl-1 and macrophage activation has not yet been explored. Thus, the main aim of this chapter was to determine if Mcl-1 has any effect on macrophage activation. The CD68 Mcl-1 transgenic mouse which over-expresses human Mcl-1 on a macrophage specific promoter is defective in apoptosis associated killing and therefore pneumococcal clearance, modelling the apoptosis defect seen in COPD (Bewley et al., 2011a; Bewley et al., 2017). I hypothesised that CD68 Mcl-1 BMDMs would have a skewed macrophage activation profile compared to wild-type cells. This hypothesis was based on the importance of Mcl-1 to macrophage mitochondrial function and the observation that COPD AMs show Mcl-1 upregulation with consequences to macrophage immune effector phenotypes.

To fulfil the first aim, wild-type and transgenic BMDMs were analysed for skewed macrophage activation marker expression before and after stimulation with activating cytokines. The secondary aims of this chapter were to firstly delineate the optimum culture conditions for murine macrophage activation in our laboratory conditions, including, peak cell culture day for

activation after seeding, optimum concentration of cytokine stimulants and ideal length of the stimulation period. Secondly, I wanted to ensure that human MDMs could be successfully activated following protocols previously described by others (Cassol et al., 2009; Sudan et al., 2015). Fulfilment of these aims feed directly into chapter 4 of my PhD project.

Initially, the stimulants which I chose to use to induce macrophage activation were IFN-γ + LPS for a CAM phenotype, IL-4 for an 'alternatively activated' (AAM) phenotype and IL-10 to induce an 'immune-regulatory' phenotype (Mosser and Zhang, 2008; Murray et al., 2014). Since classical and alternative activation can be induced using a variety of stimulants, to avoid confusion conditions are hereafter referred to as M(IFN-γ+LPS), M(IL-4), M(IL-10) following the nomenclature suggested by Murray and colleagues (Murray et al., 2014). To examine marker expression, I analysed enzymes involved in Arginine metabolism at both transcription and translation, cytokine and chemokine release and phosphorylation of the STAT family of transcription factors (Mills et al., 2000b; Mosser and Zhang, 2008; Murray et al., 2014).

3.2. Results

3.2.1. Optimisation of cell culture conditions for macrophage activation

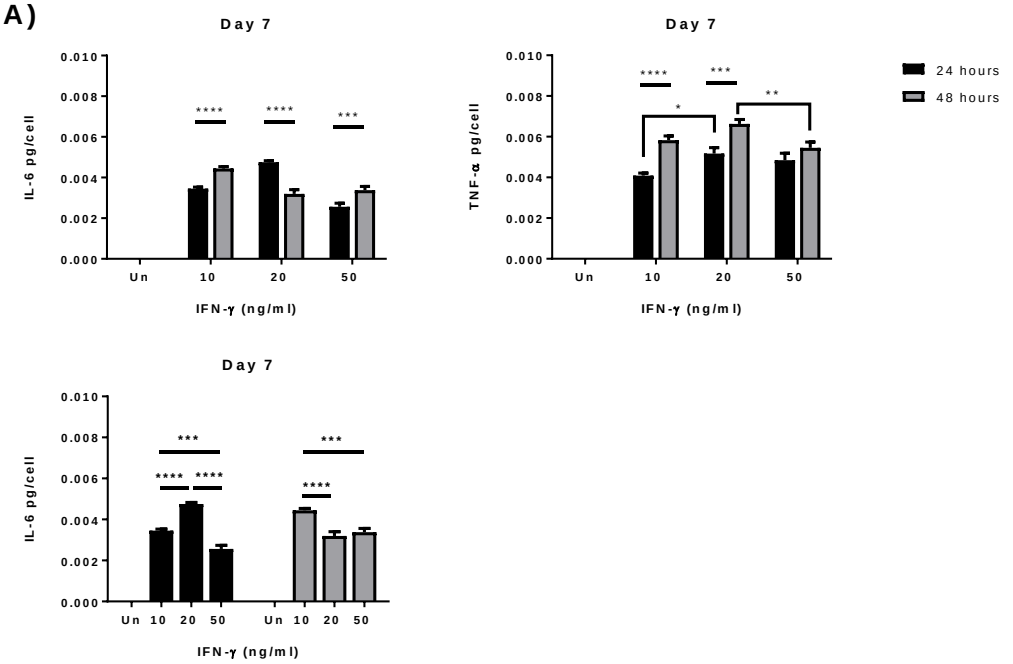
Cytokine concentration for activation of macrophages has been optimised in many different immortal and primary cell lines, including murine BMDMs (Edwards et al., 2006; Genin et al., 2015; Mulder et al., 2014). In the literature, a range of concentrations over a series of incubation periods have been suggested for induction of macrophage effector phenotypes (Mosser and Zhang, 2008). Before continuing, it was important to determine the optimum conditions for BMDM activation in our laboratory conditions. Primary murine BMDMs were stimulated with various concentrations of IFN-γ supplemented with LPS for different incubation periods. For dose optimisation, concentrations of 10, 20 or 50 ng/ml of IFN-γ were tested, all concentrations were within the ranges previously described by others, IFN-γ stimulation was supplemented with 100ng/ml LPS (Davis et al., 2013; Mosser and Zhang, 2008; Stein et al., 1992). TNFα and IL-6 levels in supernatants from IFN-γ + LPS stimulated cells were tested by ELISA at 24 and 48 hours of activation (Figure 3-1). All IFN-γ concentrations with LPS induced TNFα and IL-6 cytokine expression. iNOS protein expression was observed in IFN-γ + LPS stimulated cells at 6, 12, 24 and 48 hours of activation, however 24 hours yielded the highest density of iNOS protein (Figure 3-2). Furthermore, 24 hours of activation is sufficient for study of macrophage effector functions in response to bacterial challenge (Mosser and Zhang, 2008). For activation of M(IL-4) macrophages, a concentration of 20ng/ml was selected as this is commonly used by others (Cho et al., 2014; Jiménez-Garcia et al., 2015). Figure 3-2, shows 20ng/ml IL-4 was enough to stimulate

Arginase-1 expression after 6, 12, 24 and 48 hours of activation however 24 hours was selected as the most practical stimulation time to match the optimised conditions for M(IFN-γ+LPS). The data presented in Figure 3-2 was confirmation that 24 hours was a suitable timepoint for both classical and alternative macrophage stimulation going forward. Macrophages were activated on days 7, 10 and 14 of culture to determine which day would be optimum for macrophage activation. ELISA data showed 14 days of culture was optimum based on cytokine expression (Figure 3-1 A-C). TNFα and IL-6 were used as a measurement of M(IFN-γ+LPS) activation and IL-10 was used as a measurement of M(IL-4) activation (Gordon and Martinez, 2010) (Figure 3-1 D). It is noteworthy that BMDM optimisation data does not include IL-10 as the decision to also include M(IL-10) condition was made later during the project. 20ng/ml IL-10 was used throughout experiments in keeping with reports by others (Liu et al., 2008).

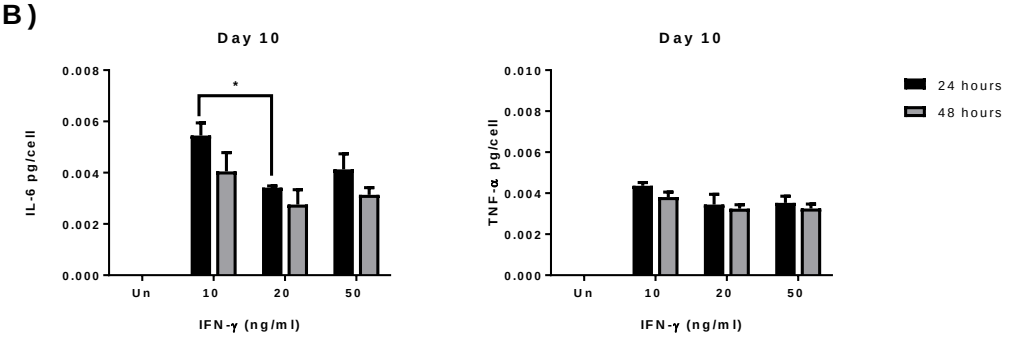
3.2.2. Transcription of macrophage markers of activation is not influenced by the CD68-Mcl-1 transgene

A fundamental method of distinguishing classical and alternative activation is analysis of L-Arginine metabolism (Mills et al., 2000b). CAMs preferentially metabolise Arginine to NO and citrulline by upregulation of the enzyme iNOS. The counterpart of this reaction, used by AAMs, is catalysed by the enzyme Arginase-1 which hydrolyses Arginine to ornithine and polyamines. To understand whether the CD68 Mcl-1 transgene might alter transcription of iNOS or Arginase-1 in activated and unstimulated BMDMs, we conducted qPCR for analysis of mRNA levels. Having previously optimised activation doses (section 3.2.1) macrophages were stimulated for a duration of 12 hours which is considered sufficient to induce gene change (Mosser and Zhang, 2008). Fold change for each gene was calculated and compared to the unstimulated control (Figure 3-3). Results demonstrated that after 12 hours IFN-γ + LPS and IL-4 stimulation successfully increased gene transcription of iNOS and Arginase-1 respectively. There was no influence of the CD68 Mcl-1 transgene on gene expression of iNOS or Arginase-1, nor was there a preference for iNOS or Arginase-1 expression in the CD68 Mcl-1 unstimulated control. This data suggests that the CD68 Mcl-1 transgene has no effect on macrophage transcriptional regulation of Arginine metabolism at 12-hours activation.

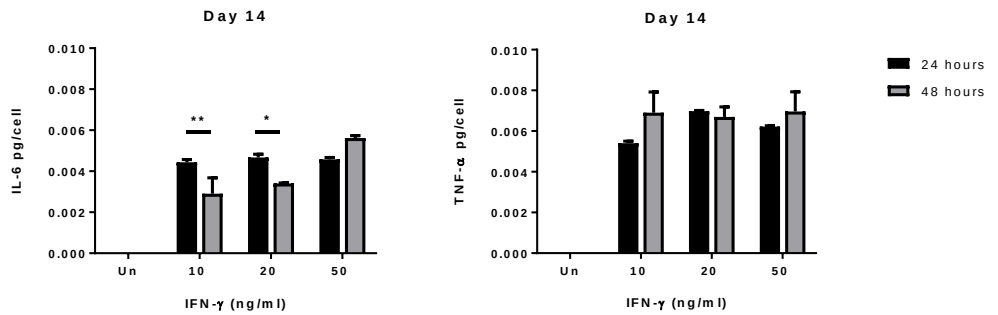
A)


Day 7
IL-6 pg/cell
0.010
0.008
0.006
0.004
0.002
0.000
****
****
***
Un
10
20
50
IFN-γ (ng/ml)
Day 7
TNF-α pg/cell
****
***
**
*
24 hours
48 hours
Day 7
IL-6 pg/cell
***
***
****
****
****
Un 10 20 50 Un 10 20 50
IFN-γ (ng/ml)

B)


Day 10
IL-6 pg/cell
0.008
0.006
0.004
0.002
0.000
*
Un
10
20
50
IFN-γ (ng/ml)
Day 10
TNF-α pg/cell
0.010
0.008
0.006
0.004
0.002
0.000
24 hours
48 hours

C)


Day 14
IL-6 pg/cell
0.010
0.008
0.006
0.004
0.002
0.000
**
*
Un
10
20
50
IFN-γ (ng/ml)
Day 14
TNF-α pg/cell
24 hours
48 hours

D)

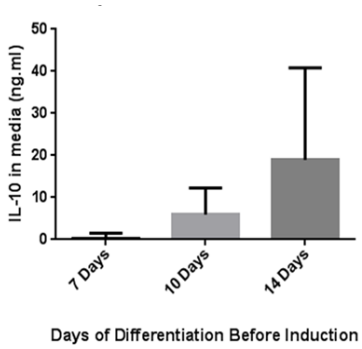

IL-10 in media (ng.ml)
50
40
30
20
10
0
7 Days
10 Days
14 Days
Days of Differentiation Before Induction

Figure 3-1: Optimisation of conditions for cytokine stimulation.

BMDM were stimulated with concentrations of 10, 20 or 50 ng/ml IFN-γ supplemented with 100ng/ml LPS for a duration of 24 or 48 hours on day **(A)** 7, **(B)** 10, **(C)** 14 of cell culture. TNF-α and IL-6 production were quantified by ELISA. Data analysed by two-way ANOVA with Sidak's post-test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. Data is represented as Mean ± SEM (n=4) **(D)** BMDM were stimulated with 20ng/ml IL-4 for 24 hours on day 7, 10 and 14 of culture and IL-10 release was quantified by ELISA. No significance was determined by one-way ANOVA with Tukey's post-test, data represented as mean ±SEM (n=3).

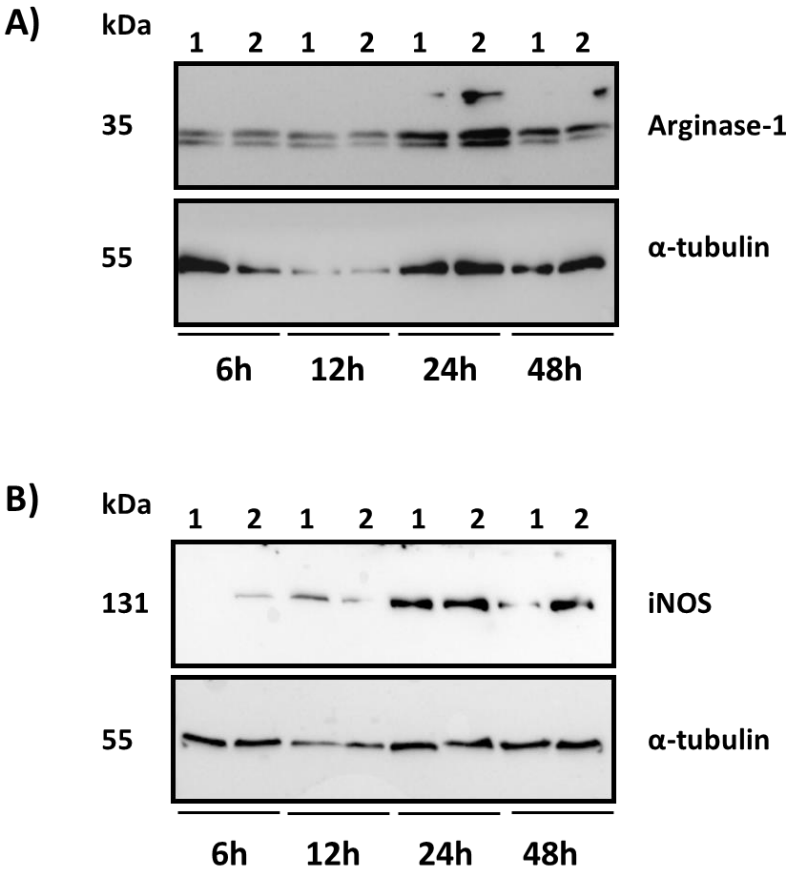


Figure 3-2: Macrophage cytokine stimulation induces protein expression of iNOS and Arginase-1 at 6, 12, 24 and 48 hours of stimulation.

Wild-type BMDMs were stimulated with **(A)** 20ng/ml IFN-γ + 100ng/ml LPS or **(B)** 20ng/ml IL-4 for a duration of 6, 12, 24 or 48 hours. BMDM were then lysed for protein and western blotted. Membranes were probed for **(A)** iNOS (131 kDa) or **(B)** Arginase-1 (35 kDa), α-tubulin (55 kDa) was used as a loading control, lysates from 2 replicate donors, 1 (mouse 1) or 2 (mouse 2) were ran side by side (n=2).

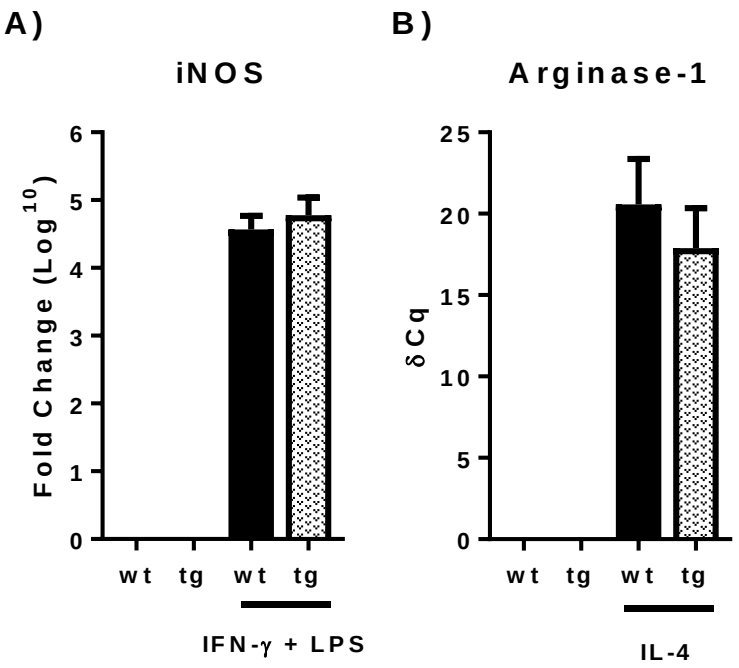


Figure 3 3: Macrophage cytokine stimulation causes increases in iNOS and Arginase-1 mRNA

Wild-type and transgenic BMDMs were unstimulated or activated with **(A)** 20ng/ml IFNγ + 100ng/ml LPS or **(B)** 20ng/ml IL-4 for a duration of 12 hours. At the time point cells were lysed and RNA isolated. qPCR was conducted using primer/probe sets for **(A)** iNOS and **(B)** Arginase-1. β-actin was used as a housekeeping control. iNOS expression is represented as fold change compared to unstimulated macrophages. Arginase-1 levels are represented as δCt due to the presence of Arginase-1 in the unstimulated control being beneath the level of detection, therefore it was not possible to calculate δδCt. No significance between wild-type and transgenic cells was confirmed using a two-tailed unpaired t-test, (n=3) data is represented as Mean ± SEM.

3.2.3. Protein expression Arginase-1 and iNOS is not effected by the CD68 Mcl-1 transgene

Since gene expression data does not always correlate with protein abundance (Vogel and Marcotte, 2012) I was also interested in comparing protein levels of iNOS and Arginase-1 in wild-type and transgenic BMDMs. Cells were incubated with cytokines for a duration of 24 hours to ensure protein induction (Mosser and Zhang, 2008). Protein was western blotted for iNOS or Arginase-1. Both wild-type and transgenic BMDMs showed induction of iNOS or Arginase with either IFN-γ + LPS or IL-4 stimulation respectively (Figure 3-4). These results were in keeping with the qPCR data in that there was no baseline difference in expression of iNOS and Arginase in the

unstimulated control nor a difference in induction of iNOS and Arginase-1 levels after macrophage activation induction. These results indicate that over-expression of human Mcl-1 does not affect protein production of iNOS or Arginase-1 after macrophage stimulation. Further it suggests that without prior stimulation (unstimulated), CD68 Mcl-1 macrophages display no strong preference for either the iNOS or Arginine metabolic pathway.

3.2.4. Pro-inflammatory cytokine release is not altered by the CD68-Mcl-1 Transgene

IFN-γ + LPS stimulation induces a complex network of signalling pathways and transcription factors which orchestrate the release of various effector molecules to mediate the M(IFN-γ+LPS) response. Transcriptional regulation of macrophage activation is vast and complex (Lawrence and Natoli, 2011) and therefore the signalling pathways which orchestrate L-Arginine metabolism are not always concurrent with those which stimulate cytokine release. I decided to now analyse the effect of the CD68 Mcl-1 transgene on pro-inflammatory cytokine release. I chose to measure IL-6, TNFα and MIP-2 which are all markers of M(IFN-γ+LPS) activation (Murray et al., 2014). BMDMs were stimulated over a time course and pro-inflammatory mediator release was quantified by ELISAs (Figure 3-5). Results demonstrated that IFN-γ + LPS treatment generated production of all three pro-inflammatory mediators at all time points tested, as expected (Mosser and Zhang, 2008) however this result was more marked at 24 and 48 hours. Levels of TNFα and IL-6 in other activation conditions remained low in comparison or beneath the limit of detection. In agreement with qPCR and western blot data, the presence of the CD68Mcl-1 transgene showed no effect on pro-inflammatory marker activation at any of the time points tested. These results indicate that the CD68 Mcl-1 transgene does not influence induction pro-inflammatory cytokine production.

3.2.5. Macrophage Activation does not change mMcl-1 protein levels

Data collection thus far indicates over-expression of Mcl-1 has no effect on macrophage activation type. To conclude this finding, I wanted to check stimuli did not affect endogenous levels of Mcl-1. BMDMs were activated for 24 hours and western blotted for murine Mcl-1. Mcl-1 protein density was then quantified using Fiji image analysis software. The ratio of Mcl-1 to α-tubulin was calculated and data plotted as fold change of each activation condition compared to the unstimulated control (Figure 3-6). Murine Mcl-1 has three isoforms, full length Mcl-1 (40KDa), Mcl-1 truncated at Isoleucine-10 (38KDa) and Mcl-1 truncated at leuceine-33 (36KDa). The full-length and the 38KDa forms of Mcl-1 are located on the outer mitochondrial membrane and are involved in the anti-apoptotic function of Mcl-1. The 36KDa form is located in the

mitochondrial matrix and is essential for normal mitochondrial bioenergetic function (Perciavalle et al., 2012). All three forms were detected by western blot in each activation condition. There was no significant effect of macrophage activation on any form of Mcl-1 expression although there was a visible increase in the IL-4 stimulated condition.

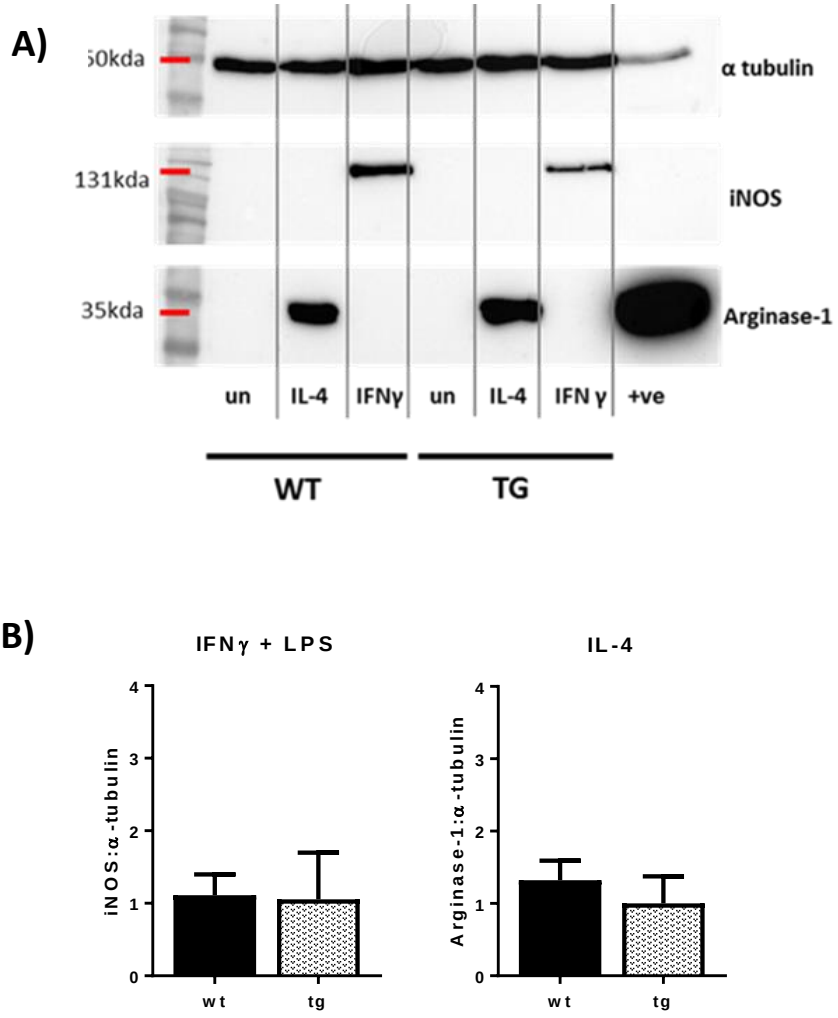


Figure 3-3: Differential macrophage cytokine stimulation causes expression of either iNOS or Arginase-1 protein

(A-B) Wild-type and transgenic BMDMs were stimulated with 20ng/ml IL-4 or 20ng/ml IFNγ + 100ng/ml LPS for a duration of 24 hours. Macrophages were then lysed for protein and western blotted. Membranes were probed for iNOS or Arginase-1, α-tubulin was used as a loading control. **(A)** representative blot pictured **(B)** Density of each antibody was quantified relative to α-tubulin intensity and expression compared between genotypes. Homogenised mouse liver from wild-type mice was used as a positive control for Arginase-1 detection. No significance was determined by unpaired Mann-Whitney test (n=3) data is represented as mean ± SEM.

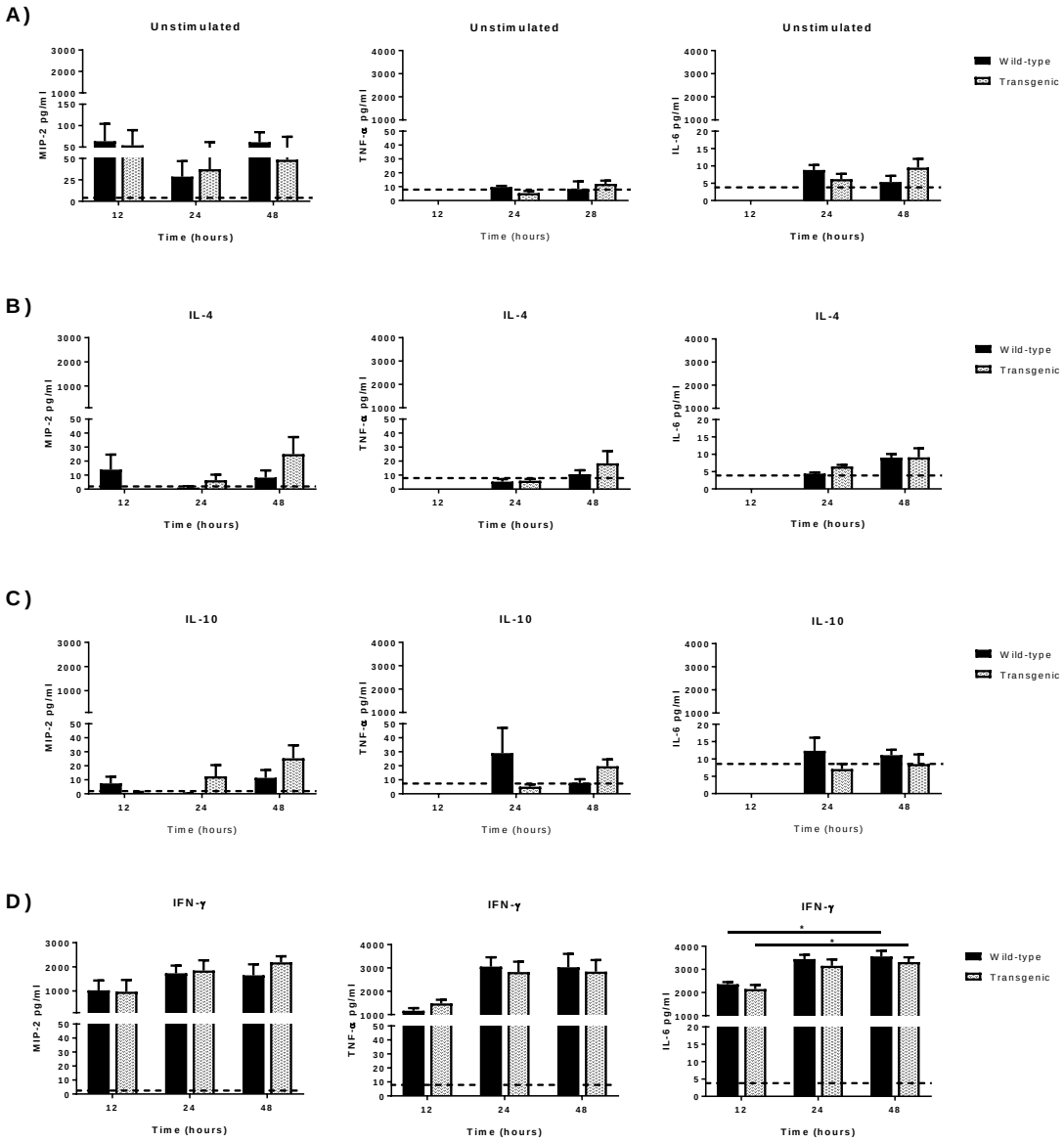


Figure 3-4: IFN-γ + LPS activation stimulates pro-inflammatory cytokine and chemokine release

Wild-type and transgenic BMDMs were left **(A)** unstimulated or stimulated with 20ng/ml **(B)** IL-4, **(C)** IL-10 or **(D)** IFN-γ + 100ng/ml LPS for a duration of 12, 24 or 48 hours. At the time point cell supernatants were collected and analysed by ELISA for MIP-2, TNFα or IL-6. Dotted line is representative of the sensitivity of the ELISA kit, MIP-2 (1.5pg/ml), TNF-α (8pg/ml), IL-6 (4pg/ml). Wild-type and transgenic responses were compared and no significant differences determined using two-way ANOVA with Sidak's multiple comparisons test at each timepoint (n=3) data is represented as mean ± SEM.

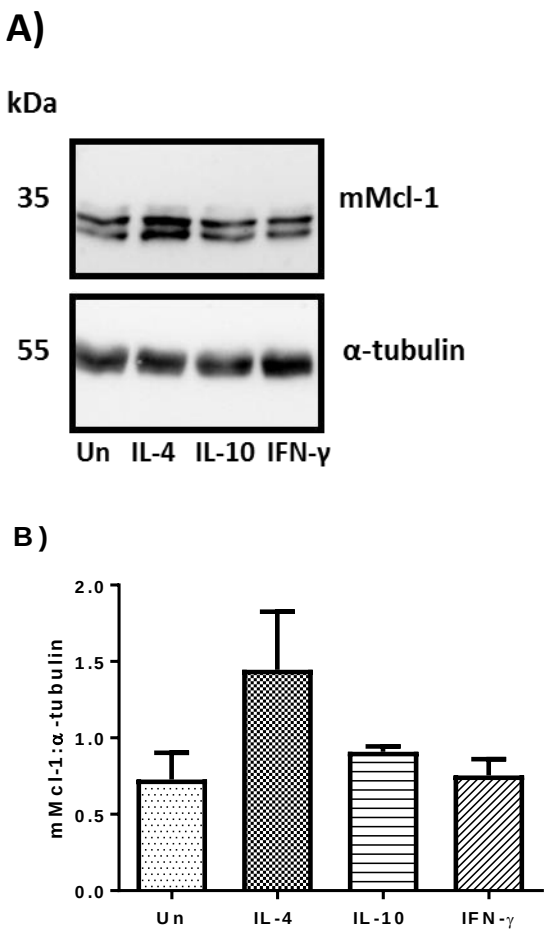


Figure 3-5: Macrophage activation has no effect on protein levels of Mcl-1.

Wild-type BMDMs were unstimulated or stimulated with 20ng/ml IL-4, IL-10 or IFN-γ for a duration of 24 hours. At the time point, macrophages were lysed and protein western blotted **(A)** Representative western blot pictured. Membranes were probed for mMcl-1 (35 kDa) and α-tubulin (55 kDa) was used as a loading control. **B)** Ratio of mMcl-1:α-tubulin was quantified by densitometry, no significance was determined by one-way ANOVA with Dunnett's multiple comparisons test. (n=4) data is represented as mean ± SEM.

3.2.6. Cell viability is affected by treatment with IFN-γ+LPS

IFN-γ + LPS are the standard stimulants used to produce a CAM phenotype (Mantovani et al., 2004), however, I found that stimulation with IFN-γ + LPS was affecting cell density (Figure 3-7) compared to all other conditions. In line with this observation, other studies state IFN-γ + LPS treatment in combination and independently can trigger cell loss (He et al., 2008; Xaus et al., 2000). To test the effect of macrophage activation on the amount of cell metabolic activity, I

decided to carry out an MTT assay which under certain circumstances can also indicate the number of viable cells. 24 hours of LPS treatment with and without IFN-γ caused a significant decrease in metabolic activity compared to the other activation conditions. IFN-γ stimulation alone showed a significant decrease although not as marked as IFN-γ + LPS. At 24 hours, results have the same level of significance for both wild-type and transgenic BMDMs, after 48 hours of stimulation, there was more variation within groups. Metabolic activity of unstimulated cells was decreased with the extended incubation time and IL-4 stimulation showed increased metabolic activity which suggests higher levels of metabolic activity, a survival advantage or proliferative capacity as demonstrated by others (Jenkins et al., 2013). In conclusion, M(IFN-γ+LPS) and M(LPS) macrophages showed reduced metabolic activity with no major differences in genotype.

3.2.7. Re-optimisation of using IFN-γ alone

Due to the effect of LPS on cell number and metabolic activity, I decided to re-optimise the stimulation of CAM to limit the variation in cell density and potential cytotoxicity caused by LPS. The main factor which I thought might be causative of the effects seen in Figure 3-7 was the concentration of stimulant. I therefore tested a range of different concentrations of IFN-γ + LPS on BMDM using the MTT assay. I did not see any difference in metabolic activity between these altered conditions and I concluded the presence of LPS too harsh for BMDMs in our culture conditions (Figure 3-8A). Furthermore, I reasoned after priming with IFN-γ, "triggering" with LPS and then further challenge with bacteria, as a second 'trigger' event (Adams and Hamilton, 1984), BMDMs might undergo apoptosis or some other form of cell death which is simply a consequence of being overwhelmed rather than being in association with macrophage apoptosis associated killing. Based on this I decided it would be beneficial for future work involving infection to prime macrophages with IFN-γ alone before addition of bacteria which will provide a sufficient 'trigger' event to induce the full classical phenotype but not too harsh to cause cell death. To ensure BMDMs could be successfully primed by IFN-γ alone and subsequently triggered with addition of Gram-positive and Gram-negative bacteria I first looked at the presence of iNOS mRNA after IFN-γ activation. Treatment with 20ng/ml IFN-γ for 24 hours successfully induced iNOS mRNA amplification compared to unstimulated cells (Figure 3-8B) in keeping with published reports (Murray et al., 2014). I then tested for pro-inflammatory cytokine release by priming BMDMs with IFN-γ and subsequently infecting with *S. pneumoniae* or NTHi. IFN-γ activation alone was not sufficient to stimulate pro-inflammatory cytokine release however addition of *S. pneumoniae* and NTHi showed enhanced pro-inflammatory cytokine

release with TNFα and an increase in IL-6 with NTHi challenge within 4 hours of infection comparable to the IFN-γ + LPS condition (Figure 3-8C).

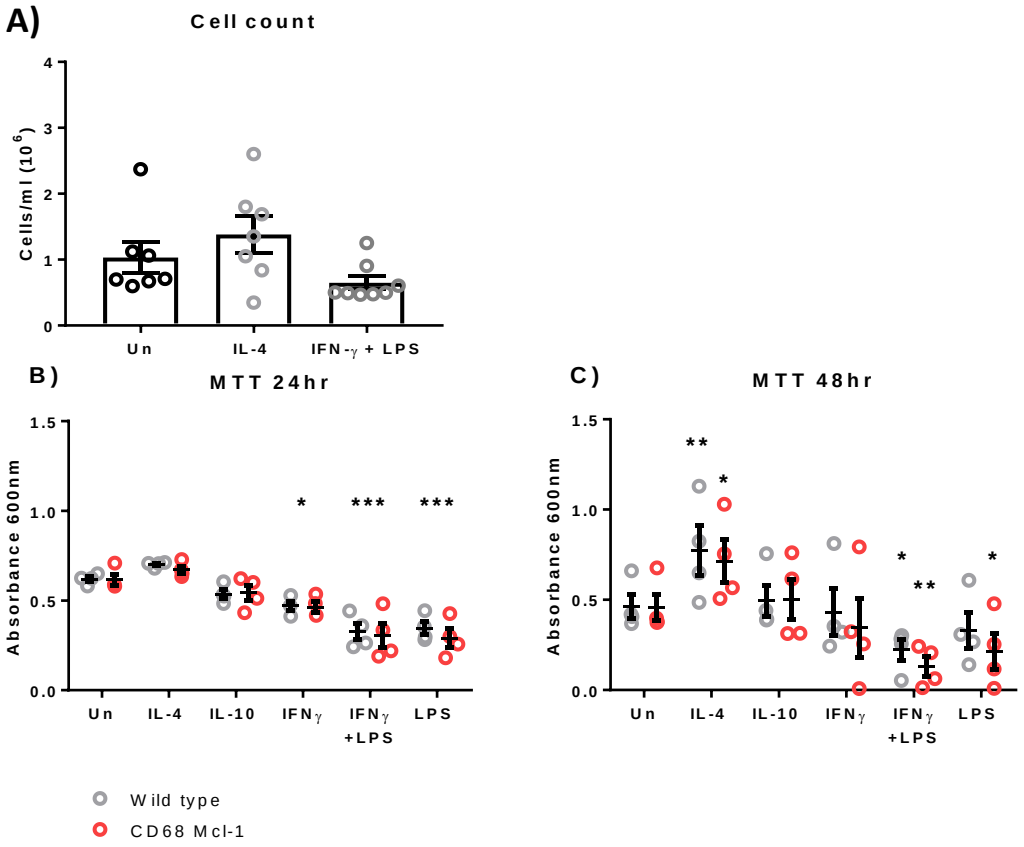


Figure 3-6: Cytokine stimulation affects macrophage viability at 24 and 48 hours.

(A) Wild-type BMDMs were stimulated were left unstimulated or stimulated with 20ng/ml IL-4, IFN-γ + 100ng/ml LPS for 24 hours and cell counts conducted. No significant difference was found between groups by Kruskal-Wallis test with Dunn's post-test, data represented as mean ± SEM (n=7). **(B-C)** Wild-type and transgenic BMDMs were seeded in a 96 well plate at a density of $1x10^5$ cells / well. Cells were stimulated with 20ng/ml IL-4, IL-10 or IFN-γ, 20ng/ml IFN-γ + 100ng/ml LPS, 100ng/ml LPS or left unstimulated for a duration of **(B)** 24 or **(C)** 48 hours. TRIVIGEN MTT assay was conducted at each timepoint. Cellular metabolic activity was determined by reading absorbance of the purple formazan product at a wavelength of 600nm. Activation conditions were compared within each genotype using a two-way ANOVA with Sidak's multiple comparisons test. *p=0.05, **p=0.01, ***p=0.001, n=4. Data is represented as mean ± SEM (n=4).

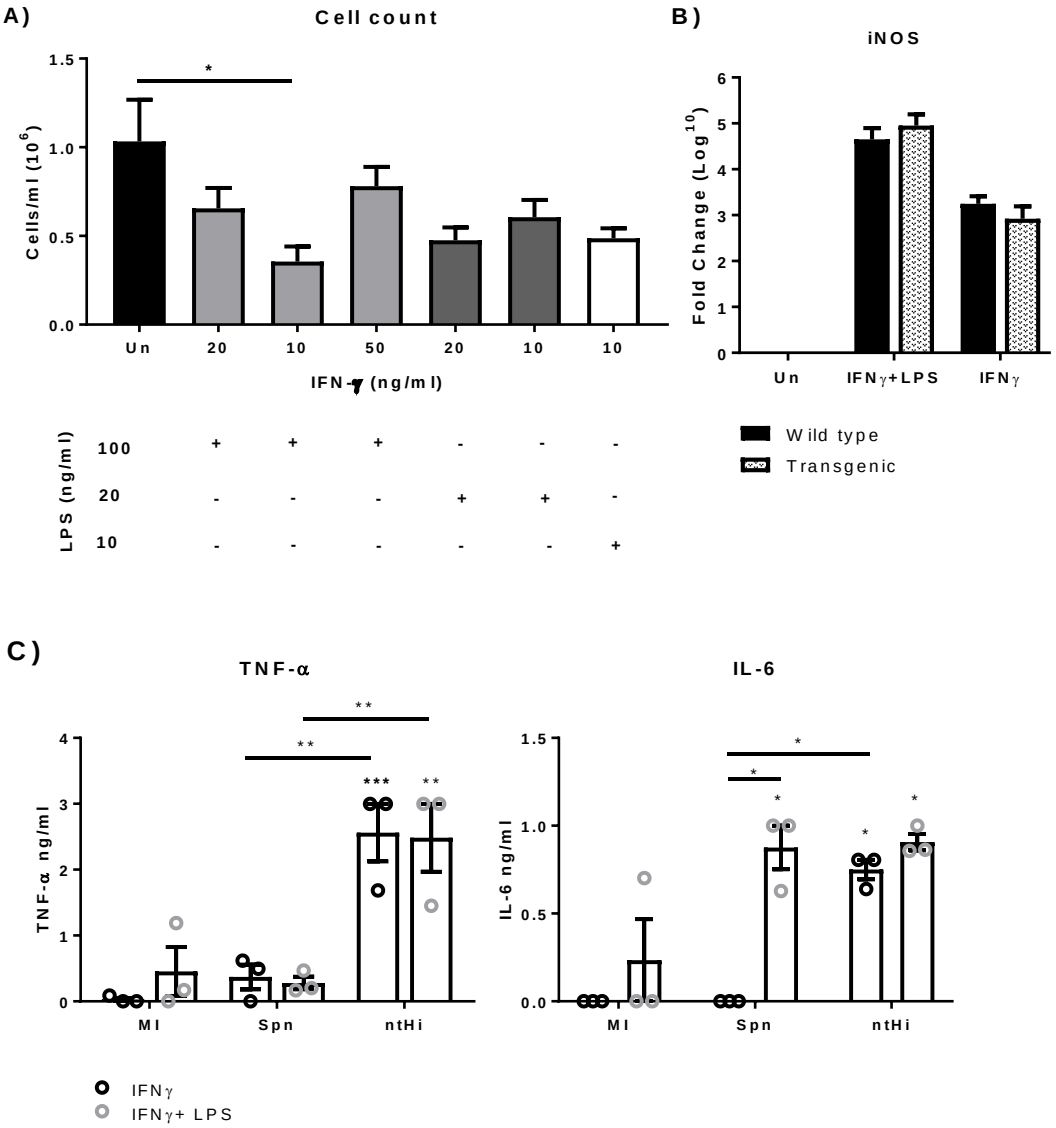


Figure 3-7: IFN-γ stimulation induces a classically activated phenotype

(A) BMDMs were activated with varying concentrations of IFN-γ + LPS for 24 hours and cell counts conducted to assess cell density (n=3-7) significance determined by Kruskal-Wallis test with Dunn's post-test *=p<0.05 **(B)** BMDMs were activated with IFN-γ, IFN-γ + LPS or unstimulated for 12 hours, mRNA levels of iNOS and were detected by qPCR. β-actin was used as a housekeeping control (n=3) **(C)** BMDMs were activated with IFN-γ, IFN-γ + LPS for a duration of 24 hours and either mock challenged or challenged with *S. pneumoniae* serotype 1 or NTHi (MOI 10) for a duration of 4 hours. Supernatants were collected and TNFα and IL-6 levels assessed by ELISA, significance was determined by two-way ANOVA with Sidak's post-test, *=p<0.05, **=p<0.01, ***=p<0.001 (n=3) data represented as mean ± SEM.

3.2.8. Human MDMs can be induced to express markers of macrophage activation

To ensure successful activation of human MDMs, defined activation markers were identified using western blotting and MSD analysis. MDMs were cultured to day 14 and activated with 20ng/ml IL-4, 20g/ml IL-10, 20ng/ml IFN-γ + 100ng/ml LPS or 20ng/ml IFN-γ alone for a duration of 24 hours as described by others (Cassol et al., 2009) (Sudan et al., 2015). Supernatants were collected and cells lysed for protein. Phosphorylation of the STAT family of transcription factors was analysed by western blotting (Murray et al., 2014). MDMs stimulated with IFN-γ showed activation of pSTAT1 at tyrosine residue 701. Stimulation with IL-4 displayed phosphorylation of STAT6, Tyr641. To demonstrate IL-10 activation a positive result was seen by detection of pSTAT3 Tyr705. pSTAT3 Tyr705 was also detectable in IFN-γ + LPS stimulated MDMs (Figure 3-9). Chemokine activation markers were studied by MSD Analysis. IFN-γ+LPS stimulation significantly enhanced release of Eotaxin, Eotaxin-3 and IL-8. Although the spread was more variable, IFN-γ+LPS also stimulated increased release of MIP1-α, IP-10, MCP-1 and MCP-4 in some donors. IFN-γ activation alone did not significantly enhance any pro-inflammatory chemokine release (Figure 3.10). MDMs activated with IL-4 showed anti-inflammatory chemokine release typical of AAM (Murray et al., 2014) including, TARC, MIP-1β and MDC (Figure 3.11). No activation of anti-inflammatory cytokine was seen in IL-10 stimulated cells. In summary, these experiments indicate human macrophage activation can influence transcription factor activity and chemokine release as described by others and reassures us that the stimulation conditions are suitable for inducing shifts in human MDM phenotype (Mantovani et al., 2004), (Murray et al., 2014).

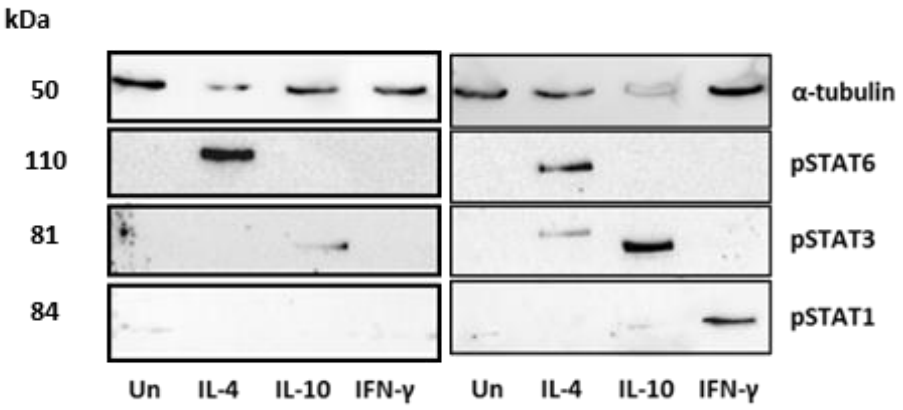


Figure 3-8: Activation marker expression in Human Monocyte Derived Macrophages

Human MDMs were stimulated with 20ng/ml IL-4, IL-10 or IFN-γ for a duration of 24 hours. Protein was collected and western blotted for markers of macrophage activation. Phosphorylated STAT-6 Tyr641 as a marker for IL-4 activation, phosphorylated STAT-3 Tyr705 as a marker for IL-10 activation and phosphorylated STAT-1 Tyr701 as a marker for IFN-γ stimulation. Representative blots are shown from two different donors, molecular weight of each antibody and phosphorylation sites are indicated. α-tubulin was used as a loading control.

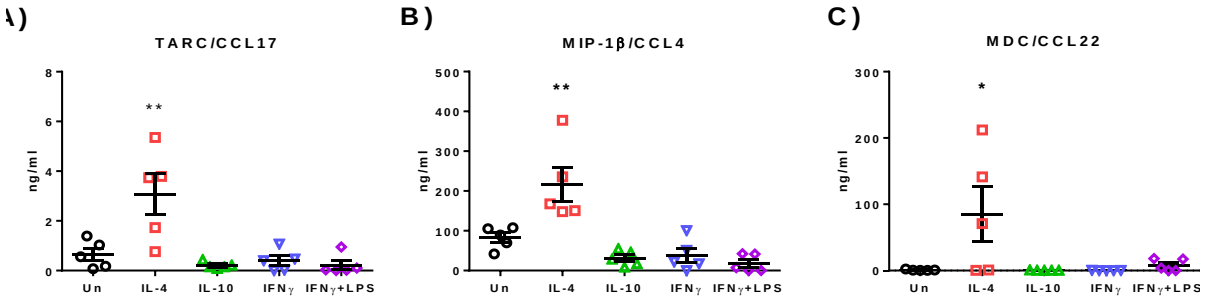


Figure 3-9: MDM IL-4 activation stimulates M(IL-4) chemokine marker production

Human MDMs were stimulated with 20ng/ml IL-4, IL-10, IFN-γ or 20ng/ml IFN-γ + LPS or left unstimulated in triplicate repeats for each donor for a duration of 24 hours. At 24 hours cell supernatants were collected and MSD Multi-spot Analysis conducted using Human Chemokine Panel 1 V-Plex™ Plate. Chemokine concentration of each sample was determined by MSD DISCOVERY WORKBENCH® analysis Software. Significant changes in chemokine concentration compared to the unstimulated control were determined by one-way ANOVA's with Dunnett's post-test. *p=0.05, **p=0.01 (n=5 donors), data is represented as mean ± SEM. Sensitivity of each analyte: TARC: 0.36 pg/ml, MIP1-β: 0.24 pg/ml, MDC: 2.59 pg/ml.

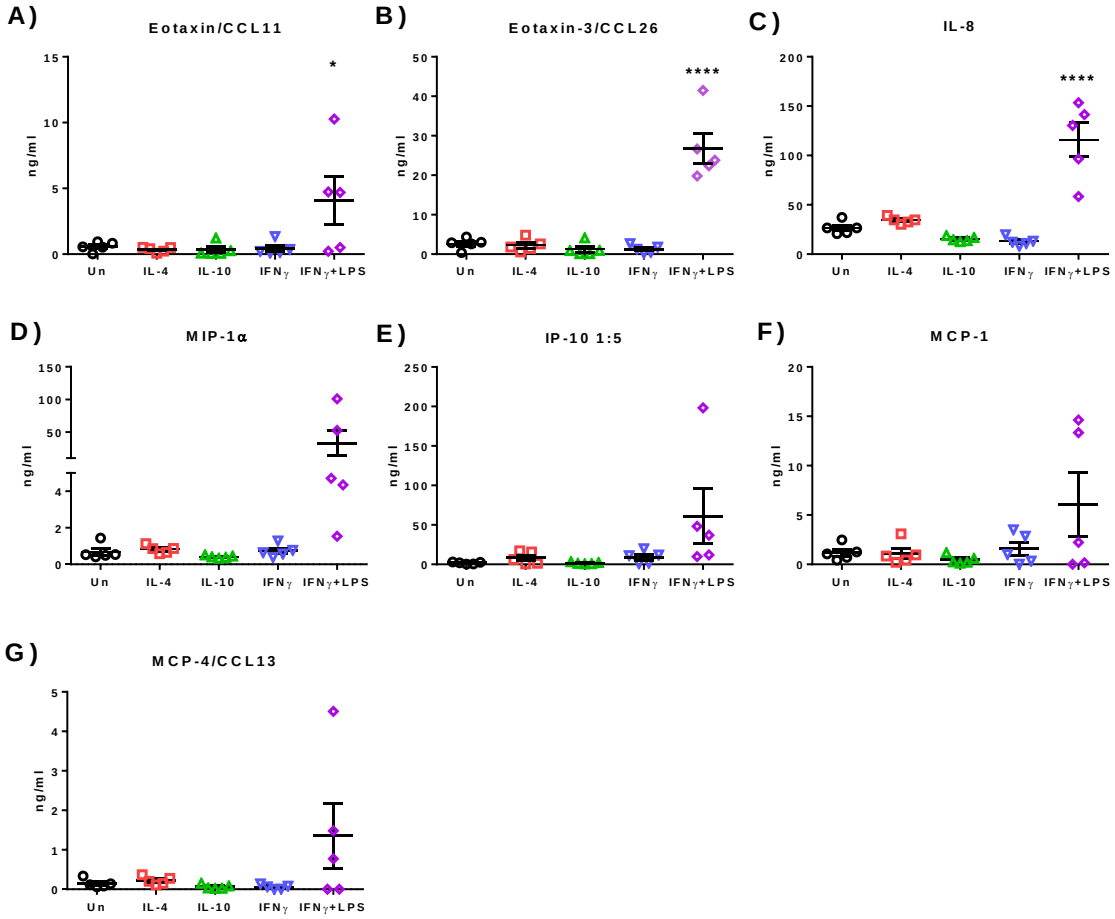


Figure 3-10: MDM IFN-γ+LPS activation causes pro-inflammatory chemokine production

Human MDMs were stimulated with 20ng/ml IL-4, IL-10, IFN-γ or 20ng/ml IFN-γ + LPS or left unstimulated in triplicate repeats for each donor for a duration of 24 hours. At 24 hours cell supernatants were collected and MSD Multi-spot Analysis conducted using Human Chemokine Panel 1 V-Plex™ Plate. Chemokine concentration of each sample was determined by MSD DISCOVERY WORKBENCH® analysis Software. Significant changes in chemokine concentration compared to the unstimulated control were determined by one-way ANOVA's with Dunnett's post-test. *p=0.05, ****p=0.0001, n=5, data is represented as Mean ± SEM. (n=5 donors), data is represented as mean ± SEM. Sensitivity of each analyte: Eotaxin: 0.36 pg/ml, Eotaxin-3: 1.21 pg/ml, IP-10: 0.58 pg/ml, MIP-1α: 0.23 pg/ml, IL-8: 18.5 pg/ml, MCP-1: 0.10 pg/ml, MCP-4: 0.16 pg/ml.

3.3. Discussion

In this chapter, I have demonstrated that wild-type and CD68 Mcl-1 BMDMs can be activated by cytokines to express markers of activation as recommended by others (Murray et al., 2014). iNOS and Arginase-1, the opposing enzymes which catalyse L-Arginine metabolism, are

considered the key markers in defining murine macrophage activation (Mills et al., 2000b). L-Arginine is either metabolised by iNOS to produce NO and Citrulline or hydrolysed by Arginase to generate Ornithine and polyamines. The end-products of these reactions hint at the function of the cell, for example, NO is a free radical with microbicidal associations, Ornithine is a pre-cursor of molecules involved in wound healing (Albina et al., 1990). I have confirmed that activation of BMDMs by IFN-γ + LPS can induce iNOS and IL-4 stimulate Arginase-1 protein expression by western blotting. I have also demonstrated that iNOS and Arginase-1 mRNA is detectable by qPCR at 12 hours post activation. Pro-inflammatory cytokine and stimulation by IFN-γ + LPS at 12, 24 and 48 hours was also confirmed by ELISA in wild-type and transgenic BMDMs. This work reassures us that wild-type and transgenic BMDMs are successfully activated by cytokine stimulation protocols.

For protocol development, a range of cytokine concentrations were tested to determine the optimum activation conditions for our BMDMs. Concentrations of IFN-γ were evaluated by iNOS expression and cytokine production. All concentrations tested initiated a response and it was decided that 20ng/ml be routinely used as this yielded consistent results and is routinely used by others (Jablonski et al., 2015). As well as concentration, duration of stimulation was also optimised. Protein and supernatants were collected from M(IFN-γ + LPS) and M(IL-4) stimulated cells over a time course and western blots and ELISAs conducted. iNOS and Arginase-1 protein expression and pro-inflammatory cytokine stimulation was seen at all time points tested. 24 hours of activation was chosen as an appropriate and practical incubation period for experiments requiring further incubation periods, i.e. infection time courses. Furthermore Reports state that 24 hours of activation is enough for full macrophage activation (Mosser and Zhang, 2008). 48 hours of stimulation showed successful induction of iNOS and Arginase-1 protein and pro-inflammatory cytokine expression however later experiments showed cell metabolic activity and therefore potentially cell viability was more variable than at 24 hours, by MTT assay. The optimal duration of cell culture for macrophage activation was also analysed. Macrophages were activated and markers of activation profile were investigated on day 7, 10 and 14 of culture. 14 days was seen to generate the highest marker expression, potentially as it is the time needed for BMDMs to reach maturity using our culture methods.

An MTT test was conducted on activated wild-type and transgenic macrophages. This test determines viability based on metabolic activity. Cells which were stimulated with IFN-γ + LPS and LPS alone showed a significant decrease in metabolic activity when compared to the unstimulated control. A decrease was also seen in IFN-γ activated macrophages however this

was not as marked. Reduced metabolic activity with LPS stimulation also coincides with a decline in cell number observed by cell counts before macrophage infection. Significant decreases in metabolic activity and induction of apoptosis in response to IFN-γ + LPS has also been shown by others (He et al., 2008). LPS causes induction of apoptosis at early time points due to autocrine production of TNFα and at later time points by endogenous NO production through iNOS expression (Xaus et al., 2000). IFN-γ has been shown to induce apoptosis through STAT1 activation however IFN-γ activation can also have proliferative effects depending on which IFN-γ receptor is activated (Bernabei et al., 2001). Although commonly used as a read out of cell viability (He et al., 2008) the MTT assay measures NADPH oxidase activity of the cell and is therefore a way of measuring metabolic activity. My data showed a decrease in metabolic activity in cells treated with IFN-γ and LPS. IFN-γ and LPS treatment are known to reduce cell viability in some scenarios, however it is also possible that in this case, the metabolic activities of IFN-γ and LPS treated cells have become saturated or that these stimuli cause cells to become less metabolically active or arrest proliferation (Xaus et al., 1999). IFN-γ and LPS stimulated cells were not found to cause cell death without additional bacterial stimuli after 24 hours of activation by analysis of nuclear morphology by DAPI staining. However, since a central part of my PhD project is based on apoptosis caused by bacterial challenge, I wanted to limit any additional cell death stimuli which might be attributable to macrophage activation conditions. Furthermore, IFN-γ + LPS was also shown to decrease cell density. Murray and colleagues describe IFN-γ stimulation alone as a more polarised state of classical activation than M(IFN-γ + LPS) (Murray et al., 2014), however others describe IFN-γ stimulation as a 'mild' stimuli, influencing gene regulation much less than 'potent' M(LPS) activation (Sudan et al., 2015). I decided to alter my CAM activation protocol to include just 20ng/ml IFN-γ and tested if stimulation would be successful in priming BMDMs to generate a heightened pro-inflammatory response conditions with Gram-positive and Gram-negative bacterial challenge (Tötemeyer et al., 2006). Although IFN-γ activation alone was not sufficient to stimulate pro-inflammatory cytokine release, IFN-γ activation did amplify iNOS mRNA 100-fold compared to unstimulated samples suggesting BMDMs were primed for pro-inflammatory responses. After bacterial infection, IFN-γ activated BMDMs showed a heightened response comparable to IFN-γ + LPS condition. This suggested M(IFN-γ) cells were primed for activation and subsequently triggered by microbial stimuli.

Both wild-type and transgenic BMDMs were used throughout optimisation experiments to assess if there are any baseline differences in macrophage activation between the two genotypes. The evidence presented in this chapter suggests there is no effect of the CD68 Mcl-

1 transgene on macrophage activation. Further to this, macrophage activation by IFN-γ + LPS, IL-4 and IL-10 appear to have no significant effect on Mcl-1 levels. This is however not to say that CD68 Mcl-1 macrophages might express a profile that differs from archetypal CAM and AAM subsets, in certain respects, involving specific differences which have not been explored here. To delineate the specific macrophage activation profiles of wild-type and transgenic BMDMs in this context it would be necessary to conduct a transcriptomic analysis. Further, to make this analysis more clinically relevant to lung disease, it would be interesting if we based our analysis on AMs, bearing in mind the heterogenous activation profiles which exist within and between tissue compartments (Davies et al., 2013; Gordon et al., 2014).

Markers of human macrophage activation are less well defined than murine models. Generation of NO by iNOS in human macrophages has long been a topic of debate (Schneemann and Schoedon, 2002). Other reports stipulate that Arginase-1 expression is restricted to neutrophils among circulating human leukocytes (Munder, 2009). To ensure that human MDMs could be successfully stimulated for future experiments, I analysed expression of the phosphorylated forms of the STAT family of transcription factors as recommended by others (Murray et al., 2014). As expected, pSTAT1 Tyr701 and pSTAT6 Tyr641 were present in IFN-γ and IL-4 stimulated macrophages respectively. pSTAT3 was expressed in IL-10 stimulated macrophages, however only due to phosphorylation at the tyrosine 705 residue. LPS activation also induced pSTAT3 Tyr705 activation. It has previously been shown that LPS can cause production of IL-10 which phosphorylates STAT3 (Carl et al., 2004) this was further explored by Murray and colleagues in an infection model which showed *Mycobacterium tuberculosis* caused TLR signalling via MyD88 to produce IL-10 and subsequently phosphorylation of STAT3 (Qualls et al., 2010). Chemokine marker expression was also analysed by MSD. Pro-inflammatory chemokine release was evident from activation with IFN-γ + LPS activation. IL-4 stimulation also caused generation of anti-inflammatory cytokines, in line with previous reports (Mantovani et al., 2004; Murray et al., 2014). However, MCP-4 (CCL13) was expected to increase with IL-4 stimulation but rather it was increased upon IFN-γ+ LPS stimulation. A recent study warns that chemokine expression is transient after activation and expression of MCP-4 and MDC can also be induced by PAMPs and interferons at earlier time points meaning they are not consistently effective markers of IL-4 activation (Sudan et al., 2015). The latter paper suggests MCP-4 is only a useful marker of IL-4 activation at 24 hours and although supernatants were collected at 24 hours, transiency is likely to be donor specific and since Sudan and colleagues only conducted analysis on one donor it is hard to say how variable expression patterns are between subjects. The straying in marker expression in my data set compared to other reports on macrophage activation and the large

spread of data for some of the chemokines tested is most likely attributable to the high donor to donor variability when using human MDMs.

In conclusion, CD68 Mcl-1 transgenic macrophages are neither biased to “M1”, “M2a” or “M2c” activation states. Further to this, the CD68 Mcl-1 transgene causes no variation in activation marker expression after cytokine stimulation, at least with the small number of responses measured here. This evidence suggests that the CD68 Mcl-1 transgene has no effect on macrophage phenotype however a more thorough analysis would be needed to confirm this finding. The experimental conditions for activation of BMDMs have been optimised and I have provided evidence that our MDMs are successfully activated *in vitro* along different activation pathways.

4. The effect of macrophage activation and apoptosis associated killing on *S. pneumoniae* challenge

4.1. Introduction

As discussed, *S. pneumoniae* is the leading cause of CAP however, the pathogenesis is incompletely defined. AMs, the resident phagocytes of the lung, are the body's first line of defence against pneumococcal infection. Opsonised pneumococci are readily ingested by AMs into intracellular compartments known as phagosomes. Maturation of the phagosome is associated with a loss of pneumococcal viability (Gordon et al., 2000) through employment of ROS and other mechanisms including NO mediated killing, antimicrobial peptides and proteases (Aberdein et al., 2013). At low doses, early killing mechanisms are sufficient to resolve infection, however, the pneumococcus has evolved many ways to subvert host defence and therefore killing by AMs is finite (Dockrell et al., 2003). When ROS and NO killing mechanisms become overwhelmed, macrophages require additional means to control infection, we have previously shown macrophages do this by undergoing apoptosis (Dockrell et al., 2001b).

Initiation of macrophage apoptosis occurs at later time points of infection (16-20 hours) and onset is proportional to the intracellular burden of bacteria (Ali et al., 2003). Therefore, it is likely that this phenomenon transpires when conventional early killing mechanisms are exhausted. Our previous work has showed that macrophage apoptosis associated killing is regulated by the Bcl-2 family member Mcl-1 (Bewley et al., 2011a; Marriott et al., 2005). The contribution of Mcl-1 and apoptosis to host defence has been further explored using a transgenic mouse over-expressing human Mcl-1 on a macrophage specific promoter (Bewley et al., 2017). Although the molecular regulation of the apoptotic program has been partially deciphered (Bewley et al., 2011a) the widespread transcriptomic changes associated with the early stages of apoptosis associated bacterial killing and Mcl-1 over-expression remain largely unexplored.

In addition, we have not yet attempted to characterise the activation phenotype of macrophages after *S. pneumoniae* infection, nor do we understand how differential macrophage activation might affect apoptosis associated killing. Conventionally, macrophages become classically activated by bacterial components which aids pathogen clearance through transcription of genes involved in phagocytosis, bacterial killing and neutrophil recruitment (Nau et al., 2002). Furthermore, the effect of *S. pneumoniae* infection on macrophage activation has not been extensively explored in the literature, particularly at late time points when apoptosis associated killing is required. As the lung environment contains a mixture of macrophage

phenotypes which are often skewed by underlying clinical disease (Byrne et al., 2015), it is important to understand the responses of differentially activated macrophages to pneumococcal infection and whether altered activation might cause ameliorated or attenuated macrophage effector functions. *S. pneumoniae* is an important pathogen in COPD where canonical macrophage activation is perturbed (Shaykhiev et al., 2009b). Although earlier in this thesis I concluded overexpression of Mcl-1 has no effect on baseline macrophage activation within the subsets explored, given the importance of the Mcl-1 regulated apoptosis defect in COPD (Bewley et al., 2017), it is interesting to consider whether the Mcl-1 transgene might effect macrophage phenotype after pneumococcal infection.

The aims of this chapter were to therefore understand how macrophage activation affects early macrophage responses to *S. pneumoniae* infection including, phagocytosis, microbicidal production and killing activity. Effects of macrophage activation at later time points of infection were also explored to delineate any relationships between macrophage phenotype and apoptosis associated killing. Macrophage effector functions were analysed in human MDMs and BMDMs. Since the CD68 Mcl-1 transgenic mouse is a model for a defect in apoptosis-associated killing, experiments were conducted in both CD68 Mcl-1 and wild-type BMDMs. To better understand how the transcriptomic landscape is altered in the CD68 Mcl-1 model during the initiation stage of apoptosis and before apoptosis is advanced, a microarray study was performed on AMs from transgenic mice at the 'tipping point' of pneumococcal infection when the ability of AMs to contain infection is becoming overwhelmed (Dockrell et al., 2003). I then attempted to validate the results of the transcriptomics study by multi-colour flow cytometry experiments which defined recruitment of specific cell subsets *in vivo* in both transgenic and wild-type mice after *S. pneumoniae* infection. *S. pneumoniae* serotype 1 was used in all of the experiments presented in this chapter as it is controlled by AMs at a low dose and a tipping point for this serotype has been well defined (Dockrell et al., 2003). Furthermore, serotype 1 is clinically relevant as it is an important cause of pneumococcal invasive disease (Harboe et al., 2010; Hausdorff et al., 2000).

4.2. Results

4.2.1. Classically activated macrophages show increased clearance of pneumococci

To understand how macrophage activation affects initial pneumococcal killing, I analysed killing ability of MDMs and BMDMs at early timepoints of infection. Although macrophage activation has not been previously explored in a *S. pneumoniae* infection model, classical activation is the

macrophage phenotype most commonly associated with bacterial infection (Nau et al., 2002). Therefore, I predicted that *S. pneumoniae* infection would be more effectively cleared in macrophages which had been pre-stimulated with classical activation stimuli compared to alternative activation stimuli or unstimulated macrophages. Macrophages were pre-stimulated with the optimised cytokine doses described in chapter 3. As early work which was done in parallel to optimisation of activation conditions included an IFN-γ + LPS condition, despite later omission, this condition has been incorporated into the figures to give a complete picture of the activation conditions explored in this thesis. Although Mcl-1 levels only become decreased at later time points of infection, CD68 Mcl-1 transgenic macrophages were also compared to wild-type BMDMs to ensure there were no early effects of Mcl-1 over-expression on macrophage killing in activated cells. Using the killing assay, both human (Figure 4-1) and murine (Figure 4-2) macrophages showed significantly increased clearance of pneumococci between 2 and 4 hours when stimulated with classical activation stimuli (IFN-γ or IFN-γ+LPS) in line with other reports detailing the importance of IFN-γ to pathogen clearance (Shaughnessy and Swanson, 2007). There was a trend towards decreased intracellular bacteria at 2 hours in both M(IFN-γ) and M(IFN-γ+LPS). This may be due to accelerated clearance of bacteria and therefore a lower intracellular burden, however, classical activation has been shown to decrease phagocytic uptake by decreasing the scavenger receptor MARCO (Metzger, 2009) and also Fcγ and complement mediated opsonic uptake (Frausto-Del-Río et al., 2012a; Schlesinger and Horwitz, 1991b), therefore I conducted a latex bead experiment on activated MDMs exposed to opsonised beads to ensure increased clearance wasn't a consequence of decreased internalisation (Figure 4-3). There was no significant difference between any activation conditions but a trend towards decreased uptake in IFN-γ + LPS stimulated macrophages was observed. This implies increased clearance of pneumococci in CAM is not due to decreased uptake in M(IFN-γ), however more experiments are needed to confirm the effect of LPS on opsonic uptake. Although no difference was found between conditions with opsonised bacteria, before a conclusion is drawn it would also be important to also test non-opsonic uptake which is an additional route for *S. pneumoniae* internalisation (Arredouani et al., 2004; Arredouani et al., 2006), though not as important for clearance as opsonic uptake in AM (Gordon et al., 2000).

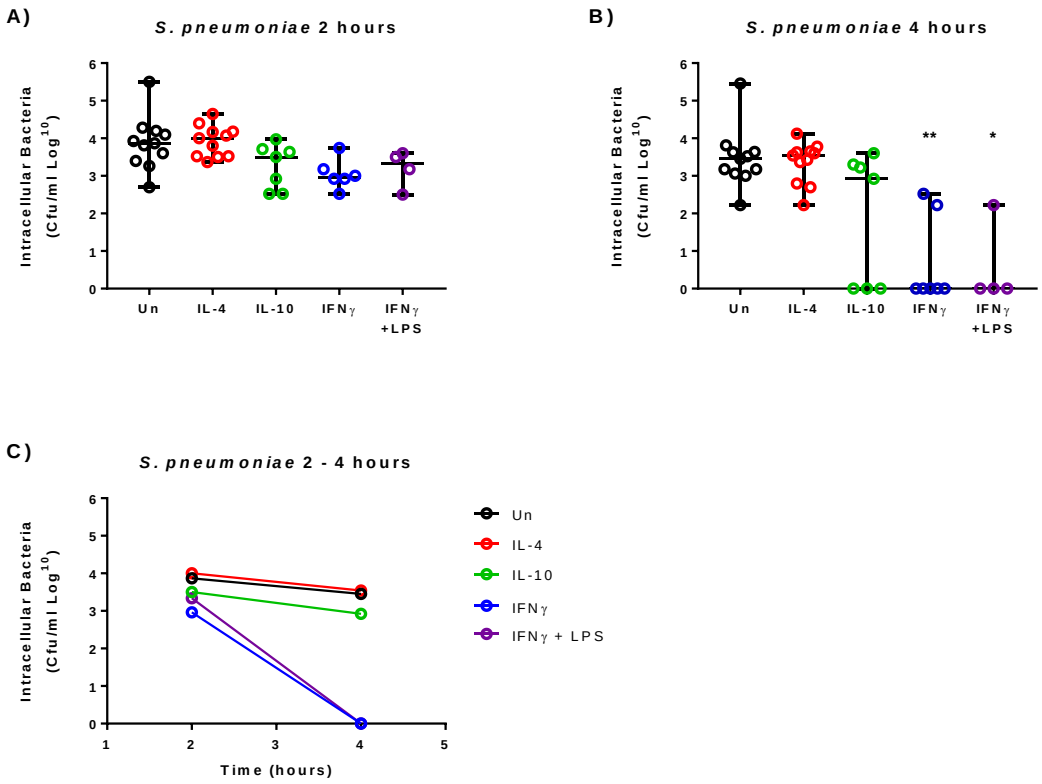


Figure 4-1: Pro-inflammatory macrophage activation enhances Killing of *S. pneumoniae* at early time points.

MDMs were activated with 20ng/ml IL-4, IL-10, IFN-γ or 20ng/ml IFN-γ + 100ng/ml LPS for 24 hours. Cells were washed and challenged with *S. pneumoniae* serotype 1 (MOI 10) for a duration of 2 hours. **(A)** At the 2-hour time point a gentamicin protection assay was performed, cells were lysed and supernatants serially diluted and plated to determine cfu. **(B)** The remaining cells were incubated in a low dose of vancomycin until the 4-hour time point and lysed to assess the killing of intracellular bacteria. **(C)** A representative plot of the macrophage killing activity between 2 and 4-hour time points. Data is represented as median ± range. Data was transformed to logarithmic and analysed by Kruskal-Wallis test with Dunn's post-test compared to the unstimulated condition (n= 4-11) *=p<0.05, **=p<0.01.

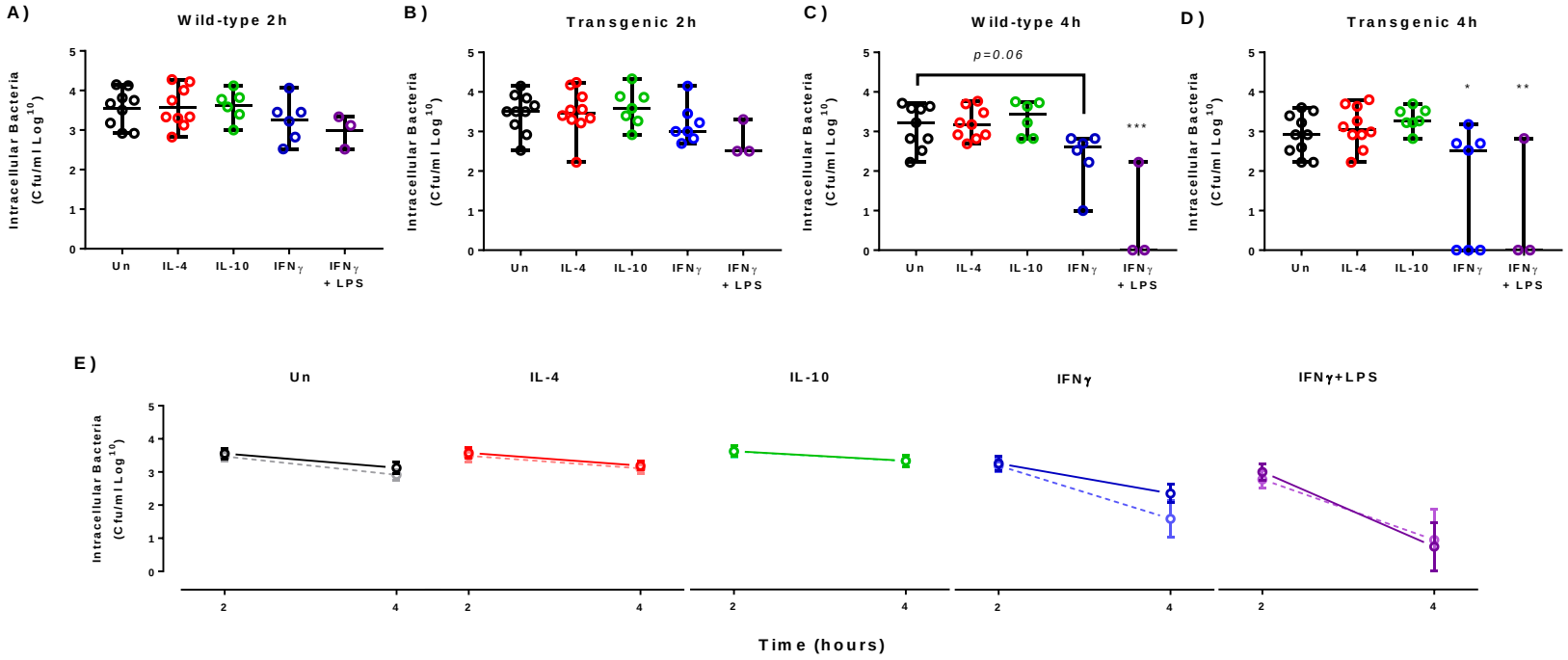


Figure 4-2: Classical macrophage activation enhances killing of *S. pneumoniae* at early time points Wild-type and transgenic BMDMs were activated with 20ng/ml IL-4, IL-10, IFN-γ or 20ng/ml IFN-γ + 100ng/ml LPS for 24 hours. Cells were washed and challenged with *S. pneumoniae* serotype 1 (MOI 10) for a duration of 2 hours. **(A-B)** At the 2-hour time point a gentamicin protection assay was performed, cells were lysed and supernatants serially diluted and plated to determine cfu **(C-D)** The remaining cells were incubated in a low dose of vancomycin until the 4-hour time point and lysed to assess the killing of intracellular bacteria. Data was transformed to logarithmic and analysed by Kruskal-Wallis test with Dunn's post-test **(E)** A representative plot of the macrophage killing activity between 2 and 4-hour time points. The dotted-line represents the transgenic mouse. Data was transformed to logarithmic and analysed by two-way ANOVA with Sidak's post-test, no significance was found between willd-type and transgenic macrophages at each timepoint *=p<0.05, **=p<0.01, ***=p<0.001(n=3-9) data is represented as median ± range.

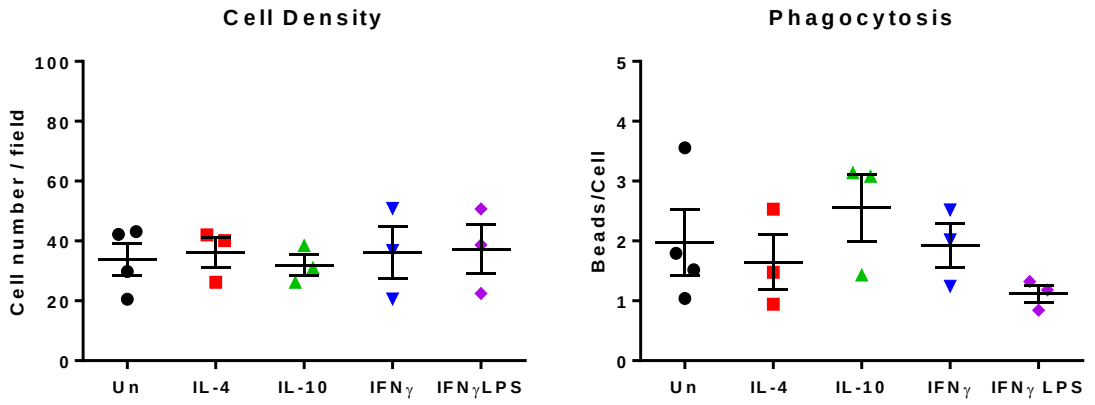


Figure 4-3: Macrophage activation does not significantly affect phagocytosis of latex beads

MDMs were activated with 20ng/ml IL-4, IL-10, IFN-γ or 20ng/ml IFN-γ + 100ng/ml LPS for 24 hours. Macrophages were washed before challenge with fluorescent latex beads (MOI 10). Beads were opsonized before challenge and cells were incubated with beads for a duration of 4 hours. At this time point macrophages were washed to remove extracellular beads and fixed with 2% paraformaldehyde. Coverslips were mounted in DAPI containing mounting medium and analysed by fluorescent microscopy. Cell density per field and number of beads per cell was quantified. No significance was determined by one-way ANOVA with Dunnett's post-test compared to the unstimulated condition (n=3) data is presented as mean ± SEM.

4.2.2. Macrophage activation has minimal effect on microbicidal production

Early macrophage killing of pneumococci takes place in the maturing phagosome and can be attributed to several different phagocyte microbicidal mechanisms including ROS, NO and RNS generation (Aberdein et al., 2013). Since CAM up-regulate genes involved in ROS and NO production (Mantovani et al., 2004) and as demonstrated in section 4.2.1 M(IFN-γ) are more effective at clearing infection, I predicted macrophages stimulated with IFN-γ would show increased ROS and NO production. MDMs were stained with DCF-DA (ROS) or DAF-FM (NO) and analysed by flow cytometry. Although macrophage exposure to bacteria caused generation of ROS in all conditions, IFN-γ activation was not found to affect levels of ROS production in MDMs (Figure 4-4). Macrophage activation also had no significant effect on NO generation after 4 hours of *S. pneumoniae* challenge but the NO signal remained low in comparison to ROS generation (Figure 4-5). Lack of significance between activation conditions was surprising as there is a marked increase in pneumococcal killing by M(IFN-γ) and the mechanism cannot be explained as due to significant changes in ROS or NO after 4 hours of challenge on the basis of these results. Wild-type and transgenic BMDMs were also analysed for ROS production (Figure 4-6). IFN-γ

activation did show enhanced ROS production and this result reached significance compared to all other activation states.

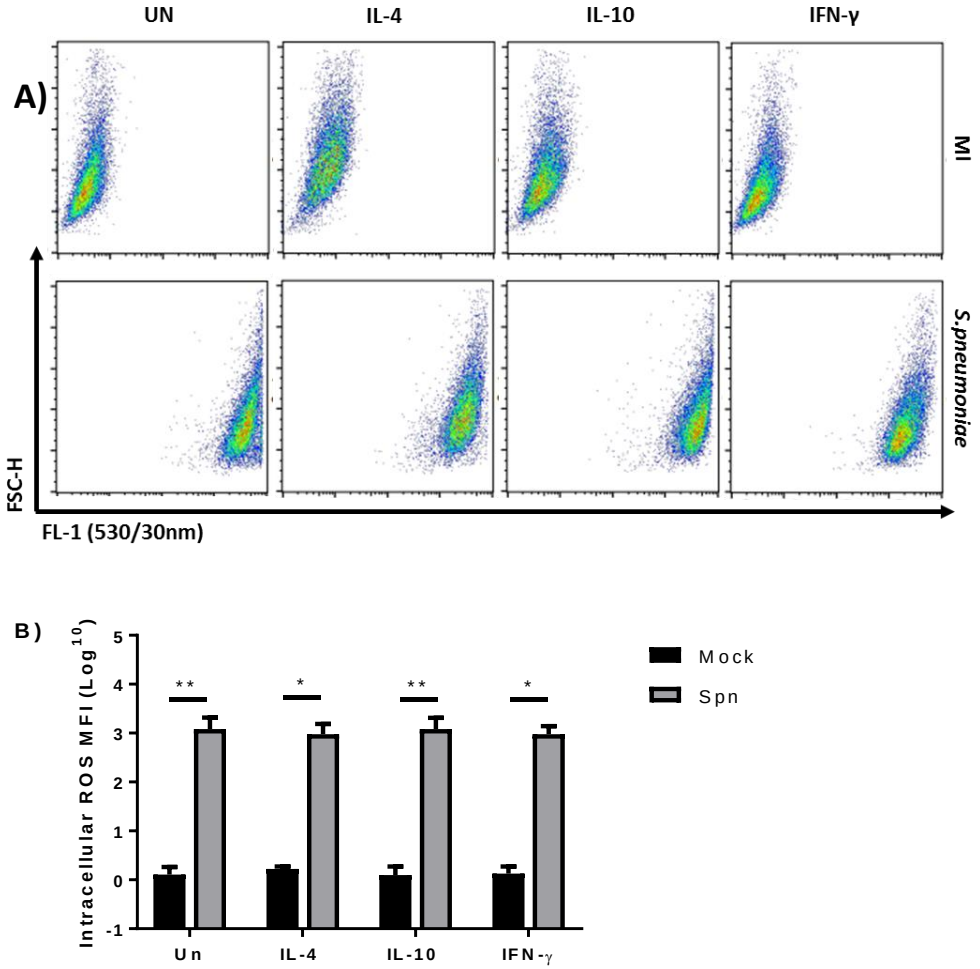


Figure 4-4: Macrophage activation does not affect intracellular ROS production after *S. pneumoniae* infection

MDMs were activated with 20ng/ml IL-4, IL-10, IFN-γ or left unstimulated for 24 hours. Cells were washed and mock challenged or challenged with *S. pneumoniae* serotype 1 (MOI 10) for a duration of 4 hours. At the time point cells were washed and stained with 10µM DCF-DA. After staining macrophages were scraped and resuspended in PBS and placed on ice protected from light until analysis by flow cytometry. Unstained data values were subtracted from stained values to account for autofluorescence. **(A)** representative flow plots pictured **(B)** Data was transformed to logarithmic and analysed by repeated measures two-way ANOVA with Sidak's post-test *=p<0.05, ****=p<0.0001, data represented as mean ± SEM (n=4).

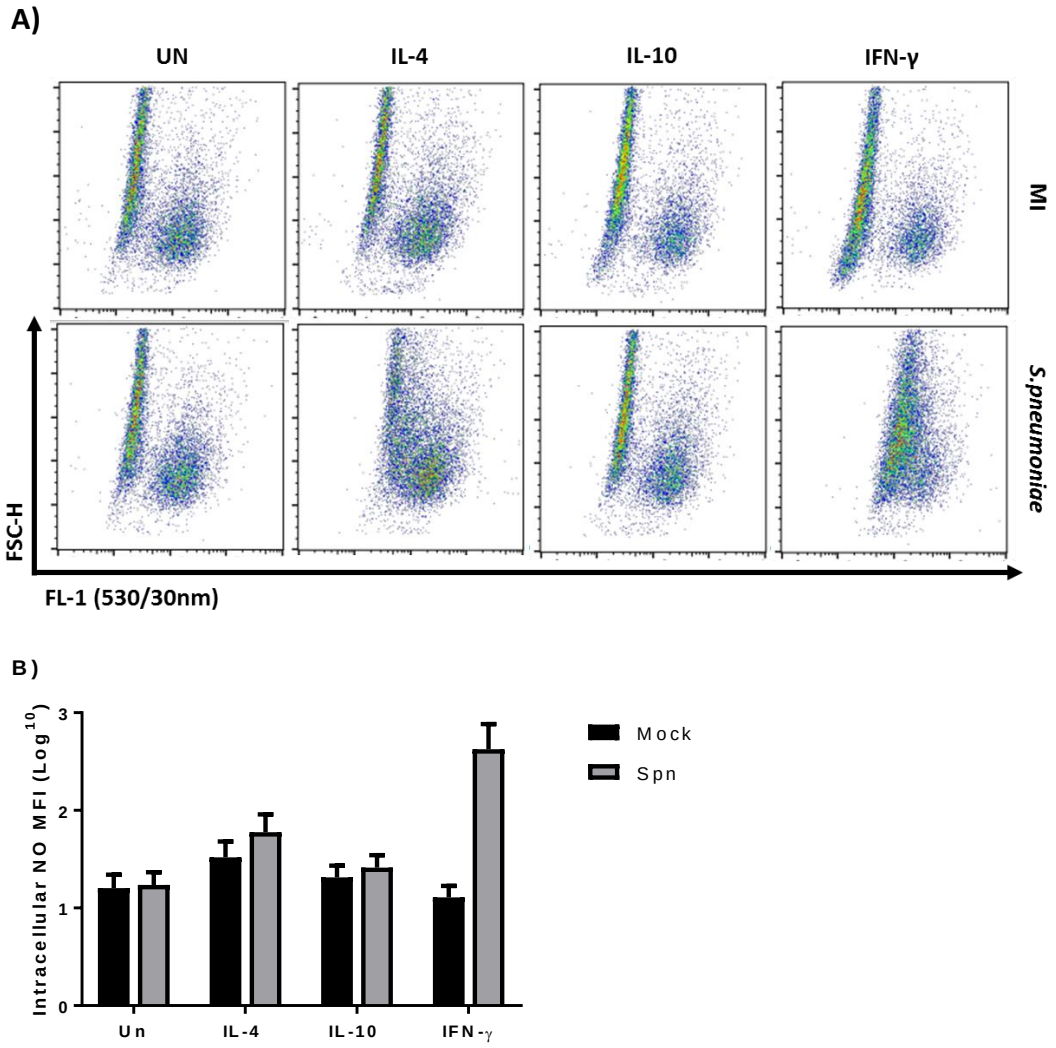


Figure 4-5: Macrophage activation does not affect NO production after *S. pneumoniae* infection

MDMs were activated with 20ng/ml IL-4, IL-10, IFN-γ or left unstimulated for 24 hours. Cells were washed and mock challenged or challenged with *S. pneumoniae* serotype 1 (MOI 10) for a duration of 4 hours. At time point cells were washed and stained with 5µM DAF-FM. After staining macrophages were scraped and resuspended in PBS and placed on ice protected from light until analysis by flow cytometry. Unstained data values were subtracted from stained values to account for autofluorescence. **(A)** representative flow plots pictured **(B)** Data was transformed to logarithmic and analysed by repeated measures two-way ANOVA with Sidak's post-test. No significance was found between activation conditions, data represented as mean ± SEM (n=6).

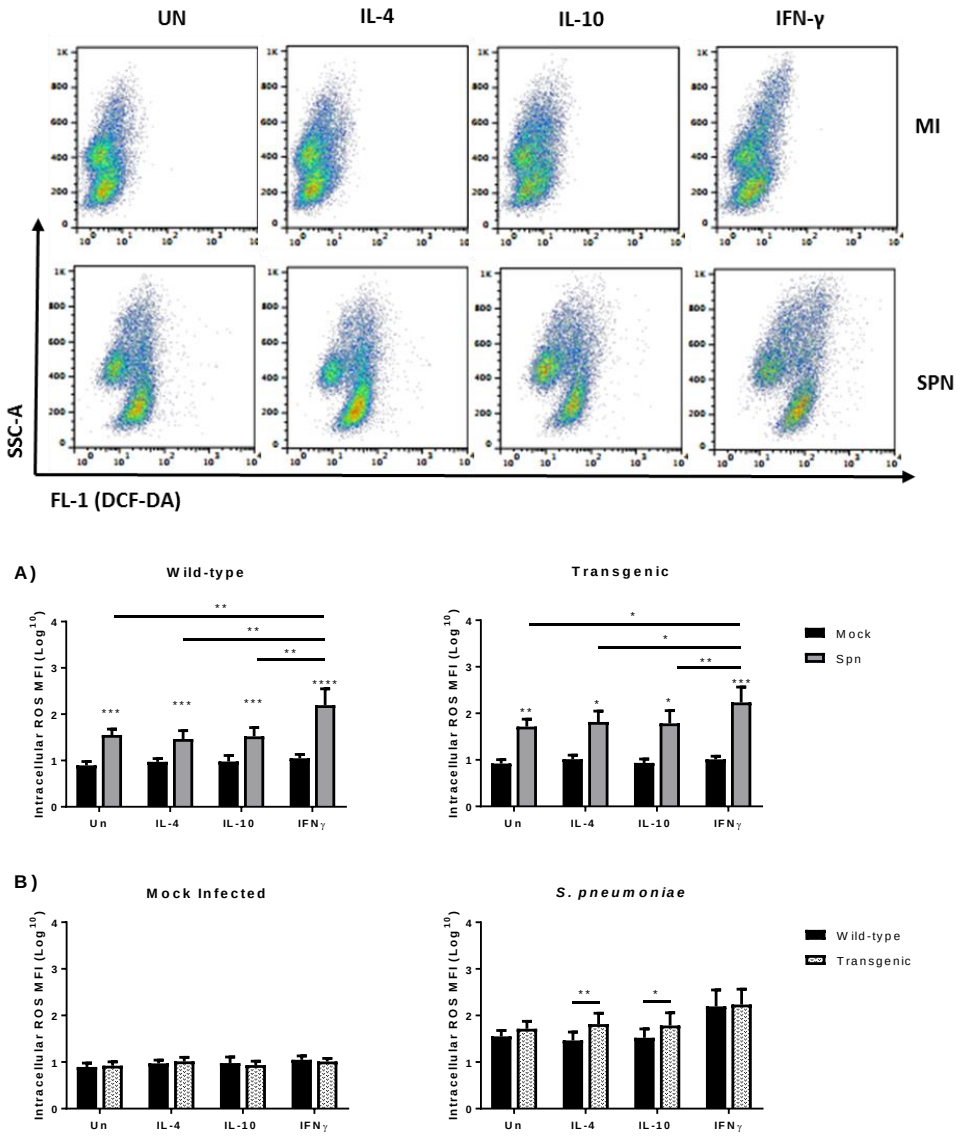


Figure 4-6: Macrophage activation does not affect intracellular ROS production after *S. pneumoniae* infection

BMDMs were activated with 20ng/ml IL-4, IL-10, IFN-γ or left unstimulated for 24 hours. Cells were washed and mock challenged or challenged with *S. pneumoniae* serotype 1 (MOI 10) for a duration of 4 hours. At time point cells were washed and stained with 10µM DCF-DA and analysed by flow cytometry. Unstained data values were subtracted from stained values to account for autofluorescence. Representative flow plots are pictured **(A)** Mock challenged and *S. pneumoniae* challenged samples are compared by repeated measured two-way ANOVA with Sidak's post-test. Stars directly above the bars indicate significance compared to the mock infected. **(B)** Wild-type and transgenic BMDMs are compared in mock infected and *S. pneumoniae* infected cells. Significance determined with repeated measures two-way ANOVA and Sidak's post-test (n=4) *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, data represented at mean ± SEM.

4.2.3. CAM show enhanced apoptosis at late time points of *S. pneumoniae* challenge

I next turned my attention to analysing late time points of *S. pneumoniae* infection when early killing mechanisms have become exhausted and apoptosis associated killing occurs (Ali et al., 2003; Dockrell et al., 2001a). Mcl-1 is a mitochondrial protein which not only regulates apoptosis but can also influence mitochondrial metabolic function and ATP generation (Perciavalle et al., 2012). As discussed elsewhere in this thesis, macrophage activation is also tightly linked to the mitochondria. Bacterial products and intracellular pathogens cause macrophages to switch from oxidative phosphorylation to metabolic pathways which favour production of NADPH derived ROS such as those associated with glycolysis or the pentose phosphate pathway (Ghesquière et al., 2014; Haschemi et al., 2012). I hypothesised macrophage activation could influence apoptosis associated killing since published work by our group shows nitrosative stress helps sensitise apoptosis via the mitochondrion (Marriott et al., 2004) and this is known to be regulated by activation phenotype (Rath M, 2014). Since activation state is linked to metabolism (Galván-Peña and O'Neill, 2014) and Mcl-1 can influence mitochondrial metabolism (Perciavalle et al., 2012) and my group has also some data that the Mcl-1 transgene can regulate oxidative phosphorylation (Bewley et al., 2017), I also explored if the transgene might modify the effects of activation state on apoptosis-associated killing. Therefore, I reasoned decreasing levels of Mcl-1 during apoptosis might also encourage switching to an alternative form of energy production such as glycolysis. A decrease in Mcl-1 protein levels typically occurs at 16 hours and continues to decrease thereafter (Marriott et al., 2005) so I decided to analyse apoptosis from 16 – 24 hours in MDMs and as macrophage apoptosis occurred slightly earlier in my experiments with BMDMs I began analysis in murine cells at 14 hours. I first examined the effects of macrophage activation on apoptosis at an early time point of 4 hours (Figure 4-7) which showed as expected negligible levels of apoptosis as confirmed by nuclear fragmentation analysis and no alteration with activation. Initial analysis included M(IFN-γ+LPS) conditions. Results show that activation of both MDMs (Figure 4-8) and BMDMs (Figure 4-9) with IFN-γ+LPS results in higher levels of apoptosis at 16, 20 and 24 hours post *S. pneumoniae* challenge. In BMDMs, significant differences are only seen in wild-type macrophages as the level of apoptosis remains low in transgenic macrophages across all activation conditions until 24 hours when apoptosis levels begin to increase in the transgenic mouse, albiet not significantly. Key time points were repeated with M(IFN-γ) stimulation and using caspase 3/7 activation as a secondary determinant of apoptosis (Figure 4-10). Analysis of nuclear fragmentation at 16 hours in MDMs showed a significant increase in M(IFN-γ) apoptosis compared to all other activation states. M(IFN-γ)

caspase 3/7 activation was significantly increased compared to M(IL-10) macrophages. At 20 hours post *S. pneumoniae* challenge, other activation states seem to have caught up with M(IFN-γ) apoptosis levels except M(IL-4) which still showed significantly lower levels of caspase 3/7 activation, indicating stimulation with IL-4 might delay onset of apoptosis. Variability in levels of apoptosis with BMDM and some higher data points in some of the unstimulated BMDM condition prevented detection of significant differences however there were again lower levels of apoptosis in the transgenic BMDM and there appeared to be higher levels of apoptosis in M(IFN-γ) than in M(IL-4) or M(IL-10) conditions.

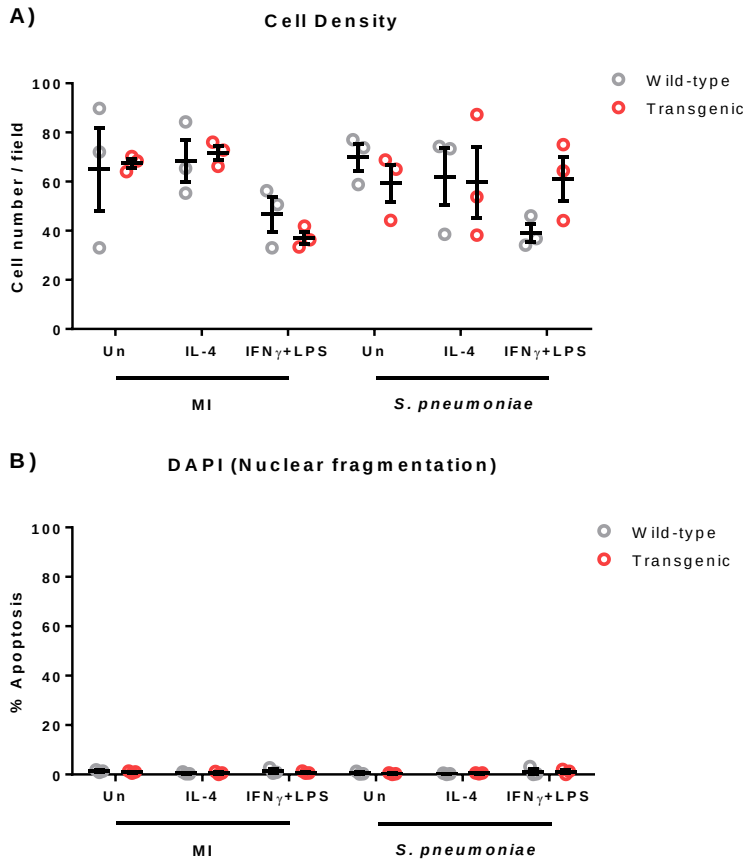


Figure 4-7: Macrophage activation does not affect apoptosis at early time points

Wild-type and transgenic BMDMs were activated with 20ng/ml IL-4, IL-10, 20ng/ml IFN-γ + 100ng/ml LPS or left unstimulated for 24 hours. Cells were washed and challenged with serotype 1, *S. pneumoniae* (MOI 10) for a duration of 4 hours and analysed by microscopy. **(A)** Cell Density per field was quantified by DAPI staining. **(B)** Nuclear fragmentation and condensation by DAPI staining was used to quantify apoptosis. No significance was determined using a two-way ANOVA with Sidak's post-test, data is represented as mean ± SEM (n=3).

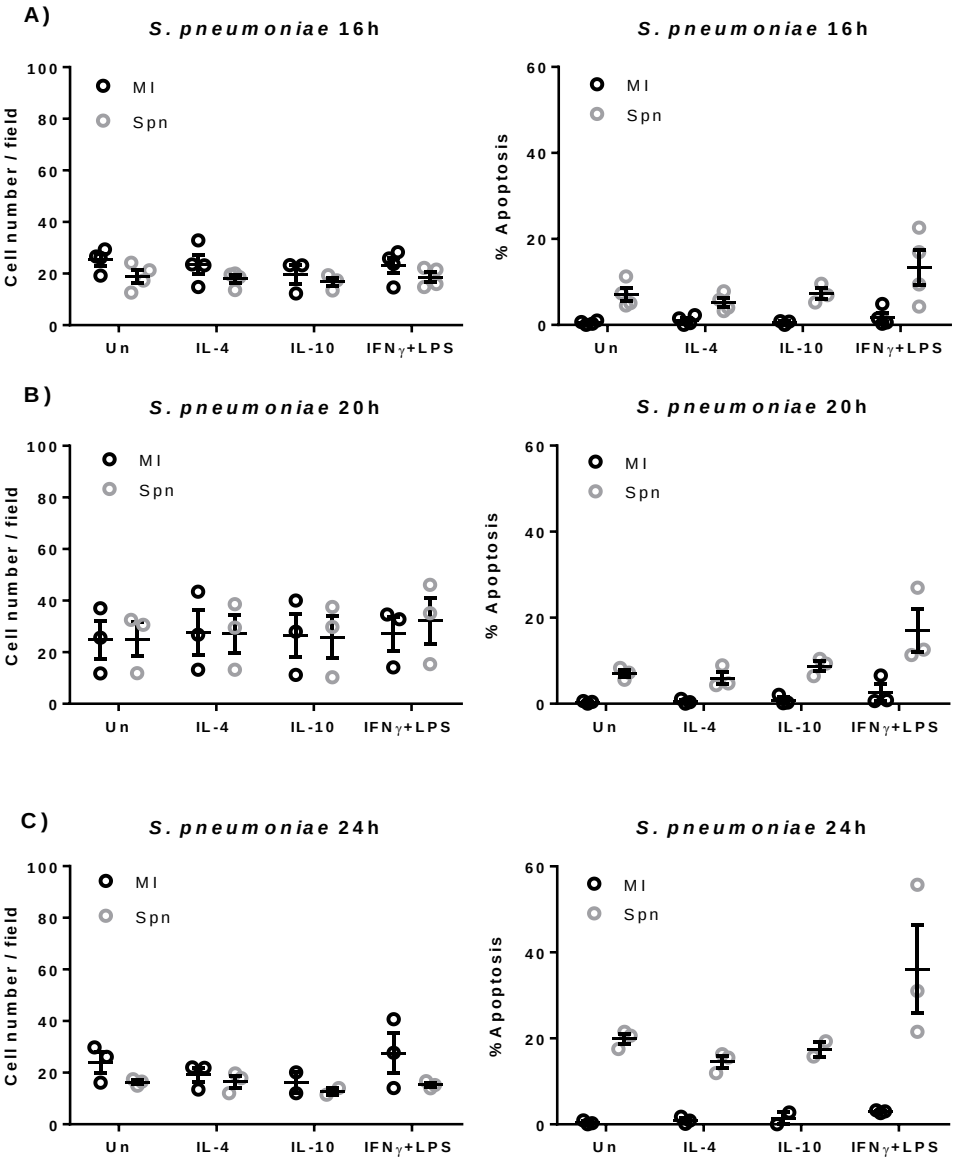


Figure 4-8: IFN-γ + LPS treatment enhances apoptosis at late time points of *S. pneumoniae* infection in MDMs

MDMs were activated with 20ng/ml IL-4, IL-10 or 20ng/ml IFN-γ + 100ng/ml LPS for 24 hours. Cells were washed and challenged with *S. pneumoniae* serotype 1 (MOI 10) or mock infected for a duration of **(A)** 16 **(B)** 20 or **(C)** 24 hours and analysed by microscopy. Cell Density per field was quantified and nuclear fragmentation and condensation by DAPI staining. Data is presented as mean ± SEM. No significance was determined between activation conditions using Kruskal-Wallis with Dunn's post-test (n=3).

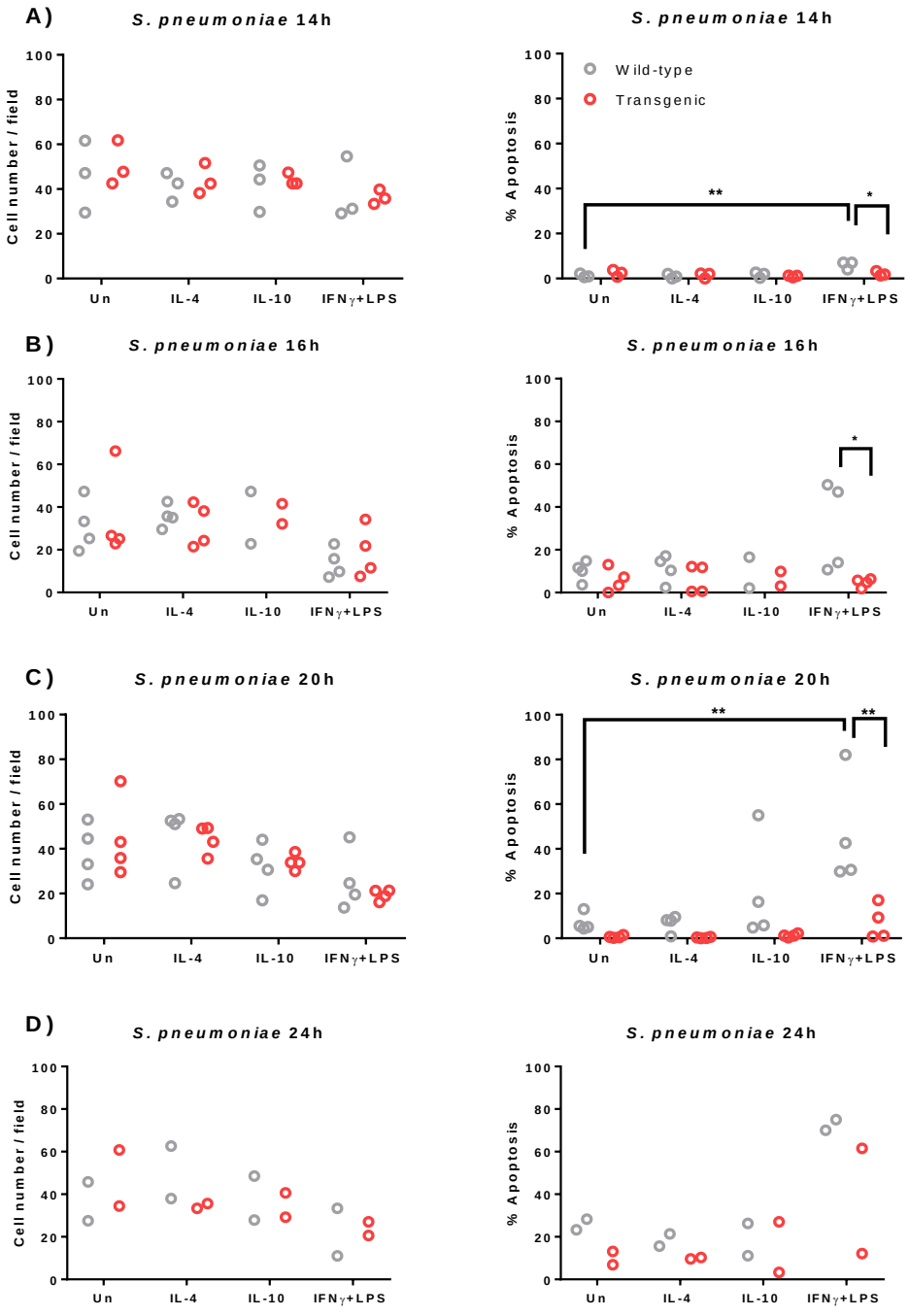


Figure 4-9: IFN-γ + LPS treatment enhances apoptosis at late time points of *S. pneumoniae* infection in BMDMs

Wild-type and transgenic BMDMs were activated with 20ng/ml IL-4, IL-10 or 20ng/ml IFN-γ + 100ng/ml LPS for 24 hours. Cells were washed and challenged with serotype 1, *S. pneumoniae* (MOI 10) for a duration of **(A)** 14, **(B)** 16, **(C)** 20 or **(D)** 24 hours and analysed by microscopy. Cell Density per field was quantified by DAPI staining. Nuclear fragmentation and condensation by DAPI staining was used to quantify apoptosis. Significance was determined using two-way ANOVA with Sidak's post-test, *=p<0.05, **=p<0.01 (n=2-4).

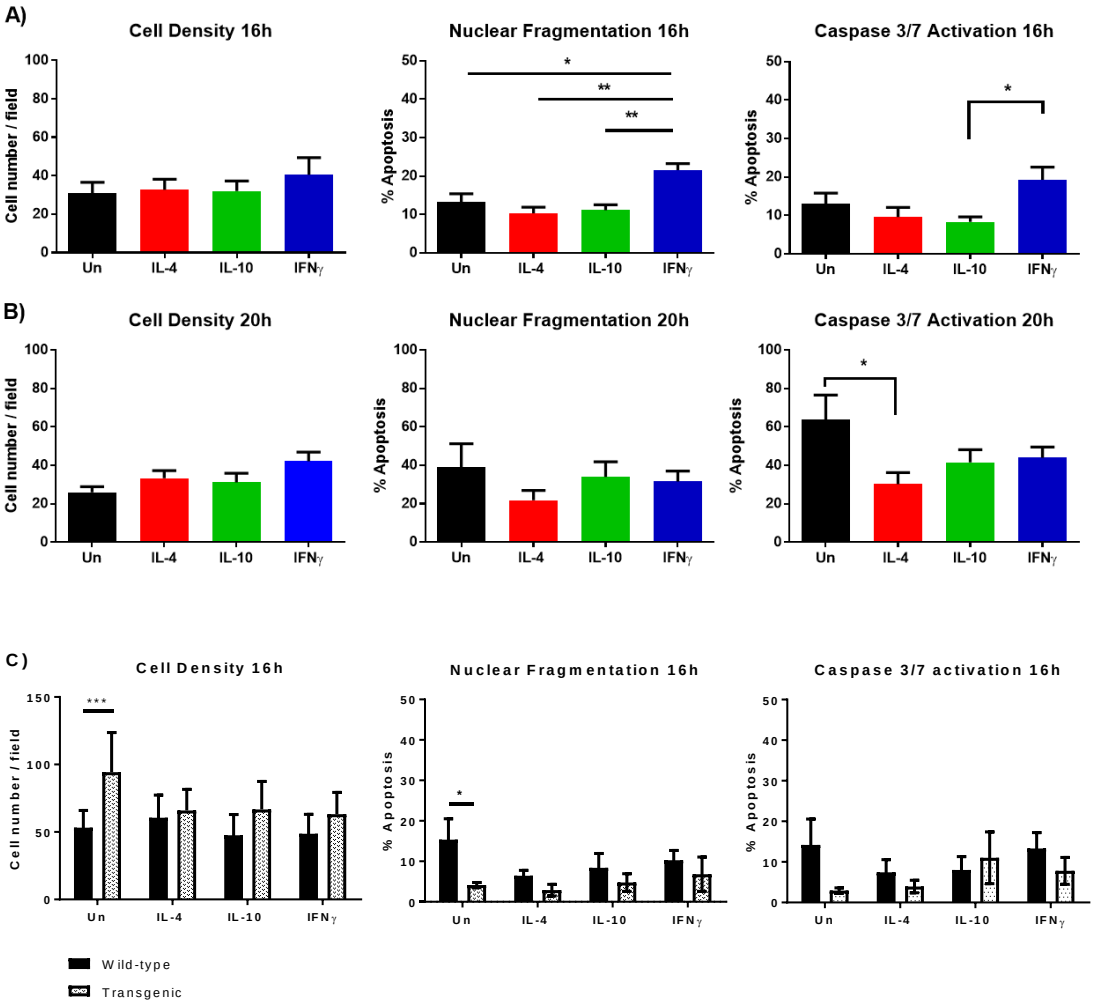


Figure 4-10: IFN-γ activation is associated with increased nuclear fragmentation and caspase 3/7 activation

Human MDMs and murine wild-type and transgenic BMDMs were unstimulated or activated with 20ng/ml IL-4, IL-10 or IFN-γ for 24 hours. **(A-B)** MDMs were washed and challenged with serotype 1, *S. pneumoniae* (MOI 10) for a duration of 16 or 20 hours, and analysed by microscopy for cell density and nuclear fragmentation (DAPI) and caspase 3/7 activation (NucView), data analysed by Kruskal-Wallis with Dunn's post-test (n=6) **(C)** BMDMs were challenged for 16 hours and analysed as above, significance was determined using a two-way ANOVA with Sidak's post-test comparing wild-type and transgenic BMDMs and activation conditions (n=4) *=p<0.05, **=p<0.01, ***=p<0.001, data is presented as mean ± SEM.

4.2.4. CAM show increased killing of pneumococci at late time points

Previously published work has shown increases in macrophage apoptosis at late time points correlate with increases in pneumococcal killing (Dockrell et al., 2001a) through re-engagement of mitochondrial microbicidal mechanisms (Bewley et al., 2017). To understand killing capacity

of activated macrophages at late time points of infection, gentamicin protection assays were conducted on and BMDMs (Figure 4-11) from 16 – 20 hours post infection. I hypothesised that M(IFN-γ) activation would show increases in killing capacity in both wild-type and transgenic BMDMs since apoptosis was increased in both. Results showed an increased trend in killing capacity for IFN-γ stimulated macrophages and this result reached significance in the wild-type BMDMs compared to IL-4 and the transgenic BMDMs compared to all other activation conditions. The increase in killing capacity of M(IFN-γ) transgenic macrophages was not fully consistent with the apoptosis data (Figure 4-10). This unexpected result could be due to insufficient replicates and time points analysed. However, it could also reflect enhanced engagement of canonical killing mechanisms independent of apoptosis (as shown to be increased in Figure 4-1 and Figure 4-2).

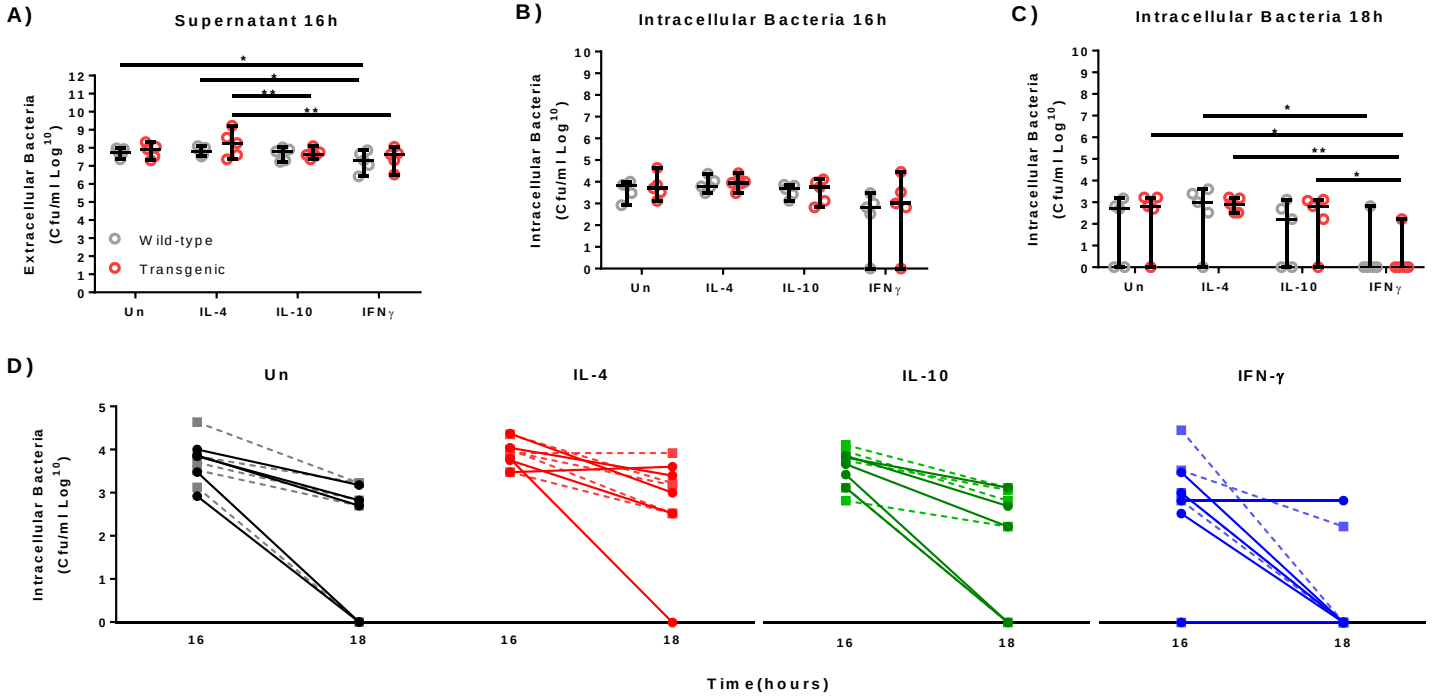
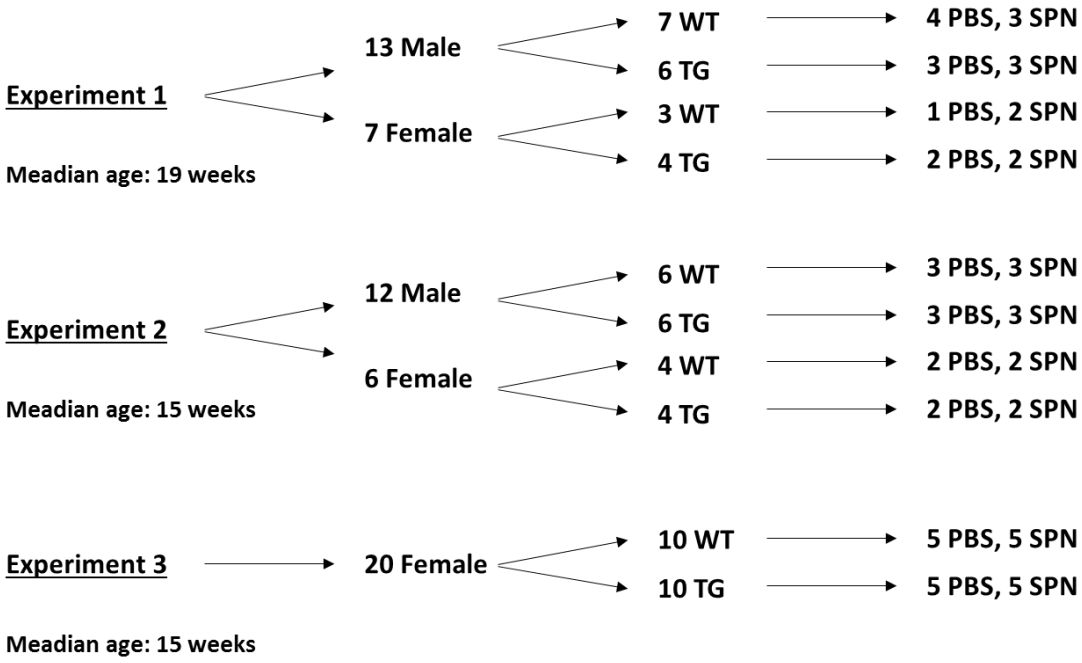


Figure 4-11: Pro-inflammatory macrophage activation enhances Killing of *S. pneumoniae* at late time points Wild-type and transgenic BMDMs were activated with 20ng/ml IL-4, IL-10 or IFN-γ for 24 hours. Cells were washed and challenged with serotype 1, *S. pneumoniae* (MOI 10) for a duration of 16 hours. **(A)** At the 16 hour time point cfu in the cell supernatant was determined by Miles-Misra. **(B)** a gentamicin protection assay was performed, cells were lysed and supernatants serially diluted and plated to determine cfu. **(C)** The remaining cells were incubated in a low dose of vancomycin until the 18 hour time point and lysed to assess the killing of intracellular bacteria. **(D)** A representative plot of the macrophage killing activity between 16 and 18hour time points. The dotted-line represents the transgenic mouse. Data was transformed to logarithmic and no significance determined by repeated measures two-way ANOVA with Sidak's post-test (n=5) *=p<0.05, **=p<0.01.

4.2.5. Transcriptomic analysis reveals T-cell signatures in CD68 Mcl-1 transgenic AMs after 16 hours of *S. pneumoniae* challenge

Some detail of the pathway of apoptosis associated killing has been previously delineated and published (Bewley et al., 2011a; Marriott et al., 2005), however, our group was yet to explore the wider genomic changes associated with apoptosis associated killing in AMs and the differences between wild-type and CD68 Mcl-1 transgenic mice at both baseline and after infection. To further understand the regulation of apoptosis associated killing and the effects of the CD68 Mcl-1 transgene on down-stream signalling, we conducted a transcriptomic analysis in collaboration with GlaxoSmithKline (GSK). RNA was isolated from AMs at 16 hours post intra-tracheal infection with *S. pneumoniae* serotype 1. BAL was initially collected from 3 individual experiments (see detail below), samples were analysed for neutrophil content and mice with >10% were excluded.



The remaining samples were then pooled according to experiment, sex genotype and infection, for example:

Experiment	Sex	Genotype	Infection
1	Male	WT	PBS
1	Male	WT	SPN
1	Male	TG	PBS
1	Male	TG	SPN

This gave a total of 8 samples for experiment 1, 8 samples for experiment 2 and 4 samples for experiment 3, leading to a total of 20 samples from which RNA was then extracted and ran on individual Affymetrix GeneChip Mouse Genome 430 2.0 Array chips and analysed as 20 individual observations.

Background noise was corrected for by robust multi-array average (RMA) correction and probe-sets cut down to give a total of 16,375 variables (Irizarry et al., 2003). Initial analysis was carried out by the bioinformatics department at GSK, including normalisation of data, determination of significant probe sets and pathway analysis. Crucially, the principal component analysis (PCA) revealed that component one accounted for the largest variable in the data-set (27.3%). This variable represents the difference between independent experiments rather than genotype or challenge (Figure 4-12). Contrary to experiment 1 and 2, the outlier, experiment 3, included only female mice. Previous studies have highlighted the importance of sexual dimorphism in immune cell recruitment (Kay et al., 2015), so it is perhaps not surprising to see a large variation in this experiment. When hierarchical clustering was conducted, on all 16,375 probes, observations clustered into individual experiments rather than infection or strain (Figure 4-12). Despite variance, only one sample from experiment 3 did not pass correlation quality control and given the small sample size, it was deemed appropriate to still include this experiment in the initial analysis. Data was fitted to linear regression and the following model used:

Model ~ strain + infection + strain: infection

Factor 1: Strain - wild-type or transgenic

Factor 2: Infection - PBS or *S. pneumoniae*

Factor 1 and 2 were crossed and a type 3 ANOVA was conducted on the data followed by individual t-tests on each of the following experiments:

1. **PBS** => Wild-type vs Transgenic
2. ***S. pneumoniae*** => Wild-type vs Transgenic
3. **Wild-type** => *S. pneumoniae* vs PBS
4. **Transgenic** => *S. pneumoniae* vs PBS

P-value data to the level of 5% significance was computed and a false discovery rate (FDR)[1] correction using the Bonferroni – Holm method was also used with a cut-off level of 5% as a method of multiple test correction, however, FDR analysis returned only one or two significantly

[1] GSK analysis initially returned FDR significant gene statistics, which were then used for pathway analysis, however the complete list of significant genes was not recieved and findings could not be exactly replicated at Sheffield.

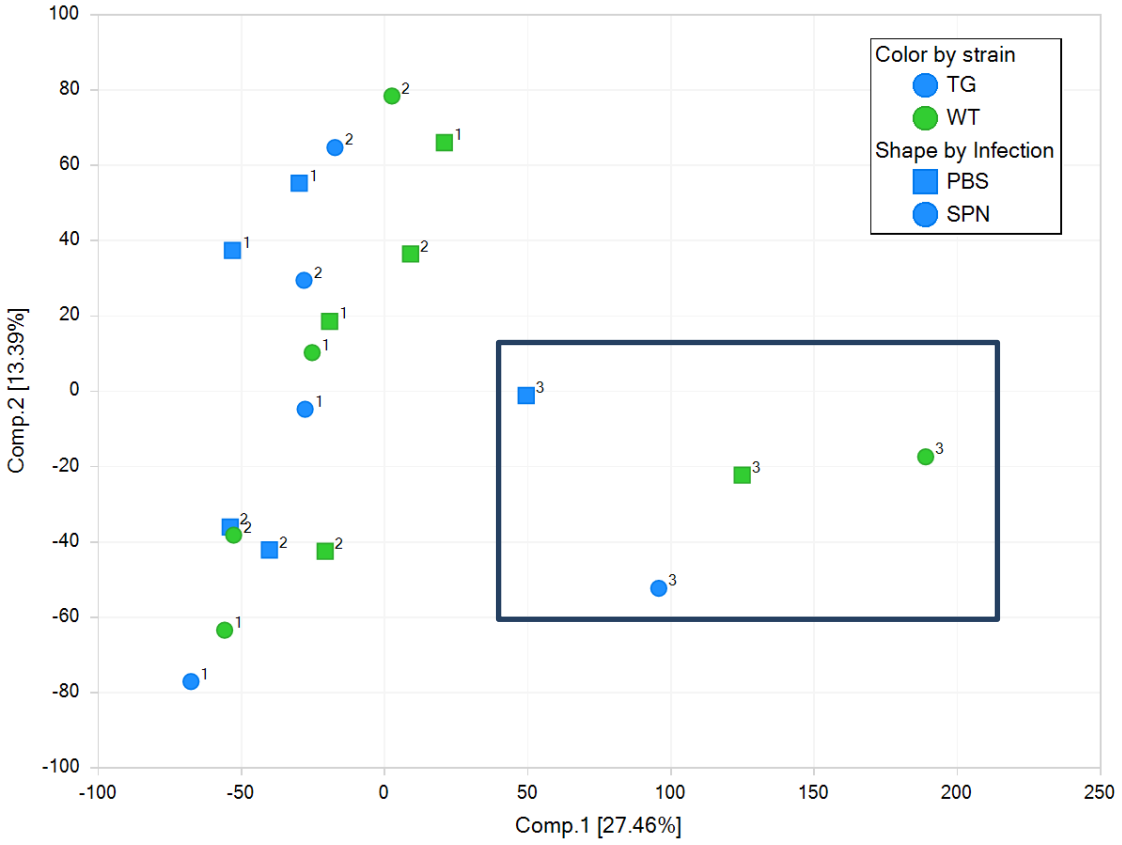
changed genes (Table 4-1) so this cut-off was not applied to this dataset and significantly changed genes were interpreted by p<0.05 with a total fold change of > +/- 1.5 visualised by volcano plots in Figure 4-13.

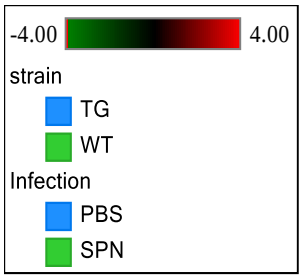
Table 4-1: Significantly altered genes

	FC>1.5_ p<0.05	**FC<-1.5_ p<0.05**	**FC>1.5_ FDR<0.05**	**FC<-1.5_ FDR<0.05**
PBS -> WT vs TG	16	79	0	0
SPN -> WT vs TG	7	38	0	1
TG -> SPN vs PBS	24	24	1	0
WT -> SPN vs PBS	19	14	0	0

Significantly altered genes from each experiment were then further analysed using pathway analysis software (KEGG, Metabase, NextBio). It was expected that significant hits would include genes involved in the macrophage immune response pathways, however results showed surprisingly that the most significantly altered genes in *S. pneumoniae* challenged wild-type vs transgenic macrophages were indicative of a T-cell signature in transgenic *S. pneumoniae* challenged samples (Figure 4-14). This result was unanticipated as cytospin analysis of BAL samples revealed no evidence of infiltrating lymphocytes. Significantly altered genes included key components of T-cell signalling pathways; CD278 – inducible T-cell co-stimulator (ICOS), CD90 - (Thy1) a pan murine T-cell marker, lymphocyte-specific protein tyrosine kinase (Lck) involved in T-cell receptor signalling, Signalling lymphocytic activation molecule 1 (Slamf-1) a T-cell surface glycoprotein, T-cell receptor β chain (TCR-β) and CD247 – T-cell receptor T3 zeta chain. It is important to note that most of these genes have also been observed in myeloid cell signalling so it is also a possibility that the T-cell signature might be attributable to macrophages expressing T-cell associated genes. There were no significant levels of T-cells present in the BAL when analysed by cytospin which supports T-cell signature gene expression by macrophages however further analysis of cell populations is needed to understand the presence and contributions of each cell type to the infection site.

A)


Color by strain
TG
WT
Shape by Infection
PBS
SPN
Comp.2 [13.39%]
Comp.1 [27.46%]
100
80
60
40
20
0
-20
-40
-60
-80
-100
-100
-50
0
50
100
150
200
250


-4.00
4.00
strain
TG
WT
Infection
PBS
SPN

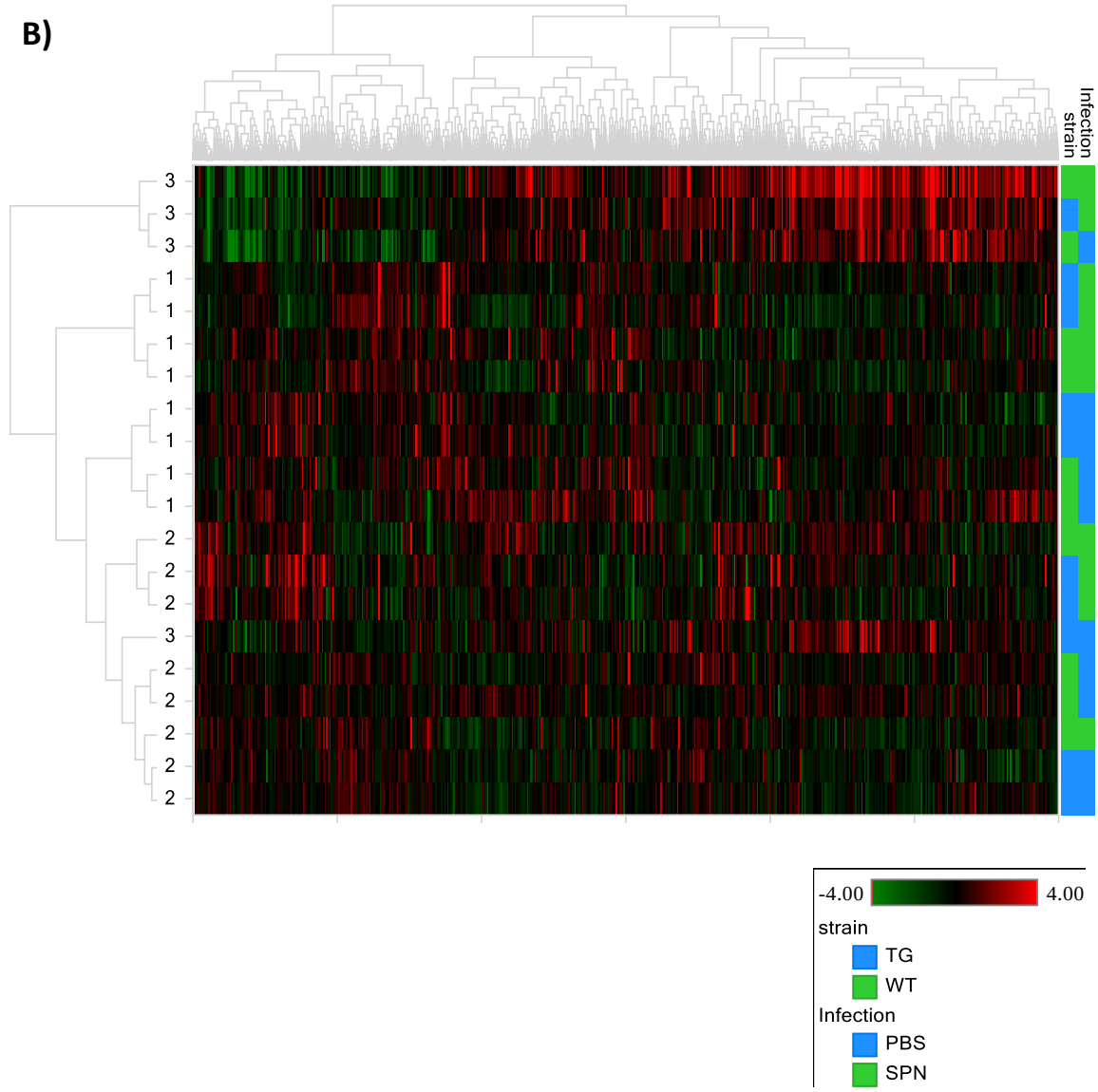


Figure 4-12: Principal component analysis (PCA) and hierarchical clustering

PCA was carried out on the complete list of experiments (20 observations) using Array Studio software. The blue box highlights experiment 3 as an outlier. Complete-linkage hierarchical clustering was observed on the RMA corrected list (16375 variables, 20 observations) using Pearson-correlation co-efficient on both the observation and variable tree. Dendrograms indicate clustering.

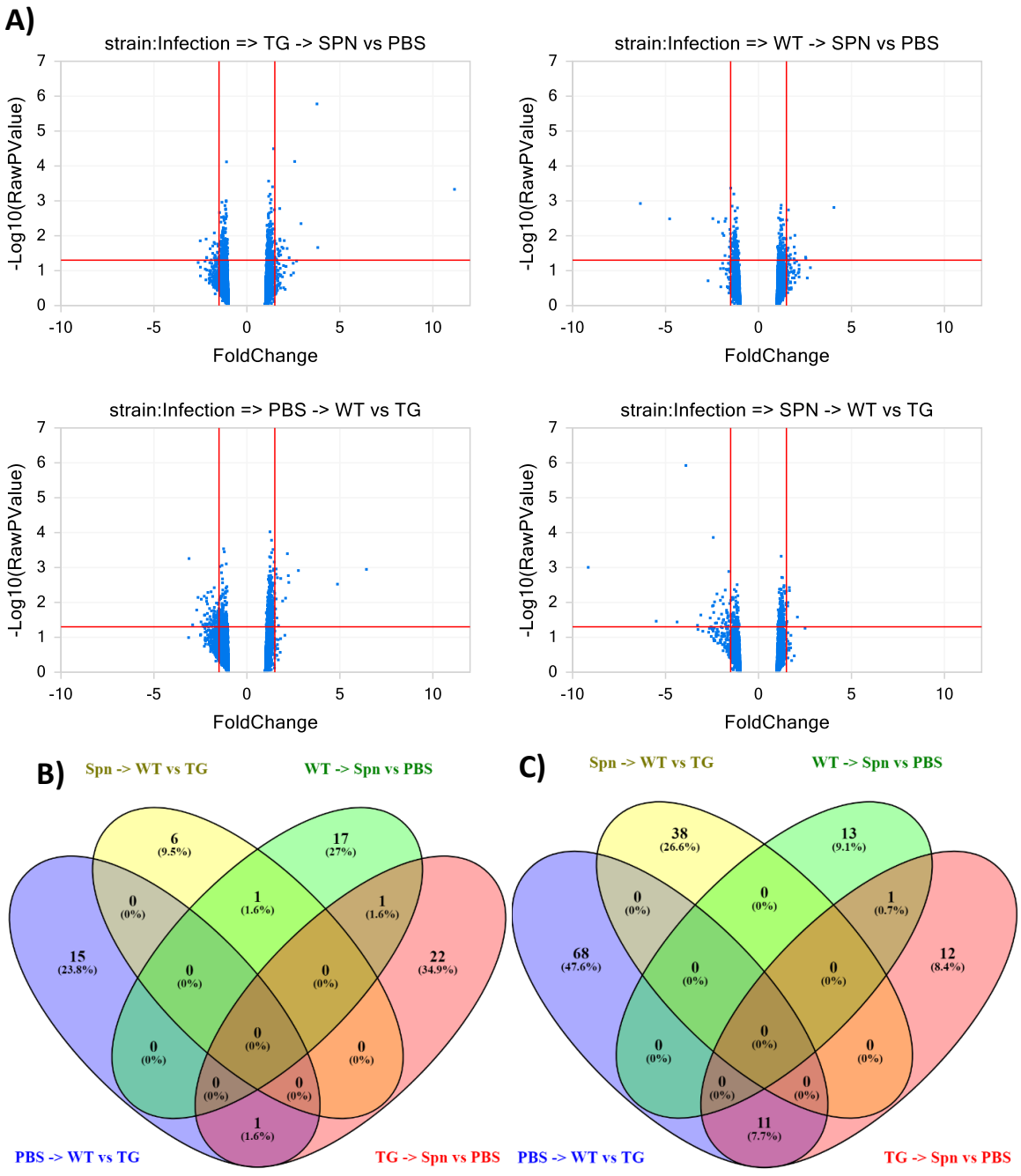
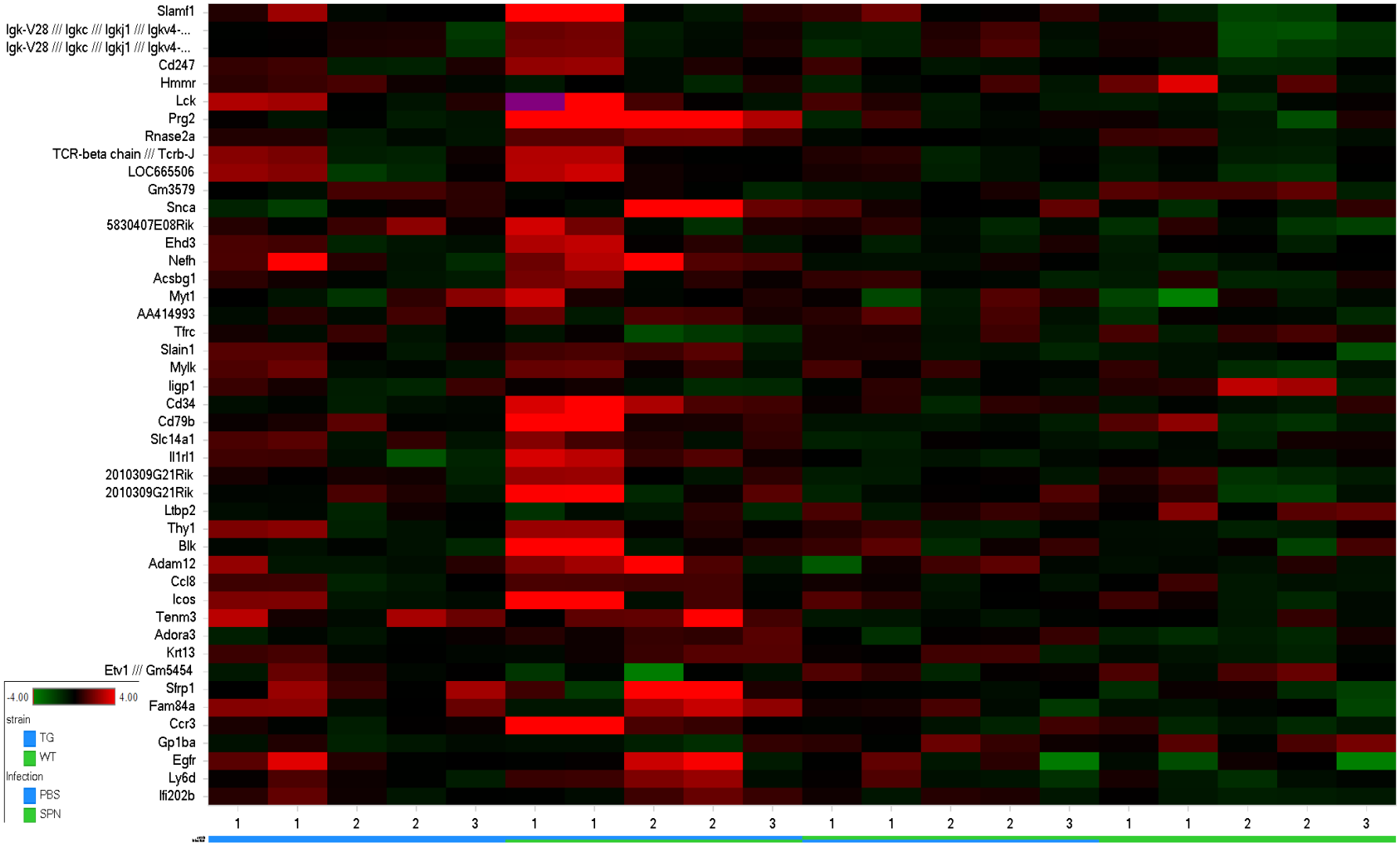


Figure 4-13: Visualisations of significantly altered genes in experiments 1-4

Data was firstly cut down from 41.5K to 16K probes and then the RMA correction method used to eliminate background noise. The data set was then analysed for significantly changed probes of p<0.05 and +/- 1.5-fold change. **(A)** volcano plots of 16K probe sets included in the report, red lines indicate p<0.05 and +/- 1.5-fold change cut offs. **(B)** Positively altered gene changes **(C)** negatively altered gene changes from each of the four experiments based on a p value of <0.05 and +/- 1.5-fold change. All data analysed using Array Studio software. Venn diagram data represented using Venny 2.1.

A)


Slamf1
Igk-V28 /// Igkc /// Igkj1 /// Igkv4-...
Igk-V28 /// Igkc /// Igkj1 /// Igkv4-...
Cd247
Hmmr
Lck
Prg2
Rnase2a
TCR-beta chain /// Tcrb-J
LOC665506
Gm3579
Snca
5830407E08Rik
Ehd3
Nefh
Acsbg1
Myt1
AA414993
Tfrc
Slain1
Mylk
Iigp1
Cd34
Cd79b
Slc14a1
Il1rl1
2010309G21Rik
2010309G21Rik
Ltbp2
Thy1
Blk
Adam12
Ccl8
Icos
Tenm3
Adora3
Krt13
Etv1 /// Gm5454
Sfrp1
Fam84a
Ccr3
Gp1ba
Egfr
Ly6d
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1 1 2 2 3 1 1 2 2 3 1 1 2 2 3 1 1 2 2 3

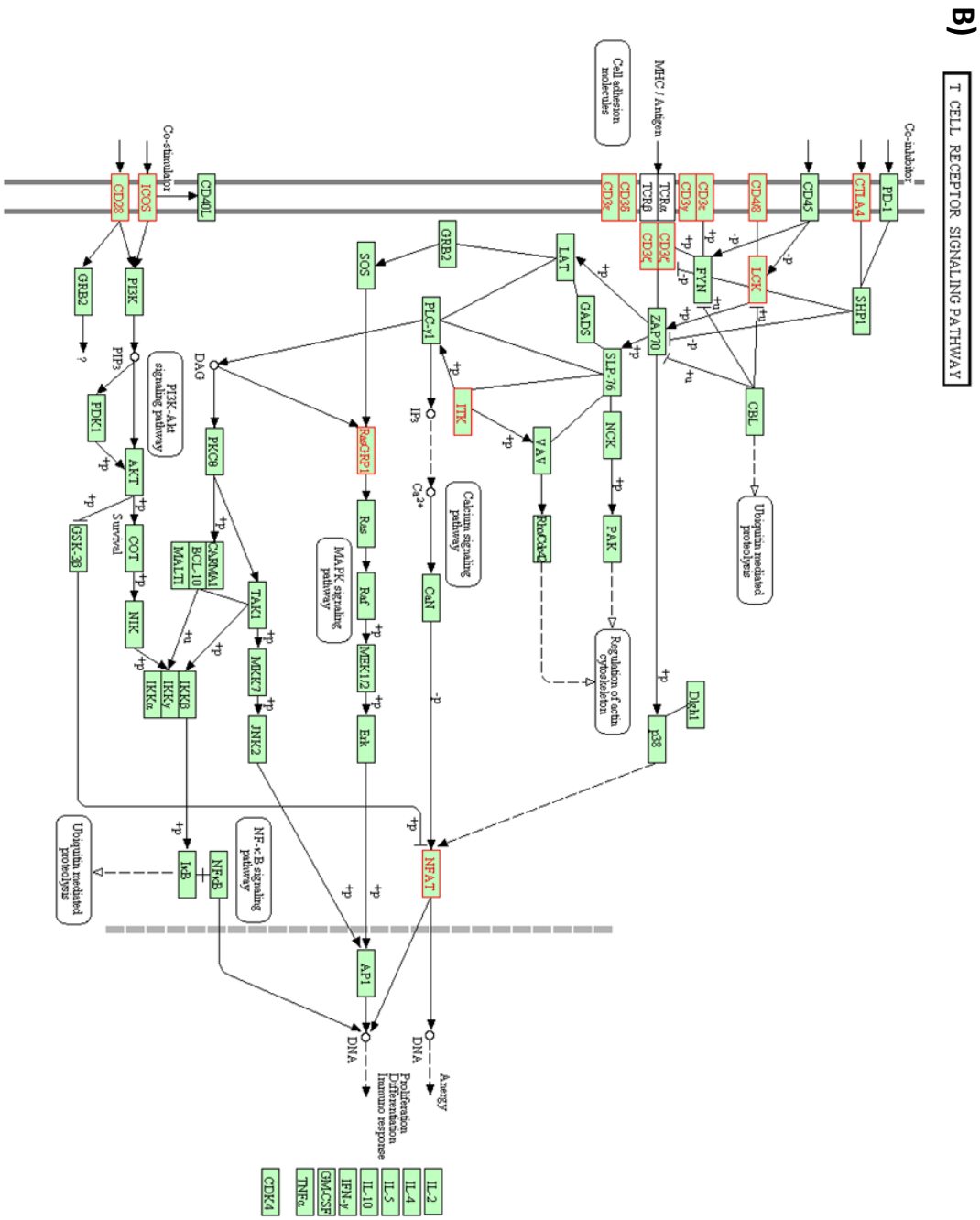
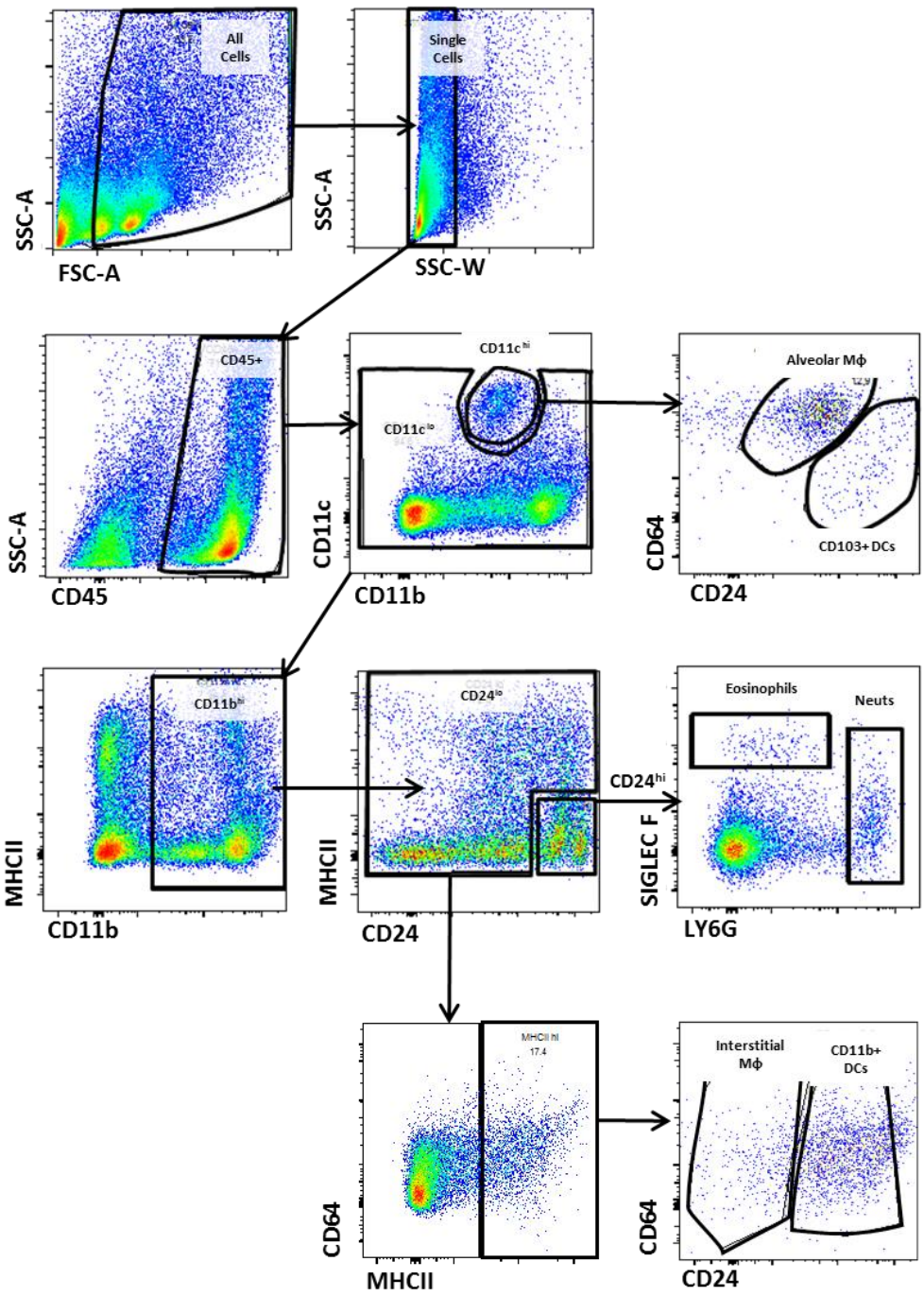


Figure 4-14: Heat map and KEGG pathway visualisation of significantly altered genes in *S. pneumoniae* => wild-type vs transgenic experiment.

(A) Significantly altered genes at the cut off level of p<0.05, fold change +/-1.5 indicate up-regulation of T-cell signalling pathways in transgenic samples. T-cell genes are highlighted in red. Samples are ordered by infection, genotype (strain) and individual experiment. **(B)** KEGG pathway analysis reveals genes involved in T-cell signalling pathways (red) – obtained from the original GSK report.

4.2.6. Multi-colour flow cytometry analysis does not identify a difference in specific cell populations between wild-type and transgenic AMs at 16 hours of *S. pneumoniae* challenge

To validate if the T-cell signal was associated with T-cell recruitment to the mouse lungs of transgenic mice, I conducted multi-colour flow cytometry analysis of myeloid and T-cell subsets in BAL fluid and lung tissue from *S. pneumoniae* challenged animals at 16 hours post inoculation. Myeloid flow cytometry analysis was based on a published panel created by Misharin and colleagues (Misharin et al., 2013), the T-cell panel was adapted from a protocol developed by our collaborators at GSK. Gating strategies for the myeloid and T-cell panel can be seen in Figure 4.15 and Figure 4.16 respectively. I hypothesised that if T-cell recruitment were present it was most likely to be Th1 or Th17 helper cells given the important role these T-cell subsets play in the resolution of pneumococcal infection and colonisation (Kemp et al., 2002; Olliver et al., 2011; Zhang et al., 2009). Furthermore, work presented earlier in this chapter indicates IFN-γ plays a central role in killing of intracellular bacteria at both early and late time points of pneumococcal infection and that IFN-γ could potentially help to restore the killing defect in the presence of the Mcl-1 transgene. It was therefore interesting to speculate whether transgenic macrophages required pro-inflammatory T-cell subsets producing pro-inflammatory agents such as IFN-γ to prime the AMs into an optimal activation state which might encourage increased killing to tackle pneumococcal infection. This hypothesis was based on the fact that bacterial clearance is impaired in transgenic AMs compared to wild-type macrophages (Bewley et al., 2017). The image below demonstrates this hypothetical model. There was not expected to be any change in myeloid recruitment between PBS treated wild-type and transgenic mice as Mcl-1 over expression is macrophage specific, however the transcriptomic study showed parts of the T-cell signature up-regulated in the PBS treated transgenic mouse (experiment 1) as well as the *S. pneumoniae* infection condition. While the presence of the CD68 Mcl-1 transgene showed no difference in any cell populations in the digested lung or the BAL fluid in either panel (Figure 4.17, 4.18), there was a high presence of neutrophils in the BAL fluid of both wild-type and transgenic infected mice. This is unusual as the low dose of infection used should not induce recruitment of neutrophils in the wild-type mouse as the presence of AMs should be sufficient to clear infection without polymorphonuclear (PMN) cell recruitment, (Dockrell et al., 2003). It is possible that recruitment of PMN indicates that a higher dose of pneumococci reached the lungs, or that the mice were more susceptible or the bacterial strain more virulent than intended and this recruitment prevented the need of a T-cell intervention to resolve the infection in transgenic macrophages. However, cfu calculations from terminal bleeds showed no evidence



of bacteraemia which is characteristic of a high dose of infection (Figure 4-19) (Dockrell et al., 2003). It is noteworthy that these experiments required use of a new stock of bacteria after failure of both the primary freezer and liquid nitrogen stores in rapid succession wiped out the original stock and a licencing change prevented use of the original strain from Statens Serum Institut. So, it is likely that this infection resulted in slightly greater stimulus for myeloid cell recruitment than intended and had exceeded the tipping point being modelled in the transcriptomic experiment, masking the ability to detect the lymphocyte recruitment.

Figure 4-15: Myeloid panel gating strategy

Cells were isolated from enzymatically digested mouse lungs. Debris events were first separated using side-scatter – area (SSC-A) and forward scatter – area (FSC-A) characteristics. Single cells were then gated using SSC-A and side scatter – width (SSC-W) characteristics. Immune cells were selected using CD45 staining. AM (Mϕ) and CD103+ DCs were then identified by isolating a $CD11c^{hi}$ population and separating based on CD64 or CD24 positive staining respectively. The $CD11b^{hi}$ population was then selected from $CD11c^{lo}$ and then the $CD24^{hi}$ population stained for Siglec F (eosinophils) or Ly6G (neutrophils). MHC class II positive cells were identified from the remaining $CD24^{lo}$ population and separated based on CD64 positivity (Interstitial macrophages) or CD24 positivity (CD11b+ DCs).

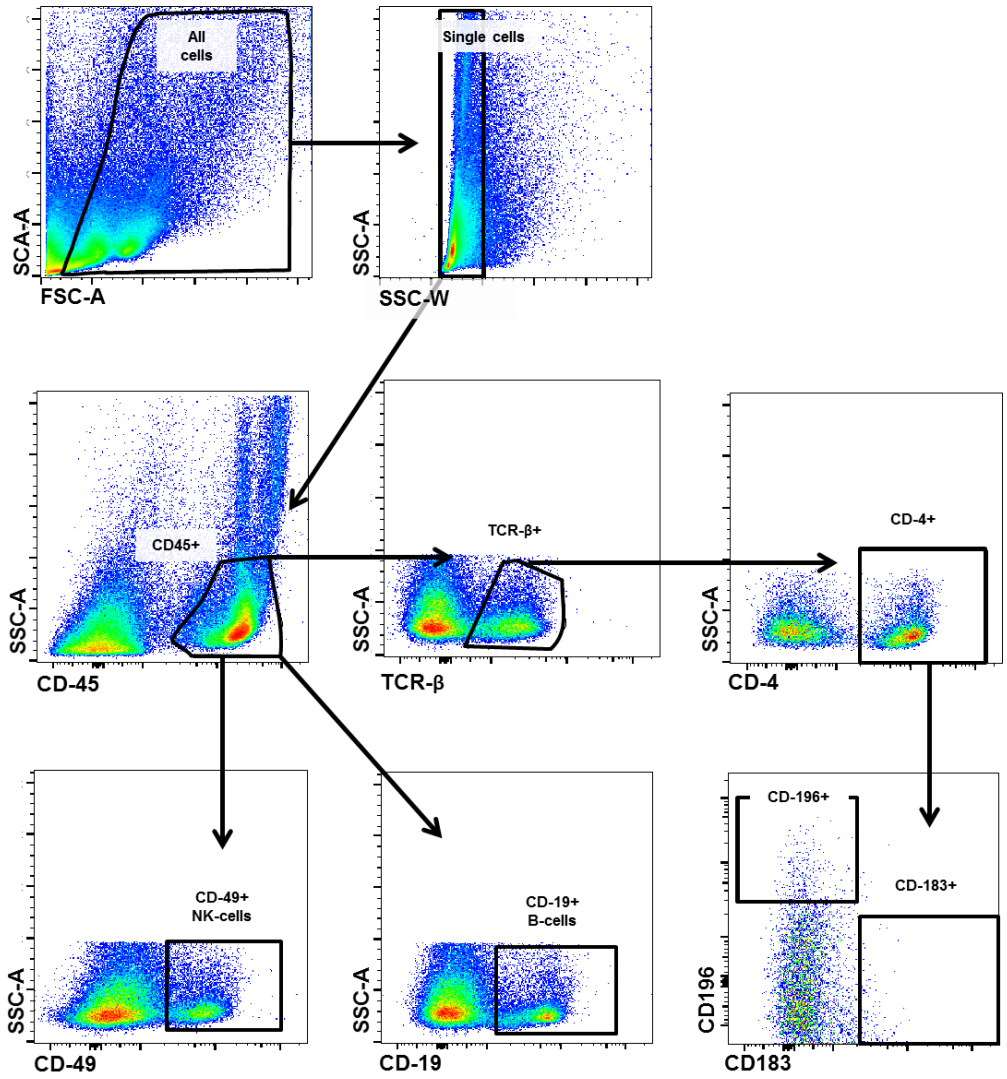


Figure 4-16: T-cell flow panel gating strategy

Cells were isolated from enzymatically digested mouse lungs. Debris events were first separated using side-scatter – area (SSC-A) and forward scatter – area (FSC-A) characteristics. Single cells were then gated using SSC-A and side scatter – width (SSC-W) characteristics. Immune cells were selected using CD45 staining. NK cells and B-cells were then identified by CD49 and CD19 staining respectively. The gating strategy to identify differential T-cell populations is as follows: T-cells were gated from CD45+ cells using TCR-β as a marker of the T-cell receptor. From the T-cell population, CD4+ T-cells were gated to identify the T-helper cell population. Cells were then separated as CD183+ (Th1 cell marker) or CD196+ (Th17 cell marker).

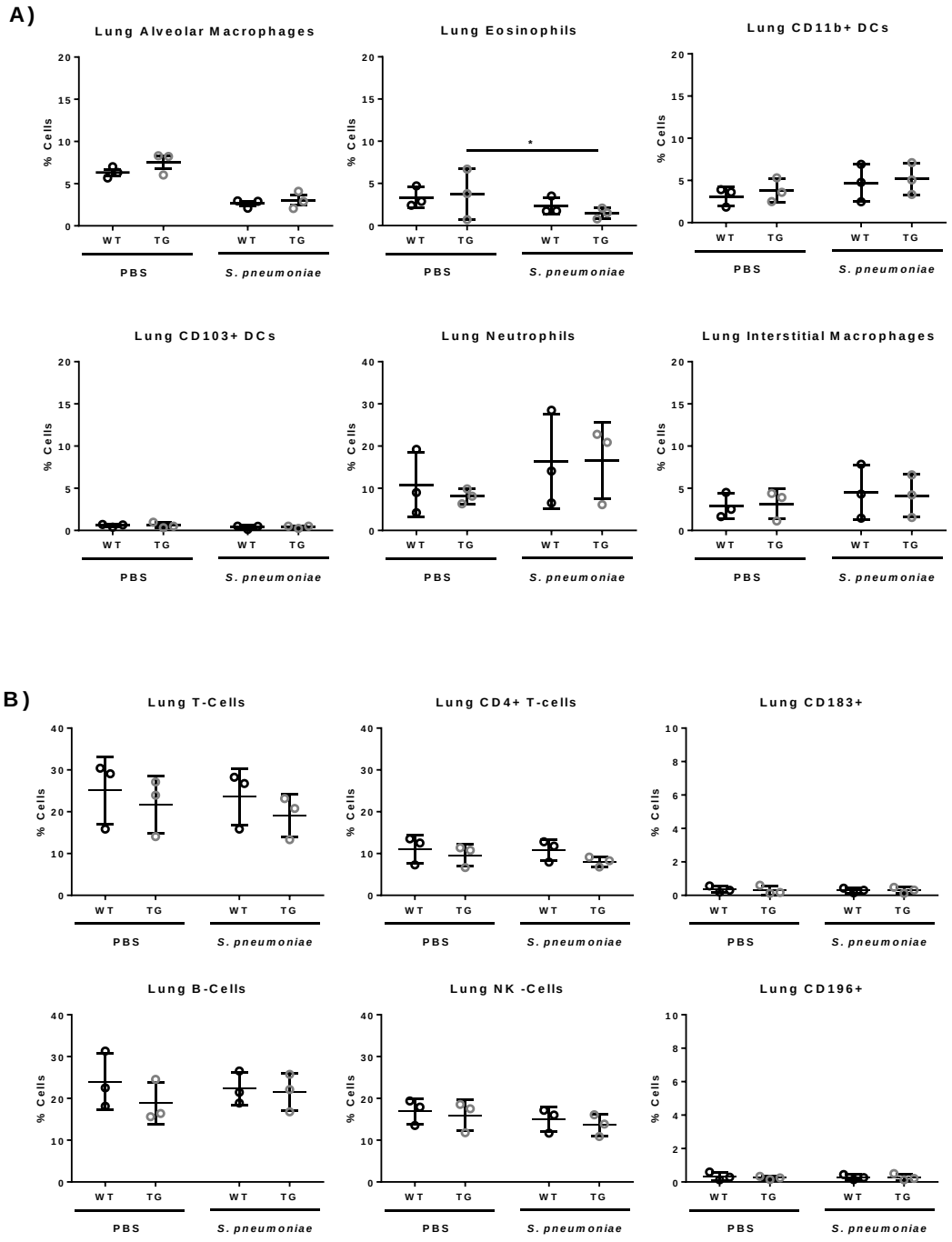


Figure 4-17: Percentage populations of T-cells and myeloid cells in enzymatically digested mouse lung.

Wild-type and transgenic mice were challenged with *S. pneumoniae* serotype 1, MOI 10, for a duration of 16 hours or mock challenged with PBS. At the time point, lungs were extracted and digested cells were extracted and stained with fluorescent antibodies and analysed on the LSRII flow cytometer. Total cell percentages from the CD45 positive population were calculated for **(A)** myeloid subsets using the myeloid gating strategy described in Figure 4-15 and **(B)** T-cell populations using the T-cell getting strategy described in Figure 4-16. Data analysed using repeated measures two-way ANOVA with Sidak's multiple comparison test, data is represented as mean ± SD (n=3) *=p<0.05.

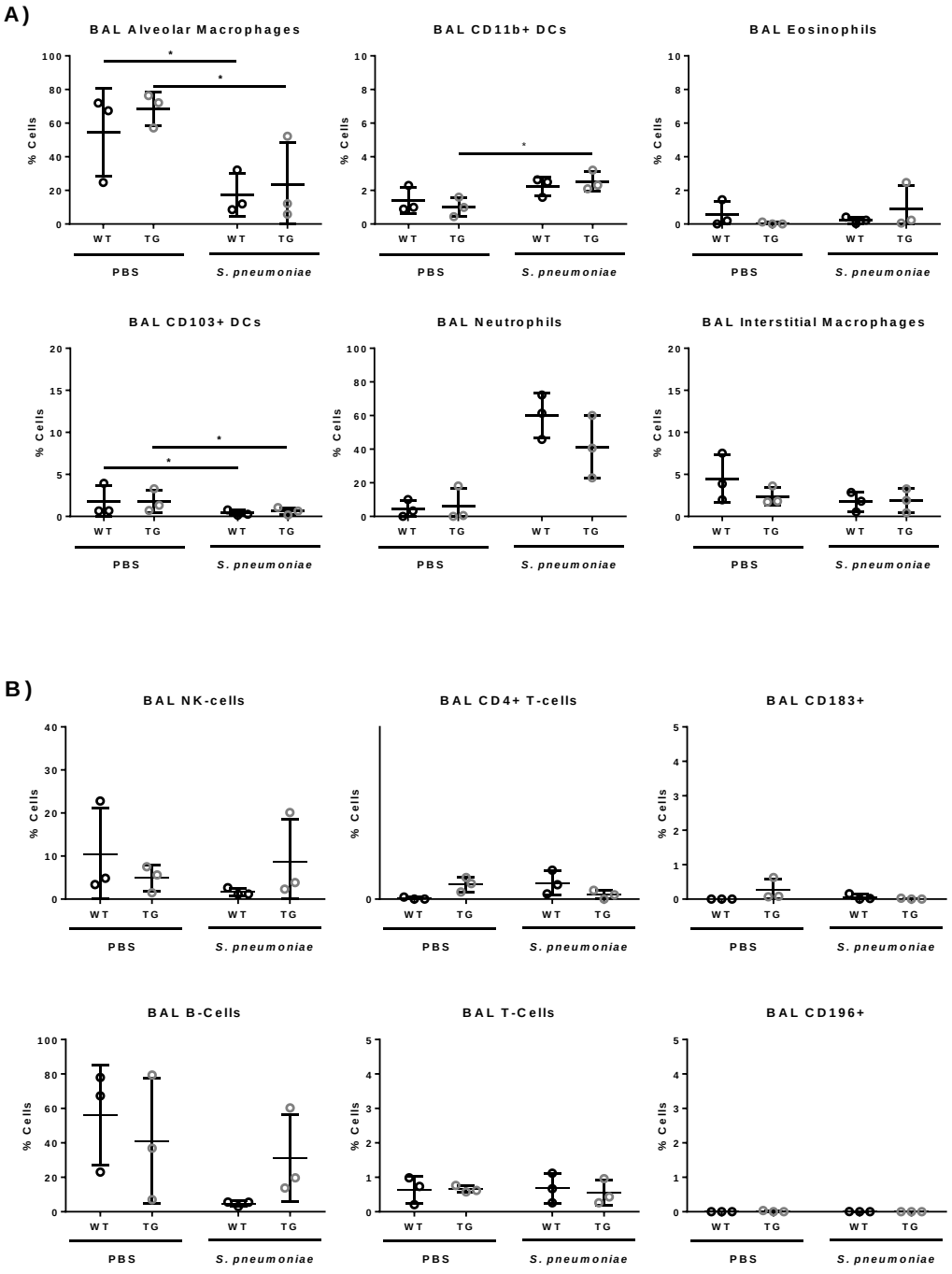


Figure 4-18: Percentage populations of T-cells and myeloid cells in murine BAL

Wild-type and transgenic mice were challenged with *S. pneumoniae* serotype 1, MOI 10, for a duration of 16 hours or mock challenged with PBS. At the time point, broncho alveolar lavage (BAL) fluid was collected. Cells were extracted and stained with fluorescent antibodies and analysed on the LSRII flow cytometer. Total cell percentages from the CD45 positive population were calculated for **(A)** myeloid subsets using the myeloid gating strategy described in Figure 4-15 and **(B)** T-cell populations using the T-cell getting strategy described in Figure 4-16. Data analysed using repeated measures two-way ANOVA with Sidak's multiple comparison test, data is represented as mean ± SD (n=3) *=$p<0.05$.

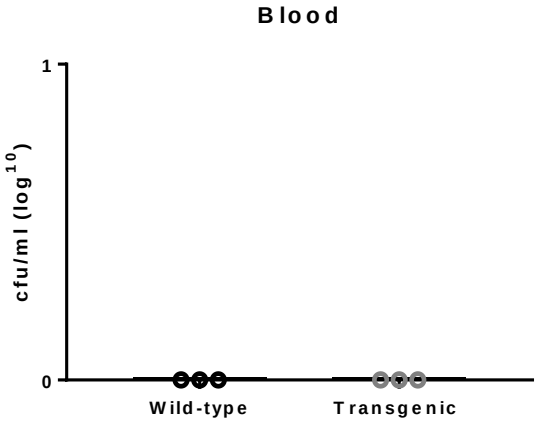


Figure 4-19: Infection does not cause bacteraemia in the wild-type or transgenic mouse.

Wild-type and transgenic mice were infected with 10^4 *S. pneumoniae* serotype 1, MOI 10, for a duration of 16 hours. At the point mice were culled and blood samples collected to assess presence of bacteria in the blood. Blood was then diluted and plated using the Miles-Misra technique. No bacterial growth was observed after overnight incubation.

4.3. Discussion

The key finding of this chapter is the demonstration of the role of IFN-γ in the innate immune response to pneumococcal challenge. Classical activation programs which are stimulated by infection with Gram positive, Gram negative and mycobacteria are considered to occur in response to bacterial infection (Nau et al., 2002). Nau and colleagues demonstrate bacterial products which are shared with *S. pneumoniae* such as lipoteichoic acid, can induce pro-inflammatory signalling pathways through TLR2 activation. *S. pneumoniae* also express peptidoglycan and lipoprotein components which are other known activators of Nod2 and TLR2 signalling (Davis et al., 2011; Koppe et al., 2012; Opitz et al., 2004). Therefore, it is reasonable to assume that *S. pneumoniae* challenge would stimulate a CAM response. However, the chemical structure of lipoteichoic acid can vary largely between bacterial species and indeed between serotypes of *S. pneumoniae* (Draing et al., 2006). Also, others have shown association of some bacterial species with alternative activation programs (Goldmann et al., 2007) either as a mechanism of host survival (Newton et al., 2000) or as a method of microbial interference to promote bacterial persistence (Benoit et al., 2008a). Regardless of the overall activation state induced by *S. pneumoniae* it is also important to determine the potential effect of differing

activation states on pneumococcal clearance, particularly as the activation state could be altered by co-infections such as parasitic infection or by diseases such as asthma, both which are associated with alternative activation, (Girodet et al., 2016; Noël et al., 2004). Therefore, I was interested in analysing how macrophage activation states would affect the host response to pneumococcal infection. As discussed elsewhere in this thesis, macrophage activation is an important contributor to host health and disease, using an *in vitro* infection model where macrophages are first activated before inoculation, which reflects the *in vivo* scenario where resident tissue macrophages are polarised to extreme phenotypes by the presence of disease and are then tasked with handling bacterial challenge. Furthermore, the presence of the CD68 Mcl-1 transgene puts this model into the context of a COPD and HIV-1 infection where there is an apoptosis defect in AM (Bewley et al., 2017). As hypothesised, evidence presented in the first section of this chapter suggests macrophages appear to clear pneumococci more effectively, at early time points, when pre-stimulated into a classical phenotype. There is no difference in clearance between wild-type and transgenic macrophages as expected as at these early time points macrophages will not be undergoing apoptosis associated killing and therefore the effect of Mcl-1 over-expression will be negligible.

There is conflicting evidence for the effect of macrophage activation on phagocytosis, owing in part to the complexity of this macrophage effector function. *S. pneumoniae* is internalised by a variety of mechanisms including Fcγ, complement and scavenger receptors (Gordon et al., 2000). IL-4 activation increases CD206 expression (Stein et al., 1992) yet some suggest IL-4 activation decreases macrophage internalisation (Martinez and Gordon, 2014; Varin et al., 2010). Other alternative stimuli such as IL-10 increase Fcγ receptor mediated uptake while IFN-γ activation has been shown to inhibit it (Frausto-Del-Río et al., 2012a). Furthermore, IFN-γ activation has been shown to decrease complement mediated and scavenger receptor mediated internalisation (Metzger, 2009; Schlesinger and Horwitz, 1991b), albeit it is important to consider that the latter study was conducted in the context of viral infection which has been shown to cause defective bacterial phagocytosis in macrophages (Cooper et al., 2016). Alternatively, there is evidence that Fc receptor expression for the ingestion of IgG opsonised particles is increased in CAM (Beyer et al., 2012). In addition, MDMs primed with recombinant IFN-γ display increased association and internalisation of *Salmonella enterica* serovar Typhimurium in the absence of opsonisation indicating IFN-γ might enhance bacterial internalisation by promoting direct interaction with non-opsonic phagocytic receptors (Gordon et al., 2005). Latex bead experiments showed no difference in phagocytosis between all activation conditions. This suggests the reason for lower recovery of pneumococci from CAM is

due to increased trafficking and killing of bacteria in the first hour of internalisation (macrophages are placed on ice for 1 hour immediately prior to inoculation to allow for bacterial adhesion, during which time no internalisation takes place), this increased killing activity is in line with other reports (Gordon et al., 2005; Mosser and Edwards, 2008). However phagocytosis was only examined in human macrophages and since there are discrepancies between human and murine phagocytic receptor expression and immunology in general, it would be beneficial to check the effects of activation on phagocytosis of *S. pneumoniae* in BMDMs also (Mestas and Hughes, 2004).

Despite increased killing, M(IFN-γ) showed no significant increase in ROS or NO levels at 4 hours after pneumococcal challenge which was unexpected given the relationship between classical activation and up-regulation of genes involved in microbicidal activity (Mantovani et al., 2004). Human macrophages have been suggested to be incapable of producing as large amounts of NO as murine macrophages do (Gordon et al., 2005; Jesch et al., 1997), and NADPH-derived ROS is not necessary for pneumococcal killing (Biggar et al., 1976; Marriott et al., 2007). Further, NO utilisation as a macrophage killing mechanism is thought to be limited until later during infection (Marriott et al., 2004) therefore we might not expect to see a significant signal at this early time point. Interestingly, members of our group have highlighted that it is not the amount of ROS generated in the cell which is key to pneumococcal killing, rather it is localisation of ROS adjacent to the phagolysosome (Bewley et al., 2017). Therefore, it is possible that classical activation by IFN-γ stimulation can induce more efficient bacterial killing by localising ROS production to the phagosome without significantly increasing overall abundance. Alternatively, my results could reflect increased production of other anti-bacterial factors such as anti-microbial peptides or proteases such as lysozyme M which has been shown to be critical for digestion of *S. pneumoniae* peptidyglycan and to subsequent stimulation of Nod2 and pathogen clearance (Davis et al., 2011). Furthermore, *S. pneumoniae* has been shown to induce release of the antimicrobial peptides, β-defensins which have been shown to be up-regulated in response to IFN-γ (Fabri et al., 2011; Scharf et al., 2012).

As described, at late time points during pneumococcal challenge macrophages utilise apoptosis associated killing as a mechanism of host defence (Dockrell et al., 2001a). Since macrophage activation is an integral part of macrophage function, I hypothesised macrophage activation might have some impact on macrophage apoptosis associated killing. IFN-γ showed increased apoptosis following pneumococcal challenge in both the presence and the absence of LPS indicating classical activation enhances apoptosis associated killing. IFN-γ and LPS have both

been shown to reduce cell viability individually and in combination in my own data (chapter 3) and in reports by others (He et al., 2008). These observations raise the question of whether IFN-γ stimulated apoptosis is linked to the apoptosis cascade we see at late time points of bacterial infection, which is triggered in response to intracellular bacteria described previously (Marriott et al., 2005), or occurs by a separate manner, which is an important consideration since there were some suggestions of increased cell death in transgenic cells after pneumococcal challenge. However, I have observed no evidence of apoptosis associated killing at early time points of mock infection in MDMs and BMDMs or challenge at 4 hours (BMDMs), indicating M(IFN-γ) is not sufficient to induce the apoptosis cascade without prolonged challenge with bacteria. Furthermore, my late time point data shows that M(IFN-γ) challenged macrophages display nuclear fragmentation with increased levels of caspase 3/7 activation which are core features of the apoptotic program associated with the apoptosis associated killing. Since IFN-γ is an inducer of microbicidal activity, stimulation may accelerate standard killing mechanisms and therefore apoptosis associated killing is not required. Further, it might be possible that the accelerated apoptosis we see in M(IFN-γ) wild-type cells which looks to also increase pneumococcal killing, begins to occur in transgenic M(IFN-γ) cells by IFN-γ related mechanisms overwhelming the inhibition of apoptosis by the transgene. In keeping with this view there was some evidence of increased apoptosis in some data points in Figure 4-9 and Figure 4-10. It would be beneficial to analyse the kinetics of killing and apoptosis over an extended time course to gain a more comprehensive idea of the exact tipping point towards apoptosis associated killing in M(IFN-γ) cells and also to include some analysis of earlier features of apoptosis such as some of the upstream mitochondrial events that could influence bacterial killing such as mROS generation (Bewley 2017).

Previously, we have shown the mechanism by which apoptosis triggered in response to intracellular bacteria re-engages the cell in bactericidal activity is by release of mitochondrial ROS (Bewley et al., 2017). The mechanism for increased killing by M(IFN-γ) is yet to be elucidated in this project however since I observe core features in common with the pathway triggered by intracellular bacteria and in particular since I observe caspase activation, which is known to trigger mROS (Ricci et al., 2003), I speculate a link to increases in mitochondrial ROS release. It is also noteworthy that IFN-γ has been shown to induce mitochondrial ROS production in other cell types (Rakshit et al., 2014; Yang et al., 2007). If this is indeed the case, it would then be interesting to discover whether IFN-γ stimulation increases bactericidal activity and cell death independently of the bacterial triggered apoptosis associated killing program or if it is at some point enhancing the cascade. One hypothesis was that stimulation with IFN-γ might cause

decreased Mcl-1 levels, however experiments conducted in the previous chapter confirmed no significant change in the baseline levels of Mcl-1 after macrophage activation. Another scenario might be that CAM downregulate Mcl-1 earlier in the infection time course, leading to earlier increases in apoptosis and killing than unstimulated macrophages. It would therefore be interesting to analyse this over a complete time course. Finally, it is possible that IFN-γ activation contributes to the apoptotic programme at some alternative stage such as increasing MOMP or caspase 3/7 activation through an alternative mechanism, such as NO sensitising the cell to apoptosis, as demonstrated in mycobacterial infection (Herbst et al., 2011). NO is known to enhance loss of mitochondrial inner transmebrane potential ($\Delta\psi_m$) and MOMP during pneumococcal infection (Marriott et al., 2004). In this regard, it was noteworthy that killing was restored by IFN-γ in Mcl-1 transgenic BMDMs which while this might have reflected apoptosis-independent mechanisms could also have been due to increased apoptosis mechanisms that overwhelmed the control regulated by Mcl-1, acting on mitochondria or caspases downstream of MOMP.

One way in which I hoped to shed more light on the specific molecular interactions during the time at which macrophages are undergoing apoptosis associated killing was by conducting a transcriptomic analysis by microarray of wild-type and transgenic macrophages with either PBS or *S. pneumoniae* challenge. I also hoped that this analysis might produce some hits involved in macrophage activation or macrophage metabolism to identify a mechanism for the effects of macrophage activation described earlier in this chapter. While the transcriptomic analysis returned few genes involved in macrophage activation, metabolism or effector functions differentially regulated by the transgene in response to bacterial challenge, it identified the presence of multiple signals associated with T-cell proliferation and recruitment. While the difference between individual experiments was the largest variable in the data set, the transcriptomics section of this thesis is viewed as a screen which requires further validation. My findings could not be validated by initial multi-colour flow cytometry after analysing myeloid and T-cell panels however, experiments were conducted using a different strain of *S. pneumoniae* serotype 1 obtained from the SHEILD consortium rather than Statens Serum Institut. This occurred due to loss of bacterial stocks when both the -80 and liquid nitrogen stores were compromised and obtaining new stocks from the Statens Serum Institut was not possible due to changes in this institute's arrangements for sharing the strain, which would have resulted in signing over any intellectual property to the institute, which was not acceptable to the University. This variation in strain could lead to changes in the low dose infection model which was optimised using the previous strain. Furthermore, the mice were infected using the

intranasal technique rather than intratracheal infection as done in the transcriptomics study due to changes required in the Home Office Licence to make it compatible with all SHIELD partners. Variations in the infection technique can lead to differential amounts of inoculant reaching the lungs (Morales-Nebreda et al., 2014). The flow experiments should therefore be repeated after optimisation of infection using the new strain by the intratracheal method to ensure the correct inoculation dose is being used to achieve low dose infection and the tipping point originally studied was replicated. Furthermore, it would be ideal to rearrange the T-cell panel so we can also include antibodies for CD44 and CD62L to identify activated CD4+ T-cells ($CD44^{hi}$ $CD62L^{lo}$). By first gating activated T-cells, I can say with more certainty that Th1 and Th17 populations are present and those cells which are CD183 and CD196 low can then be identified as Th2 cells. It also remains possible that components of the signal are also derived from cells other than lymphocytes for example, CD4, TCR (CD3) and ICOS have all been reported to show expression on macrophages and other myeloid subsets (Aicher et al., 2000; Baba et al., 2006; Fuchs et al., 2017), CD90 has also been shown to be expressed on haematopoietic stem cells (Craig et al., 1993) and CD247 in NK cell maturation (Beecher et al., 1994).

Perhaps of more concern, the T-cell signature is stronger in experiment 1 than experiment 2 and 3 and there is a possibility that the signature is an effect of a defect in the immunity of the mice used in this experiment, or an unrecognised minor viral illness, given that parts of the T-cell signature are also present in PBS treated mice. However, some T-cell markers are present throughout all three experiments.

In conclusion, this chapter demonstrates the various effects of macrophage activation on effector functions after pneumococcal challenge, highlighting an important role for classical macrophage activation in clearing infection and in the enhancement of apoptosis in response to pneumococcal infection. Transcriptomics data has revealed an interesting T-cell signature which is yet to be fully validated by use of multi-colour flow cytometry. The results also suggest that situations in which apoptosis associated killing is compromised by high levels of Mcl-1 could be potentially overcome by IFN-γ, which might represent a useful strategy when this mechanism is altered in conditions such as COPD and HIV (Bewley et al., 2017).

5. Analysis of macrophage effector functions in response to NTHi challenge

5.1. Introduction

Carriage of NTHi is common and most often asymptomatic, existing in around 75% of the population at any one time without causing infection (Murphy and Apicella, 1987; Turk, 1982). Despite its relative innocuousness, NTHi is an opportunistic pathogen associated with disease in the vulnerable such as those at the extremes of age; as a cause of otitis media in children and respiratory tract infections in elderly adults (van Wessel et al., 2011). Additionally, NTHi invasive infections including pneumonia, meningitis and bacteraemia can occur alongside co-morbidities such as COPD and HIV-1 infection (Van Wessel et al, 2011).

Research into NTHi infection has been somewhat neglected after introduction of the *H. influenzae* type B (Hib) vaccine in the 1990's which was successful in largely reducing the number of infections caused by *H. influenzae* across European countries (Van Eldere et al., 2014). However, recent surveillance studies suggest that the prevalence of invasive infection caused by non-type b encapsulated and unencapsulated *H. Influenzae* is rising in the post-vaccine era (Ladhani et al., 2010; Van Eldere et al., 2014; van Wessel et al., 2011). NTHi is now the most common cause of invasive *H. influenzae* infections previously associated with Hib (van Wessel et al., 2011). It is therefore valuable to further explore the host-pathogen interactions of NTHi with the innate immune system and uncover new mechanisms of resolving infection which can potentially avoid the use of antibiotics given the growing resistance of NTHi to β-lactams (Van Eldere et al., 2014).

Macrophages are essential for clearance of NTHi from the respiratory tract (Foxwell et al., 1998a). As the resident immune cells of the lung, macrophages are responsible for recognition, ingestion and killing of NTHi by phagolysosomal trafficking (Martí-Lliteras et al., 2009). They are also critical for neutrophil recruitment and subsequent T-cell activation to bring about the second phase of bacterial killing (Foxwell et al., 1998c)

As described earlier in this thesis, in response to *S. pneumoniae* challenge, macrophages undergo apoptosis as a mechanism of host defence. This so-called "apoptosis-associated killing" allows reengagement of macrophage microbiocidal killing mechanisms after the initial phase of bacterial killing has been exhausted (Bewley et al., 2011a; Dockrell et al., 2001b; Marriott et al., 2005). This phenomenon has also been described in the macrophage response to *E.Coli* and Hib (Webster et al., 2010). Apoptosis in this situation occurs by the mitochondrial pathway: MOMP

followed cytochrome c release and caspase 3/7 activation which ultimately results in cell death (Bewley et al., 2011a). The key mediator in this apoptotic cascade is the anti-apoptotic protein Mcl-1 (Marriott et al., 2005).

Given the rising importance of antibiotic resistance and the need for further insight into the interactions of NTHi with the innate immune system, I decided to explore the possible apoptotic responses of macrophages to NTHi infection. As NTHi is a human pathogen, the main model for this work has been human MDMs. When necessary, I have also assessed macrophage responses to NTHi in wild-type and transgenic Mcl-1 BMDMs which over express human Mcl-1 on a CD68 (macrophage specific) promoter. Where applicable I have compared these responses to those of *S. pneumoniae* infection, a bacterium for which apoptosis associated killing has been fully characterised. In attempt to better understand the role of macrophage activation in NTHi infection, I have tested the effects of cytokine stimulation on phagocytosis and clearance of NTHi in both human and murine primary cells. Finally, I have repeated my key findings using an alternative NTHi lab strain considering the high levels variability seen between *H. influenzae* strains (Craig et al., 2001).

5.2. Results

5.2.1. Optimisation of NTHi challenge

Since NTHi challenge had not already been optimised in our laboratory is was firstly important to determine the correct antibiotic concentrations for killing assays which require pulse antimicrobials (killing of extracellular bacteria) and maintenance antimicrobials (prevention of extracellular regrowth and subsequent re-internalisation of bacteria). Gentamicin was selected as an appropriate antimicrobial as it has previously been shown to be effective in killing extracellular NTHi (Ahrén et al., 2001; Kratzer et al., 2007). A concentration of 40µg/ml gentamicin was used as a pulse dose as this was the concentration found to largely eradicate extracellular NTHi (Figure 5-1A). Although 40µg/ml was not completely sufficient to kill all extracellular bacteria at the 4 hour time point, it did significantly reduce extracellular bacteria from 10^8 to 10^2 which is the equivalent of only one or two CFU per plate. Half of the cells were lysed at 4 hours and NTHi internalisation increased marginally with an increasing MOI. To the remaining cells which had received the pulse antibiotic treatment but had not been lysed, a chase dose of 2µg/ml gentamicin was applied for the remaining 16 hours of challenge to prevent any extracellular regrowth. After pulse/chase treatment, wild-type and transgenic macrophages fully cleared the 10^4 - 10^5 bacteria internalised at 4 hours by the second timepoint, lysis at 20 hours, at all MOIs tested. Overnight incubation with a 2µg/ml gentamicin chase killed all

extracellular bacteria and no cfu, extracellular or intracellular were recovered at 20 hours (Figure 5-1B). Complete clearance was unexpected – particularly in the transgenic mouse which, assuming NTHi activates macrophage apoptosis associated killing, I hypothesised would not fully clear such a large intracellular burden overnight. Furthermore, it was necessary to optimise a condition in which intracellular bacteria survived until a late time point as intracellular bacteria is necessary to drive macrophage apoptosis associated killing (Ali et al., 2003). Gentamicin has previously been shown to permeabilise the cell membrane and exhibit intracellular bactericidal activity at 50μg/ml over a time course of 2 hours although thorough washing with sterile PBS after short incubations was found to be sufficient to prevent the effect of gentamicin on intracellular bacterial killing (Drevets et al., 1994). However, since in the current model gentamicin was being applied to cells for a long incubation period over which it could potentially accumulate I attempted to identify the minimum concentration capable of preventing extracellular regrowth in order to minimise gentamicin interference with intracellular bacteria. I first tested a low dose of 0.075μg/ml gentamicin. Doses as low as 0.01μg/ml gentamicin have previously been shown to increase bacterial killing (Drevets et al., 1994) however 0.075μg/ml resulted in the regrowth of bacteria in the extracellular media at 20 hours (Figure 5-1C). I next tried an intermediate dose of 0.5μg/ml (Figure 5-1D) however, again, all bacteria were cleared by the 20-hour time point. To avoid potential problems which overnight incubation with gentamicin might cause, including entry to the cell, I decided to test a different experimental design. Since the aim of this optimisation experiment was to retain intracellular bacteria, MOIs of 10, 50 and 100 were tested in two different experimental designs, one in which cells were washed 3 times in 1ml sterile PBS at 4 hours to reduce the extracellular burden (Figure 5-1E) and another where cells were left post challenge until the 20 hour time point without any washing or antimicrobial treatment (Figure 5-1F). Both conditions involved no antimicrobial exposure until the 20 hour time point where a higher dose of 60μg/ml Gentamicin was used in the 30 minute pulse step to ensure removal of all extracellular NTHi. Both the designs in Figure 5-1E and Figure 5-1F resulted in the survival of equal amounts of intracellular bacteria, so I decided to maintain the protocol presented in Figure 5-1F due to practicality. BMDMs were initially used for optimisation with the view to explore the effect of the CD68 Mcl-1 transgene and the increased availability of this cell type compared to human MDMs. Later, the same time course was conducted in MDMs.

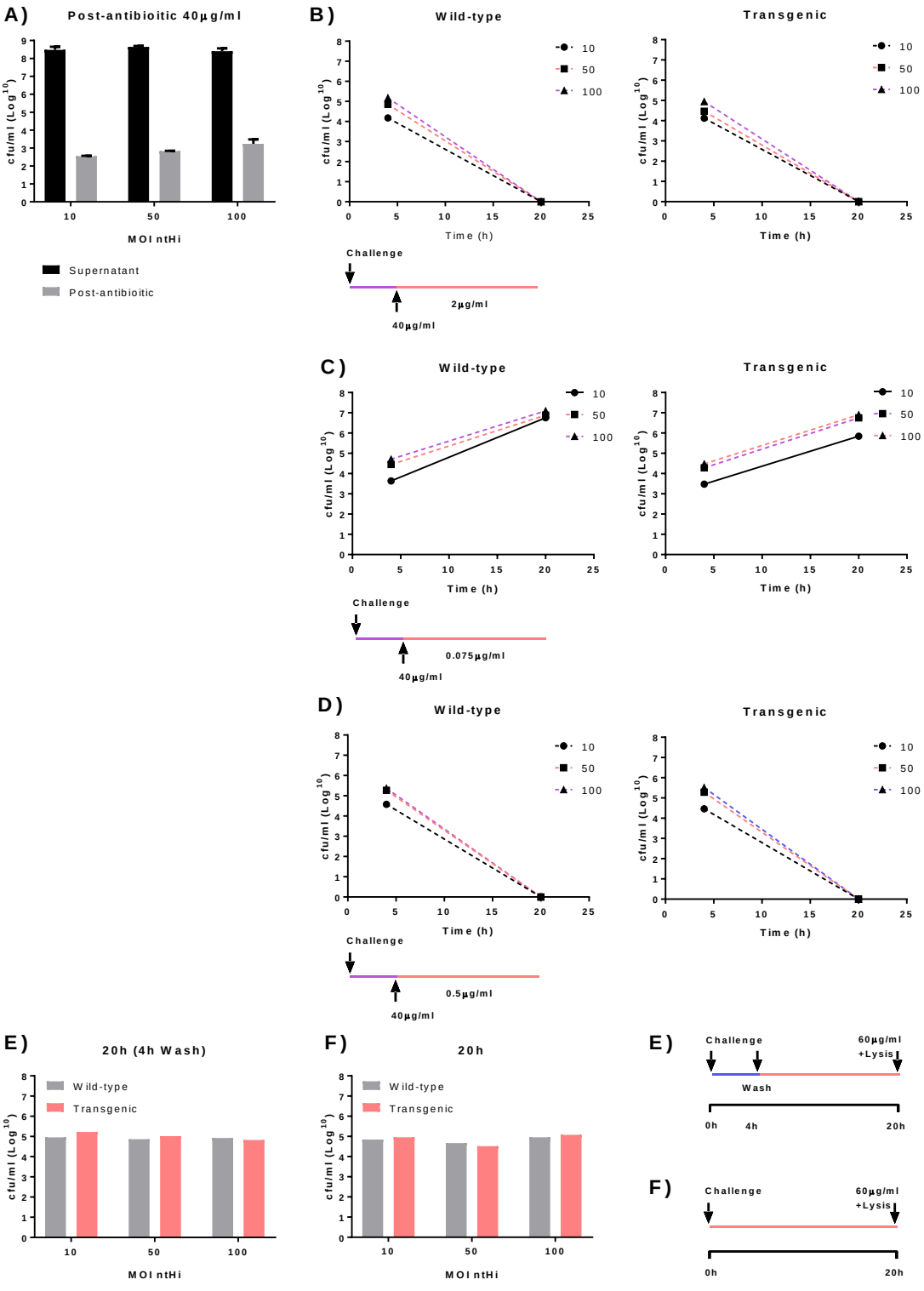

A)
Post-antibioitic 40µg/ml
cfu/ml (Log10)
MOI ntHi
Supernatant
Post-antibioitic
B)
Wild-type
Transgenic
10
50
100
Time (h)
Challenge
2µg/ml
40µg/ml
C)
Wild-type
Transgenic
Time (h)
Challenge
0.075µg/ml
40µg/ml
D)
Wild-type
Transgenic
Time (h)
Challenge
0.5µg/ml
40µg/ml
E)
20h (4h Wash)
Wild-type
Transgenic
MOI ntHi
F)
20h
E)
Challenge
60µg/ml
+Lysis
Wash
0h
4h
20h
F)
Challenge
60µg/ml
+Lysis
0h
20h

Figure 5-1: Optimising NTHi challenge to ensure intracellular stress at later time points of exposure

(A-D) Wild-type and transgenic BMDMs were challenged with NTHi at a MOI of 10, 50 or 100 for a duration of 4 hours **(A)** cfu counts from supernatants at 4 hours of challenge before and after being washed and incubated with 'pulse' antimicrobial (40μg/ml gentamicin) for a duration of 30 minutes. **(B-D)** After pulse antimicrobial treatment, half the cells were lysed and supernatants plated to determine intracellular bacterial content. Macrophages were then maintained in low doses of gentamicin 'chase' until the 20 hour time point. **(B)** BMDMs were incubated with a dose of 2μg/ml **C)** 0.075μg/ml and **D)** 0.5μg/ml gentamicin during the chase period. **(E)** Washing BMDMs at 4 hours in sterile PBS to reduce the number of extracellular bacteria and pulsing with a higher dose of 60μg/ml gentamicin was also tested or **(F)** BMDMs were incubated without washing until 20 hours and intracellular cfu estimated. Schematic diagrams illustrate the experimental design, representative of at least 3 independent experiments.

5.2.2. Prior activation does not alter macrophage response to NTHi

Previous studies show that classical activation of macrophages by IFN-γ treatment can enhance microbicidal activity and pathogen clearance (MACKANESS, 1964; Murray et al., 2014). To understand whether clearance of NTHi can be accelerated in this manner, macrophages were activated with cytokines, IL-4, IL-10 and IFN-γ (as previously defined in chapter 3). I hypothesised that stimulating macrophages with IFN-γ would enhance pathogen clearance through upregulation of genes enhancing microbicidal responses (Benoit et al., 2008b) and as shown in chapter 4 with *S. pneumoniae* challenge. After 24 hours of activation, macrophages were challenged with NTHi and killing assays conducted at the 2 – 4 hour time point (Figure 5-2). After 2 hours of bacterial challenge culture supernatants were collected and cfu estimated to assess for differences in bacterial load between conditions (Figure 5-2A). At this early time point, activation of MDMs showed no effect on internalisation and killing of NTHi (Figure 5-2B). The microbicidal response was also assessed at the 4 hour time point using DCF-DA and DA-FM to assess intracellular ROS and NO levels respectively (Figure 5-3). In general, NTHi infection caused a large increase in ROS production (2-3 log decades) (Figure 5-3A) compared to the mock infected control (Figure 5-3B) however prior macrophage activation had no effect on intracellular ROS generation, suggesting it was well induced and not influenced by the activation status of the macrophage. NO production was more modest, a higher response was seen in IFN-γ stimulated cells, although this result was not significant (Figure 5-3C). IFN-γ activation is known to increase amounts of iNOS which is the enzyme responsible for the shift from L-Arginine metabolism to nitric oxide production (Mills et al., 2000b). However, mock infected IFN-γ

stimulated macrophages did not show heightened levels of NO. This may be because IFN-γ only works to prime the macrophage and a further stimulus such as TNFα or bacterial products (LPS) are needed to generate a detectable response (as described in chapter 3). In addition NO production is often a more delayed response to intracellular bacteria and significant production at this timepoint is often not observed (Marriott et al., 2004). Also it has been debated how much of human macrophage NO production is through iNOS as opposed to other sources such as endothelial nitric oxide synthase (eNOS) (MacKenzie and Wadsworth, 2003; Schneemann and Schoedon, 2002). Further, human MDM donor variability may have contributed to the large amount of variability in the results and limited the power to detect significance.

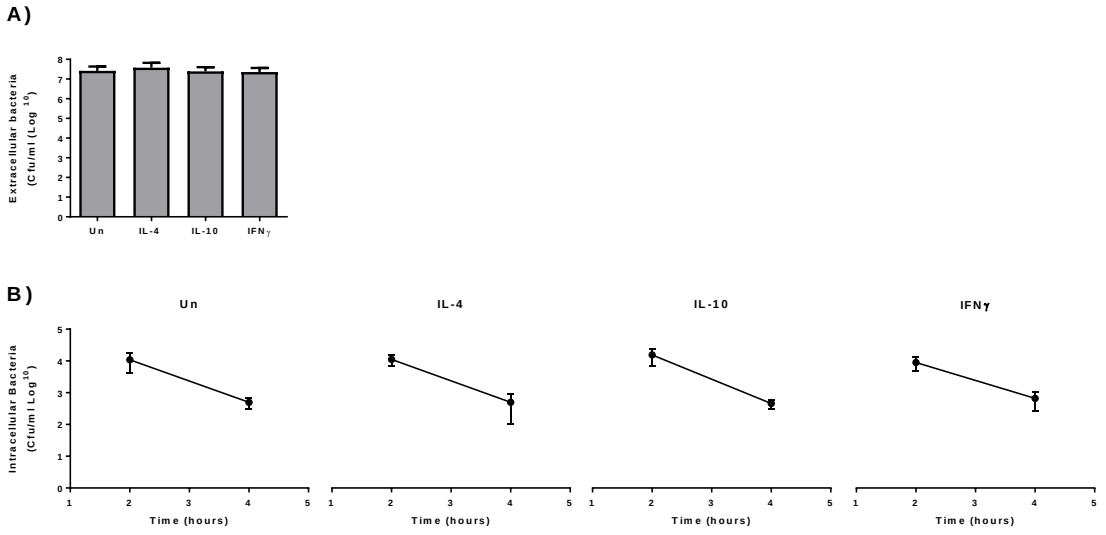


Figure 5-2: Macrophage activation has no effect on macrophage internalization and killing of NTHi at early time points.

MDMs were activated for 24 hours with 20ng/ml IL-4, IL-10 IFN-γ or unstimulated. MDMs were challenged with NTHi at an MOI of 10 for 2 hours. **(A)** The extracellular cfu from each condition was estimated, no significance was determined by one-way ANOVA with Tukey's post-test (n=3) **(B)** At 2 hours, intracellular killing of NTHi was assessed by gentamicin protection assay no significant difference was determined with two-way ANOVA and Sidak's post-test (n=4) data is represented as median ± range.

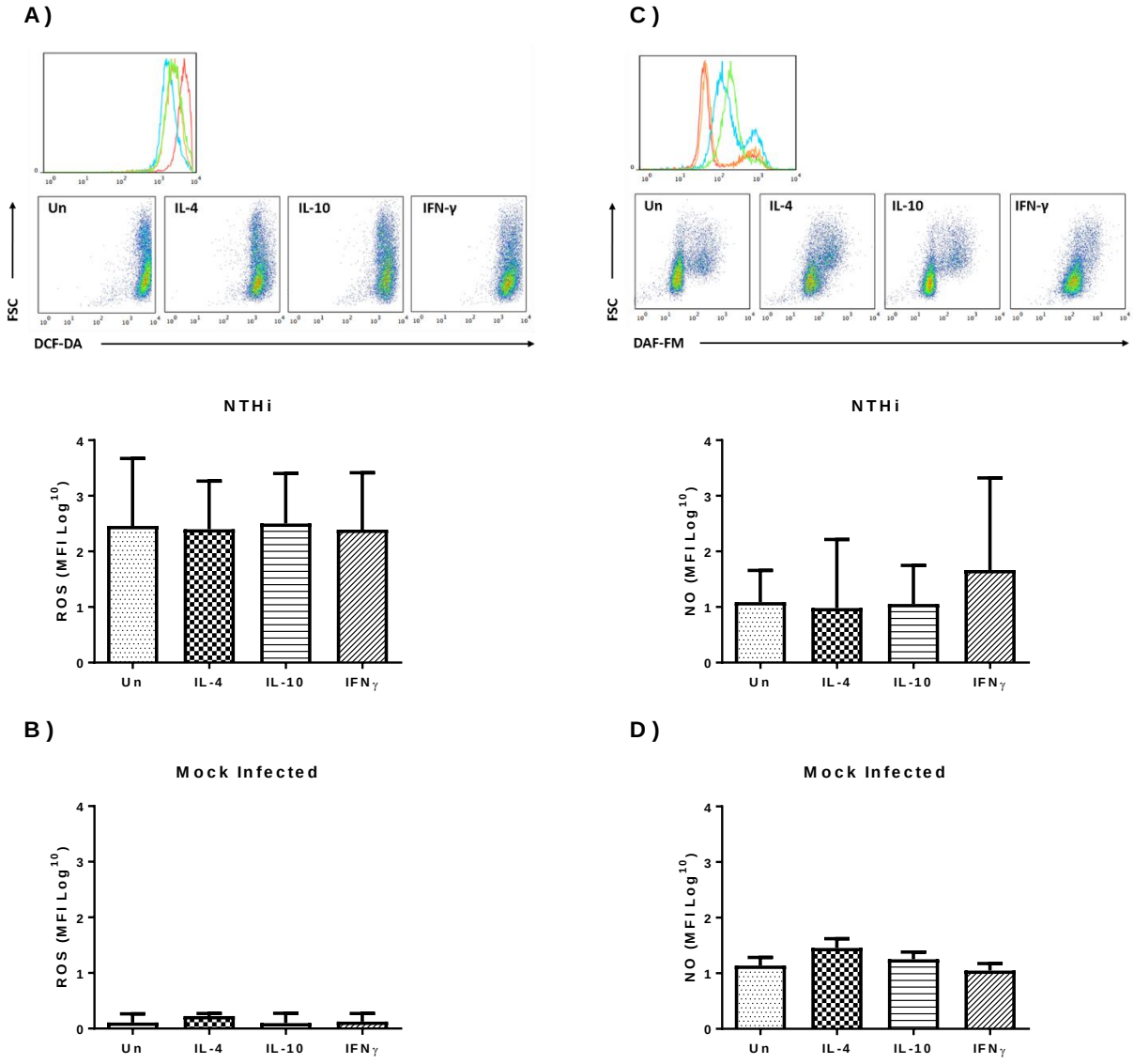


Figure 5-3: Macrophage activation has no effect on microbicidal generation in response to NTHi

Representative flow histograms show unstimulated (red), IL-4 (orange), IL-10 (blue) and IFN-γ (green). MDMs, activated with 20ng/ml IL-4, IL-10, IFN-γ or unstimulated, were challenged with NTHi or mock infected for a duration of 4 hours, flow cytometry analysis recorded the median fluorescence intensity (MFI) of **(A-B)** reactive oxygen species (ROS) production by DCF-DA staining, no significance was observed by one-way ANOVA with Tukey's post-test (n=7) and **(C-D)** nitric oxide (NO) production by DAF-FM staining no significance was observed by one-way ANOVA with Tukey's post-test (n=7). Data is represented as median ± range in all graphs. The value of the unstained control was subtracted from each condition to account for auto-fluorescence.

5.2.3. The Mcl-1 transgenic macrophages display significantly less apoptosis at high MOIs and apoptosis increases with increasing MOI in BMDMs and MDMs

As apoptosis associated killing occurs at around 20 hours with *S. pneumoniae* infection (Dockrell et al, 2001) and macrophage apoptosis is proportional to the intracellular burden, (Ali et al., 2003), I hypothesised that the residual intracellular burden could drive apoptosis and transgenic macrophages would have decreased apoptosis. Furthermore, I thought that an increasing MOI might affect the level of apoptosis, as shown previously in *S. pneumoniae* infection (Ali et al., 2003). Significant cell loss was apparent compared to the mock infected control, for all conditions, but no difference in cell density was seen with increasing MOI (Figure 5-4A). After 20 hours of challenge with NTHi I observed low levels of apoptosis in BMDMs. At an MOI of 10 apoptosis was seen in approximately 5% of cells confirmed by nuclear fragmentation analysis and caspase 3/7 activation demonstrated in Figure 5-4B and Figure 5-4C respectively and the result was significant for the nuclear morphology analysis. A rise in nuclear changes indicative of apoptosis was seen with increasing MOI in wild-type BMDMs, which reached significance at MOI of 100 versus MOI of 10. Whilst apoptosis in the transgenic mouse remained constant even with higher MOIs (Figure 5-4B). The increase in apoptosis did not reach significance when analysed by caspase activation, although there was a trend towards increased levels at higher MOI, however the number of repeat experiments was much lower and therefore further repeats are needed to determine significance. These results indicate that as the severity of the bacterial insult increases, macrophages increase their apoptotic response and this response is not reproduced to the same level in the transgenic macrophages. Cell density between the two genotypes and bacterial doses remained constant, perhaps owing to the modest levels of apoptosis and the fact that cells were only starting to become apoptotic at the time analysed (Figure 5-4A).

Apoptosis was also examined in human MDMs. Macrophages were again challenged with NTHi for a duration of 20 hours before analysis of apoptosis measured by nuclear morphology or caspase activation. As the MOI increased, MDMs showed a significant decrease in cell density, which became significant at an MOI of 100 indicating cell loss (Figure 5-5A). MDMs demonstrated similar levels of apoptosis as BMDMs and showed increasing apoptosis with increasing MOI (Figure 5-5B-C).

Levels of apoptosis in our lab strain (ATCC 49247) challenged MDMs were compared to cells challenged with *S. pneumoniae* serotype 1, Hib (Eagan strain: H636) and an alternative NTHi

strain (ATCC 53600 – referred to here as NTHi 1479) a clinical isolate from the sputum of a patient with chronic bronchitis. Both *S. pneumoniae* (Dockrell et al., 2001a) and Hib have been previously described as inducers of macrophage apoptosis associated killing (Marriott, H.M., unpublished data). The aim of this experiment was to directly compare levels of macrophage apoptosis induced by two different NTHi strains to bacteria which are known stimulators of this phenomenon. Since both *S. pneumoniae* and Hib possess a polysaccharide capsule, they were treated with human immune serum before infection to aid internalisation. All bacteria were added for a duration of 20 hours which is the time point at which we begin to see significant levels of apoptosis in *S. pneumoniae* and Hib challenged cells. MDMs challenged with *S. pneumoniae* demonstrated around 30% apoptosis as previously described (Dockrell et al., 2001a) (Figure 5-6B-C) however an increase in apoptosis was not associated with an increase in cell loss (Figure 5-6A), if macrophages are in the early stages of apoptosis, there may not yet be a significant reduction in cell density. MDMs challenged with NTHi 49247, showed significantly less apoptosis than *S. pneumoniae* (around 10%), however the level of nuclear fragmentation was significantly elevated compared to the mock infected control, and caspase 3/7 positivity almost reached the level of significance ($p=0.08$). Levels of apoptosis induced by Hib and NTHi 1479, although clearly evident (around 5%), were lower than those induced by NTHi 49247 and not significant compared to the mock infected control. NTHi strains have previously been described as very variable in their ability to persist within macrophages for extended infection periods (up to 72 hours) (Craig et al., 2001). Overall, this suggested that the levels of apoptosis induced by the NTHi strains are lower than those induced by *S. pneumoniae* and there may be variability in the level of apoptosis with NTHi strains.

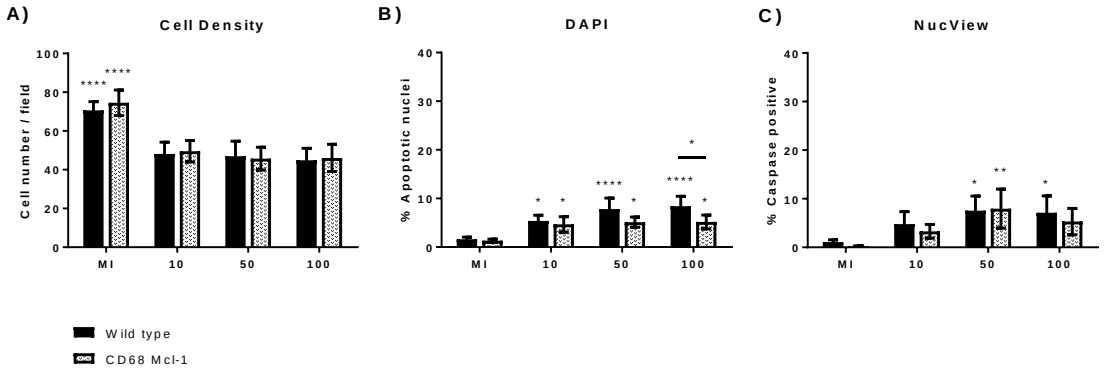


Figure 5-4: NTHi induce low levels of apoptosis after 20 hours of challenge

BMDMs were mock infected or challenged with NTHi for 20 hours at an MOI of 10, 50 or 100. **(A)** Average cell density / field of view at x100 magnification of each well was quantified by DAPI staining and **(B)** apoptotic cells were then quantified by change in nuclear morphology (n=12) significance determined by two-way ANOVA with Sidak's post-test compared to the mock infected for each genotype and between genotypes in each condition **(C)** Caspase 3/7 positive cells determined by NucView staining (n=3) significance determined by two-way ANOVA with Sidak's post-test as above. Data represented as mean ± SEM. *p=<0.05 **p=<0.01 ***p=<0.001 ****p=<0.0001.

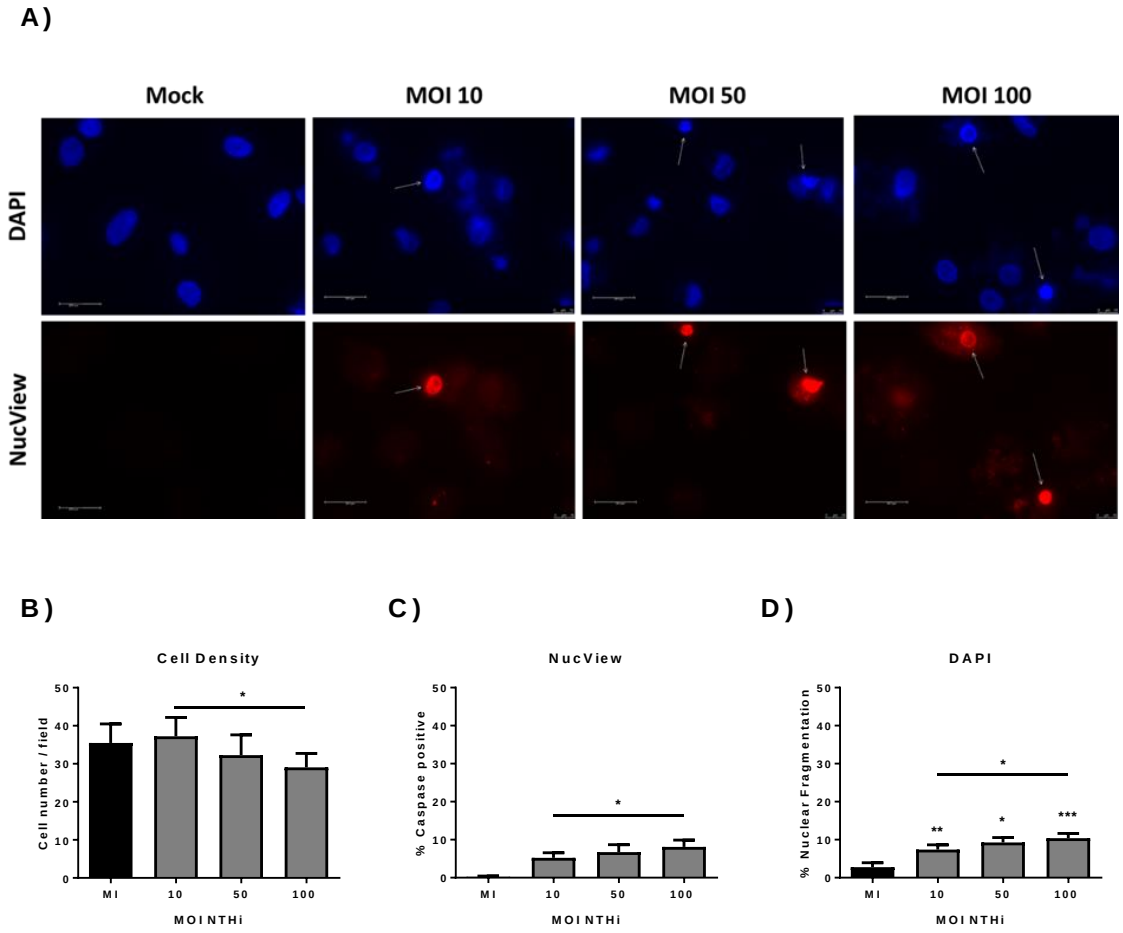


Figure 5-5: NTHi challenge causes modest cell loss and apoptosis with increasing MOI

MDMs were mock infected or challenged with NTHi for 20 hours at an MOI of 10, 50 or 100 **(A)** Representative images were captured using Leica AF6000 fluorescent microscope (x100 objective lens). Scale bar measures 20µM and white arrows indicate apoptotic cells **(B)** cell number / field counted by DAPI staining, significance determined by one-way ANOVA with Tukey's post-test, *=p<0.05 (n=7) **(C)** Quantification of number of cells with caspase 3/7 positivity by NucView staining, significance determined with one-way ANOVA and Tukey's post-test *=p<0.05 (n=4) **(D)** Quantification of number of cells with nuclear fragmentation and condensation by DAPI staining, significance determined by one-way ANOVA with Tukey's post-test (n=7) *=p<0.05, **=p<0.01, ***=p<0.001 compared to mock challenged. Data represented as mean ± SEM.

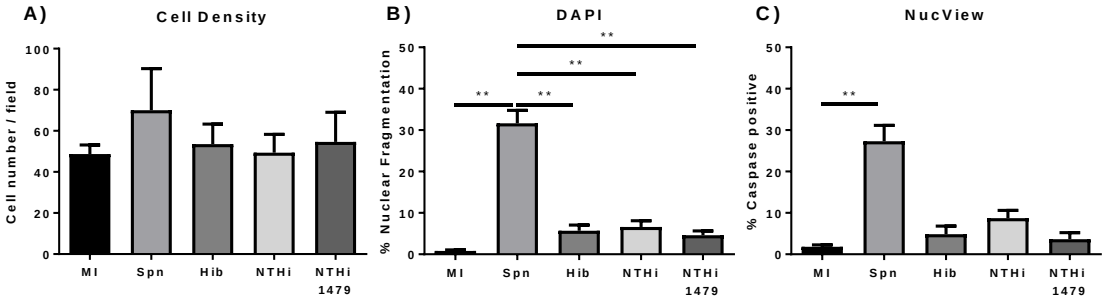


Figure 5-6: Macrophages undergo only low levels of apoptosis after 20 hours of exposure to NTHi.

Human monocyte derived macrophages (MDM) were mock challenged or challenged with *S. pneumoniae* serotype 1 (Spn), *H. influenzae* type B (Hib), NTHi (laboratory isolate - 49247) or an alternative clinical strain of NTHi (NTHi 1479) at an MOI of 10 for a duration of 20 hours. **(A-B)** Macrophages were stained with DAPI and **(A)** cell density estimated per field at 100x microscope objective, no significance was determined by one-way ANOVA and Tukey's post-test **(B)** number of cells with nuclear fragmentation and condensation counted, significance was determined by one-way ANOVA and Tukey's post-test **(C)** quantification of the number of cells with caspase 3/7 positivity by NucView staining. Significance was determined with Friedman test and Dunn's post-test, *=$p<0.05$, **=$p<0.01$ All data is represented as mean ± SEM (n=5).

5.2.4. Apoptosis makes a limited contribution to clearance of intracellular NTHi at the 20 hour time-point.

Having established some evidence for increased apoptosis following NTHi challenge using the infection model described above (Figure 5-1F) I next addressed whether the Mcl-1 transgenic macrophages showed any evidence of increased intracellular burden, indicative of reduced killing associated with lower levels of apoptosis-associated killing for which there was some evidence in Figure 5-4. Increasing MOI caused no significant difference in the number of viable intracellular bacteria in wild-type BMDMs, however, at MOIs of 50 – 100 the transgenic mouse showed up to half a log increase in viable intracellular NTHi suggesting the inability to undergo apoptosis may be preventing bacterial clearance, although with the small series studied this difference was not statistically significant (Figure 5-7A). Human MDMs internalised a similar amount of NTHi as BMDMs and the MOI showed no effect on the level of intracellular bacteria, suggesting that killing ability of macrophages at this time point may be saturated (Cannon and Swanson, 1992) and therefore increased apoptosis makes no effect on the microbicidal activity of the cell, although I cannot exclude the possibility that the increased apoptosis might be

helping to stop further increases in intracellular burden (Figure 5-7B). However, levels of apoptosis are much lower than those seen with *S. pneumoniae* infection (Dockrell et al., 2001a) suggesting either that at the 20 hour time-point at an MOI of 100, NTHi are not inducing enough of a challenge for the cell to trigger high levels of apoptosis, or alternatively NTHi may be better able to prevent induction of apoptosis unlike the host response to *S. pneumoniae* infection.

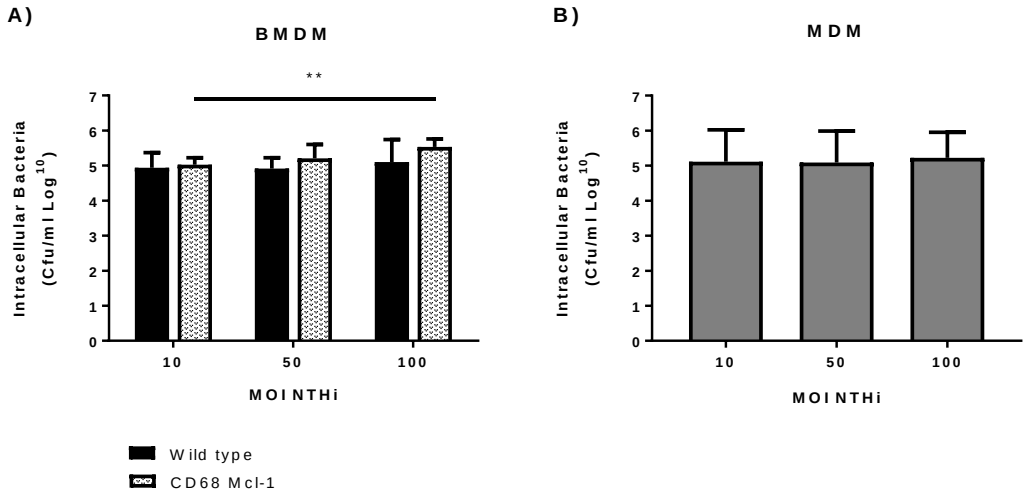


Figure 5-6: Limited impact of apoptosis on intracellular bacterial killing at 20 hours

Macrophages were challenged with NTHi for a duration of 20 hours at a MOI of either 10, 50 or 100. At the time point, internalisation of NTHi was assessed by gentamicin protection assay in **(A)** wild-type and transgenic BMDMs (n=7) two-way ANOVA with Sidak's post-test determined significance between transgenic MOI 10 vs MOI 100 **=p<0.01 and no significance was determined between wild-type and transgenic genotypes at each MOI **(B)** MDMs, no significance determined by one-way ANOVA with Tukey's post-test (n=6). Results are represented on a logarithmic axis as median ± range.

5.2.5. Macrophage activation has no effect on NTHi clearance at late time points

Next, I examined whether macrophage activation might influence the delayed bacterial killing occurring at 20 hours after bacterial challenge. Since apoptosis associated killing is associated with later time points of infection, clearance of NTHi was previously assessed at these later time points. In Figure 5-7 I determined intracellular bacterial viability at 20 hours, in a model without antimicrobial treatment, and under conditions which were associated with low levels of apoptosis, as shown in Figure 5-4 to Figure 5-6. However, at this time-point I saw little effect of increasing the MOI on killing ability, suggesting bacterial internalisation was saturated. I

therefore selected an earlier time point at which Mcl-1 would typically begin to decrease in other infections (Marriott et al., 2005) but at which the bacterial burden wasn't so high as to potentially mask an effect of macrophage activation on killing. To understand the kinetics of killing and to help select the most appropriate time to analyse whether activation could alter bacterial killing I assessed killing activity of activated macrophages at 4 time points over a 4 hour period between 14 and 18 hours in MDMs (Figure 5-8) and wild-type and transgenic BMDMs (Figure 5-9). IFN-γ activated macrophages were expected to show increased clearance of NTHi compared to other activation conditions, as seen with *S. pneumoniae* infection (chapter 4), assuming the clearance capacity had not become saturated. Furthermore if killing was not saturated I predicted wild-type macrophages would clear internalised bacteria more efficiently than transgenic macrophages (Bewley et al., 2017). Contrary to my hypothesis, following MDM NTHi challenge (Figure 5-8) there was no significant difference in the extracellular supernatant and intracellular bacteria between activation conditions, despite evidence macrophages were still clearing intracellular bacteria and killing capacity was not exhausted. Although not significant, IFN-γ stimulated BMDMs showed enhanced clearance compared to other activation conditions, in most experiments all bacteria were cleared between 16 – 18 hours, however, there was no significant effect of the Mcl-1 transgene on bacterial clearance. Again, the small numbers limited statistical analysis, even though there was a trend towards a greater effect in the wild-type BMDM, but at least for the BMDM there was some evidence of enhancement of clearance with IFN-γ. Critically, however, for both MDM and BMDM there was evidence of continued reduction in intracellular viability which showed that intracellular killing capacity was not exhausted. This contrasts with what my host group has previously found for *S. pneumoniae* at comparable times and intracellular bacterial loads (Preston et al, in submission) or for *S. aureus* (Jubrail et al., 2016). This suggests that macrophages retain an extended period over which intracellular killing is achieved with relatively modest impact of IFN-γ and that since killing is not exhausted there may be less stimulus to initiate alternative processes such as apoptosis-associated killing.

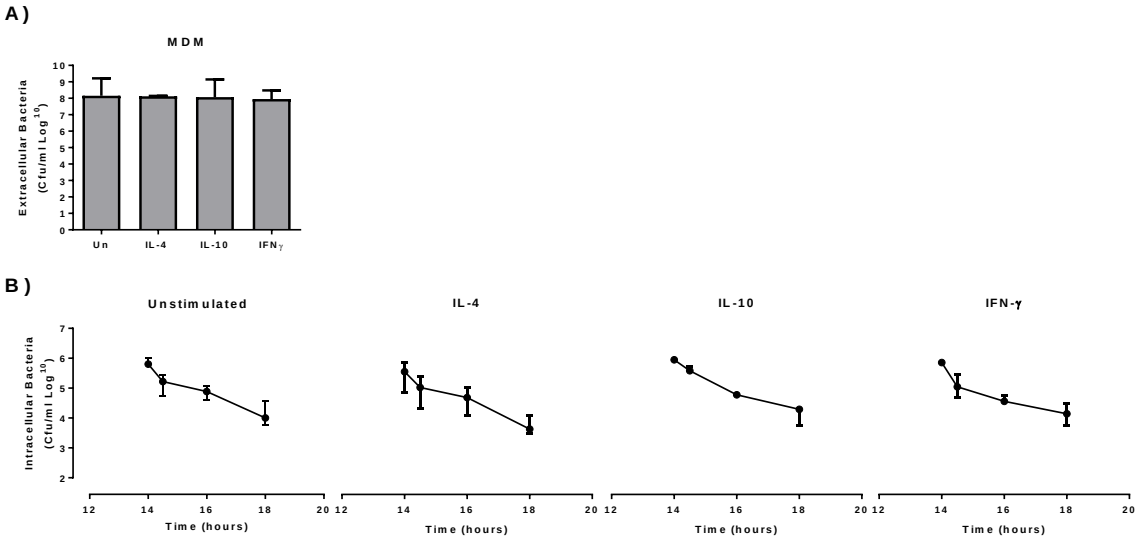


Figure 5-7: Macrophage activation has no effect on macrophage internalization and killing of NTHi

MDMs were unstimulated or activated with 20ng/ml IL-4, IL-10 or IFN-γ for a duration of 24 hours. Macrophages were challenged with NTHi at an MOI of 10 for a duration of 14 hours. **(A)** extracellular cfu were estimated at the 14 hour time point (n=3) No significance observed by one-way ANOVA with Tukey's post-test. **(B)** Intracellular killing of NTHi was estimated between 14 and 18 hours by gentamicin protection assay (n=3). No significance observed by one-way ANOVA with Tukey's post-test. Data is represented on logarithmic axis as median ± range.

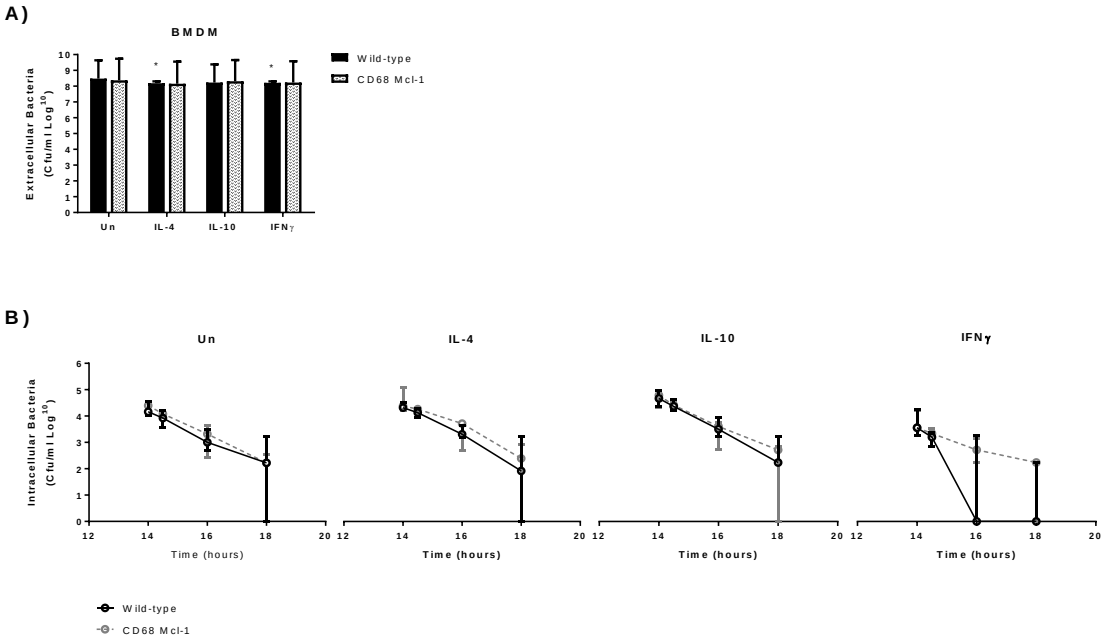


Figure 5-8: BMDM activation in the wild-type and transgenic BMDM does not affect survival of NTHi

BMDMs were activated for 24 hours with IL-4, IL-10, IFN-γ or unstimulated before challenge with NTHi at an MOI of 10 for 14 hours. At the time point **(A)** cfu in the cell supernatant were estimated for each condition (n=4) significance determined compared to the mock infected control by two-way ANOVA with Sidak's post-test and **(B)** Intracellular killing of NTHi was estimated between 14 and 18 hours by gentamicin protection assay (n=4) no significance between activation conditions at each time point determined by two-way ANOVA with Sidak's post-test. Data represented as median ± range.

5.2.6. NTHi stimulate prominent pro-inflammatory cytokine release from macrophages

Another potential reason for the lack of significant variation between activation conditions might be that NTHi stimulates a strong pro-inflammatory macrophage profile which overrides prior cytokine stimulation and causes all macrophages to switch to a classically activated phenotype. Pro-inflammatory cytokine stimulation was analysed in human MDMs challenged with NTHi or *S. pneumoniae* over a time course of 4 – 24 hours by ELISA (Figure 5-10). MDMs challenged with NTHi produced higher levels of TNFα than *S. pneumoniae* challenged cells at all time points tested. NTHi also induced higher levels of IL-6 than *S. pneumoniae* although the results were not as marked. This is line with previous studies which demonstrate the strong pro-inflammatory response of the innate immune system to *H. Influenzae*, potentially due to the specific profile of the outer membrane (Berenson et al., 2005). Since apoptosis associated killing

is associated with resolution of inflammation as well as infection, it is also possible that the limited levels of apoptosis I observed following NTHi challenge also resulted in higher cytokine levels with this bacterium than occur with *S. pneumoniae.* My results demonstrated significantly higher levels of TNF-α at 4, 16, 20 and 24 hours of NTHi challenge (Figure 5-10A). IL-6 levels also appeared increased however this result did not reach significance (Figure 5-10B)

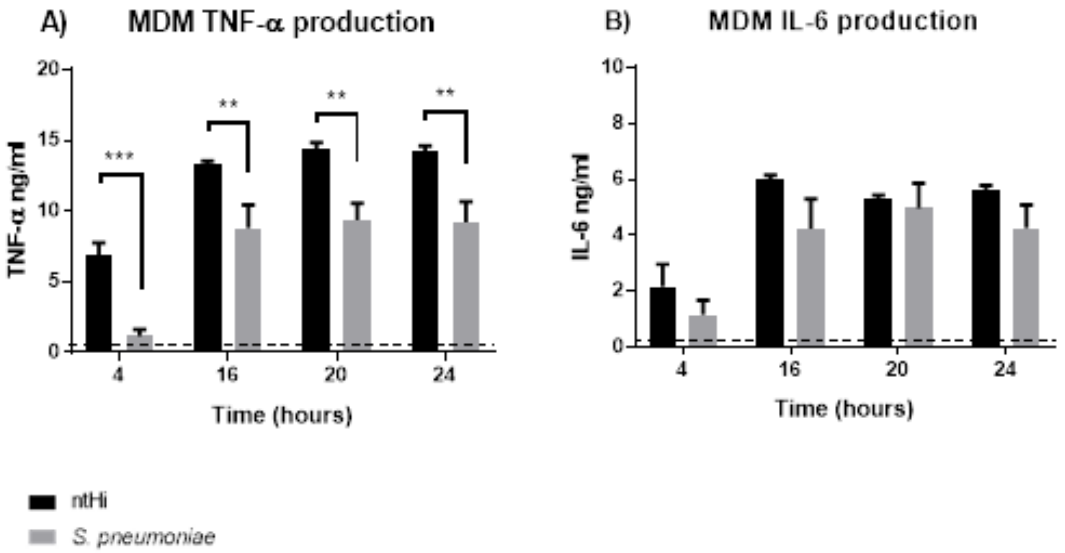


Figure 5-9: NTHi induces a larger pro-inflammatory cytokine response than *S. pneumoniae*

MDMs were challenged with NTHi or opsonized *S. pneumoniae* for a duration of 4 – 24 hours at an MOI of 10. Supernatants were collected from mock infected and NTHi challenged cells at each time point and diluted 1:10 **A)** TNFα, (limit of detection 4pg/ml), and **B)** IL-6, (limit of detection 2pg/ml), cytokine concentration was determined by ELISA (n=4), data is represented as mean ± SEM. Significant data determined by two-way ANOVA with Sidak's post-test, **p<0.01, ***p<0.001. Dotted line is representative of the mock infected control.

5.2.7. Cell loss increases after 48 - 72 hours exposure to NTHi.

Since the macrophage response to NTHi challenge had only been measured up to the 20 hour time point, and apoptosis appeared not to have been optimally engaged I reasoned that macrophage killing mechanisms through ROS and RNS had not yet become exhausted. In keeping with this was the evidence for continued killing of NTHi by apoptosis-independent mechanisms shown in Figure 5-8 for MDM and Figure 5-9 for BMDM 14-18 hours after bacterial challenge. I therefore, next questioned whether macrophage apoptosis was engaged at later

time points and sought evidence for apoptosis over a longer period, until 72 hours. This is a relevant time frame since it is a time frame for which NTHi has been shown to survive in the intracellular environment (Craig et al., 2001). Macrophages were challenged with NTHi at an MOI of 10, 50 or 100 for a duration 48 or 72 hour (Figure 5-11). At each time point, cells were assessed for caspase activation and for nuclear morphology to detect features of apoptosis and to allow estimation of cell density as quantified by microscopy. At 48 hours post infection over 50% of cells were lost across all conditions following bacterial exposure compared to a loss of 5-10% at the 20 hour time point. At 72 hours post infection, cell number did not decrease much more than that seen at 48 hours, however levels of apoptosis were increased. Quantification of caspase positive and nuclear morphological features at 48 and 72 hours showed high levels of apoptosis defined by morphology and evidence of increased and cytoplasmic NucView staining, potentially as the nuclear membrane lost integrity.

5.2.8. Mcl-1 protein levels remain high in response to NTHi challenge

A decrease in Mcl-1 expression is required to allow execution of the apoptotic programme associated with macrophage apoptosis during the response to pneumococcal challenge (Marriott et al., 2005). To assess if apoptosis after NTHi challenge is also associated in reduction in Mcl-1 level, protein was analysed from human MDMs challenged with NTHi (strain 49247) (Figure 5-12A) or NTHi strain 1479 (Figure 5-12B). MDMs were assessed over a 16 – 72-hour time course, since the data in Figure 5-11 suggested apoptosis might be delayed till the 48-72 hour time point. Protein was analysed by western blotting and Mcl-1 levels were normalised to α-tubulin level and fold change at each time point was compared to the mock infected level. In both NTHi 49247 and NTHi 1479 exposed cells, protein levels for Mcl-1 remained high from 16 hours to 20 hours. NTHi 1479 challenge saw a decrease in levels at 48 hours, slightly than NTHi 49247 which peaked at 20 hours and then began to decrease towards 72 hours. However, in both instances, levels of Mcl-1 were still increased or equal compared to the mock infected control for each time point. Changes at each time point did not reach statistical significance, despite clear increases – perhaps due to high donor variability, however for NTHi 48247 levels of Mcl-1 were significantly increased compared to the 72 hour timepoint and the mock infected control. A similar pattern was also seen in wild-type BMDMs challenged with NTHi 49247 over the same time course of 72 hours (Figure 5-13). In this case levels of Mcl-1 whilst always equal or higher than the mock challenged control, increased and peaked at 48h. Levels then proceeded to decrease closer to the level of the mock infected control by 72 hours. This data suggests that

NTHi infection causes an initial increase in Mcl-1 protein which is extended for sustained time period. This increase is significant versus mock-infected for 20 hours with NTHi 49247 in MDM and 48 hours in BMDM, after which when it begins to decrease to baseline levels but does not fall below mock-infected levels.

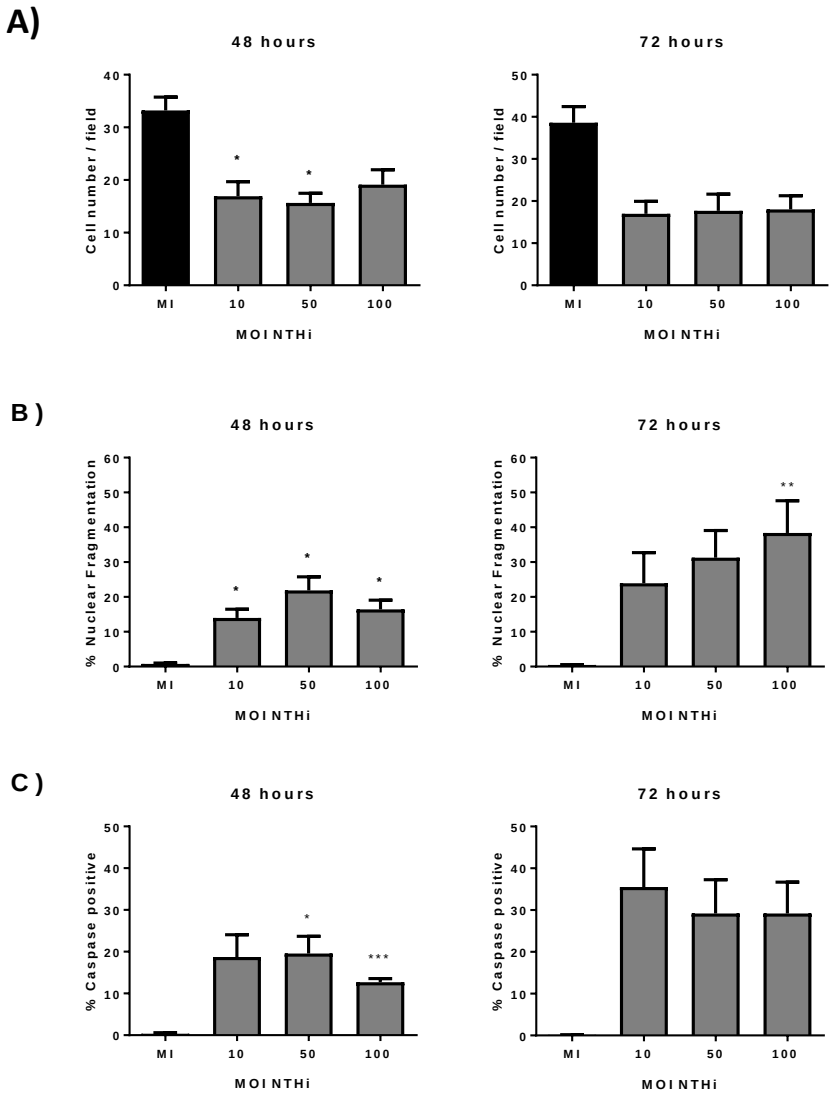


Figure 5-10: NTHi challenge causes apoptosis at later time points

MDMs were challenged with NTHi for 48 (n=5) or 72 hours (n=4) at an MOI of 10, 50 or 100 or mock infected. **(A)** Average cell density / field of view at x100 magnification of each well was quantified by DAPI staining and **(B)** apoptotic cells were then counted by change in nuclear morphology, analysed by DAPI staining and **(C)** caspase 3/7 activation by NucView staining. Significance was determined by one-way ANOVA with Tukey's multiple comparisons test, stars indicate significance compared to the mock challenged *=p<0.05, **=p<0.01, ***=p<0.001, data represented as mean ± SEM.

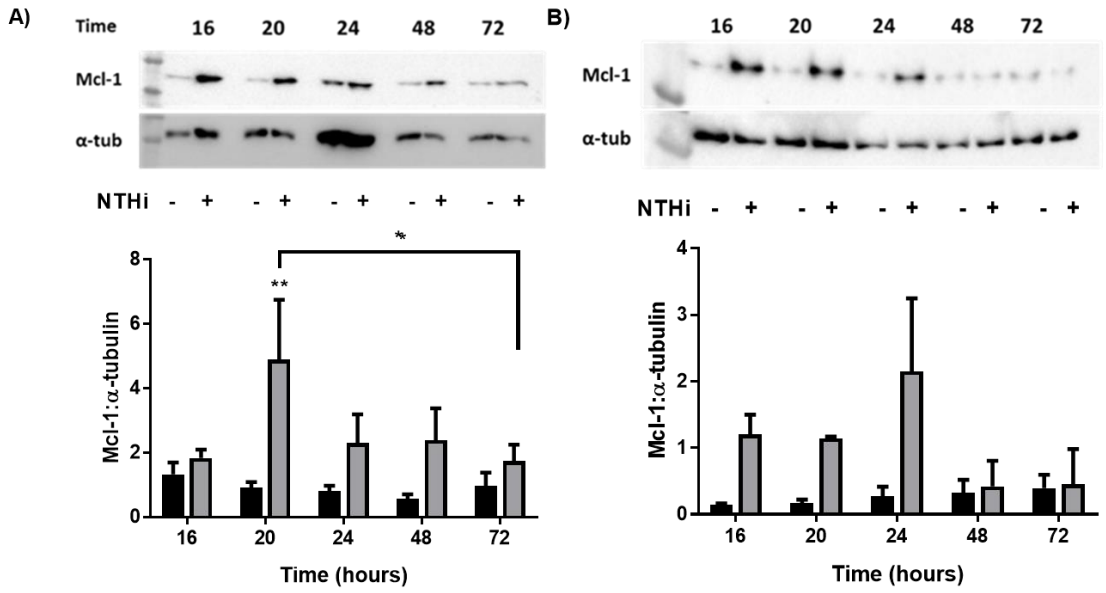


Figure 5-11: Mcl-1 levels remain high in MDMs challenged with NTHi

MDMs were challenged with **A)** NTHi 49247 (n=10), data represented as mean ± SEM, or **B)** NTHi 1479 (n=2), data represented as mean ± SD, at an MOI of 10 for a time course of 0 – 72 hours. Protein was collected from mock infected and NTHi challenged macrophages at 16, 20, 24, 48 and 72 hours post infection. Cells were lysed for protein and Mcl-1 expression measured by western blot. Membranes were stripped and re-probed for α-tubulin as a loading control. Data is represented as fold-change compared to the mock infected. A representative blot and densitometry bar chart is shown. NTHi 49247 (n=4) NTHi 1479 (n=2). Significance was determined using a two-way ANOVA with Sidak's post-test, *p=0.05, **p=<0.01 stars above bars indicate significance compared to the mock challenged control.

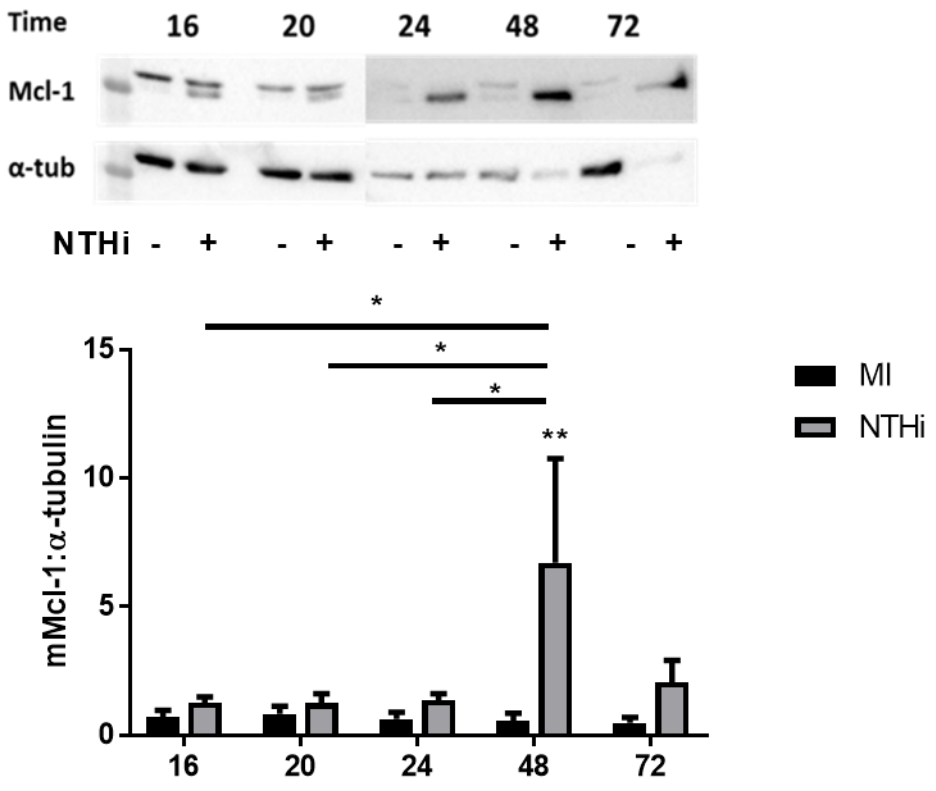


Figure 5-12: Mcl-1 levels remain high in BMDMs challenged with NTHi

BMDMs were challenged with NTHi 49247, data represented as mean ± SEM, at an MOI of 10 for a time course of 0 – 72 hours. Protein was collected from mock infected and NTHi challenged macrophages at 16, 20, 24, 48 and 72 hours post infection. Cells were lysed for protein and Mcl-1 expression measured by western blot. Membranes were stripped and re-probed for α-tubulin as a loading control. Data is represented as fold-change compared to the mock infected. A representative blot and densitometry bar chart is shown, significance was determined using a two-way ANOVA with Sidak's post-test (n=9) stars above bars indicate significance compared to the mock challenged control.

5.2.9. NTHi infection causes increased levels of Nitric Oxide at 24 hours of challenge

Data so far indicates macrophages undergo low levels of apoptosis in response to NTHi challenge until very delayed time points and this is associated with Mcl-1 upregulation for prolonged periods (16-72 hours) following challenge. There was no evidence of Mcl-1 protein decline, that normally acts as a switch to initiate apoptosis following pneumococcal challenge (Marriott et al., 2005) but rather, Mcl-1 protein levels are maintained at early time points with a decrease in levels post 20-48 hours (strain 49247) or 24 hours (strain 1479). However, large increases in cell loss occur at 48 hours of NTHi challenge. NO is produced by macrophages in response to bacterial infection as a mechanism of microbicidal activity. Furthermore, NO is required during apoptosis associated killing for MOMP and inhibition of NO shifts apoptosis to necrosis (Marriott et al., 2004). I therefore wanted to analyse NO production at 24 and 48 hours of NTHi challenge to see if increasing NO levels co-inside with decreasing levels of Mcl-1 protein at 20-48 hours onwards (Figure 5-12 and Figure 5-13). NO was measured by flow cytometry using DAF-FM (Figure 5-14). Both MDMs (Figure 5-14A) and BMDMs (Figure 5-14B) showed 5-fold and 2-fold shifts in response to NTHi challenge at 24 hours respectively, however increasing MOI had no effect on overall NO production. NO levels dropped at 48 hours to baseline indicating macrophages were no longer producing as much NO at these later time points. Interestingly, there seemed to be an overall shift in the whole population at 24 hours of challenge in MDM compared to the mock infected condition and a population of NO “high producers” was apparent for both MDM and BMDM. It would be interesting to further explore this population to see if they are also highly active at bacterial killing or undergoing apoptosis or the two in combination.

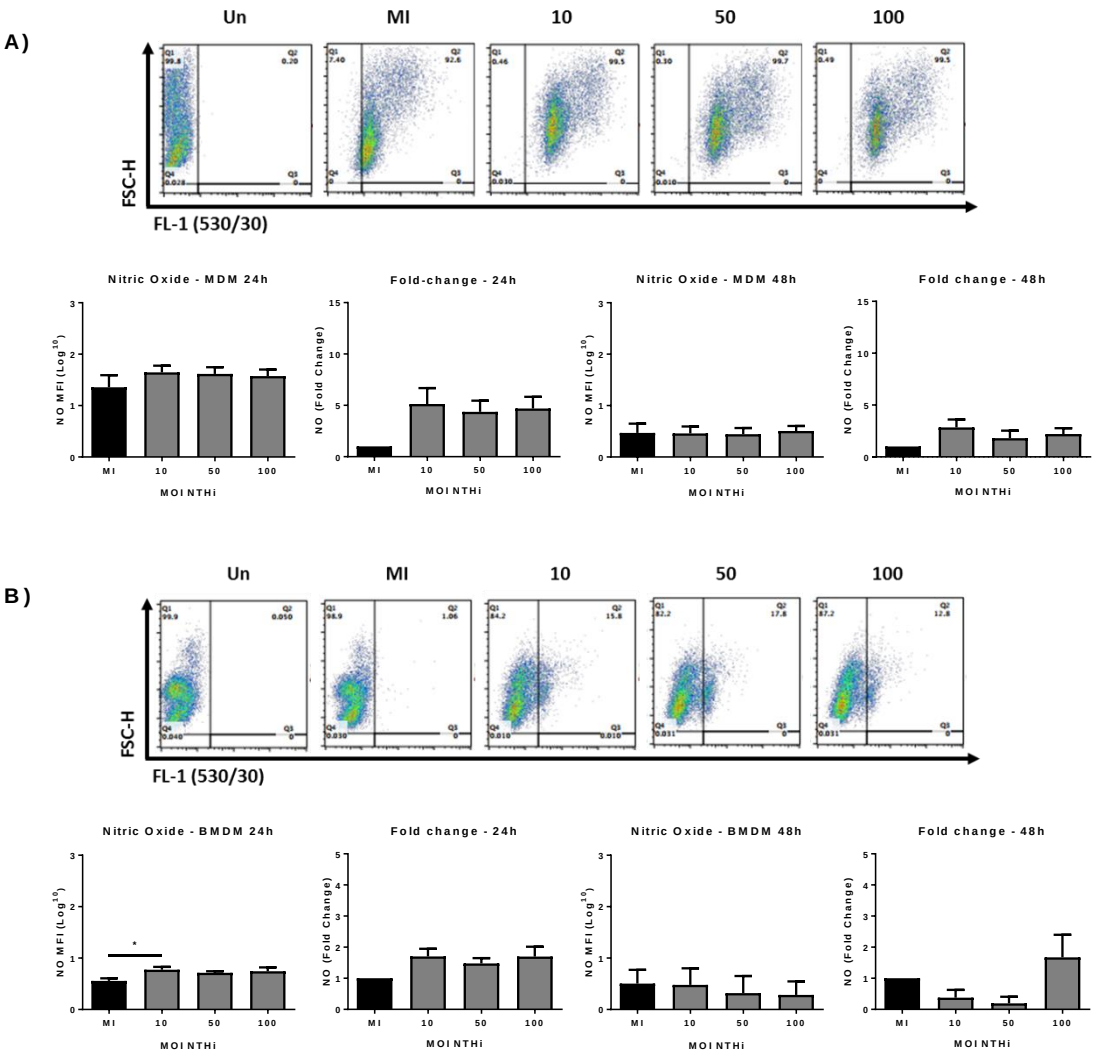


Figure 5-13: NTHi challenge increases NO generation at 24 hours.

Representative flow cytometry plots of 24 hour challenge data, Un (unstained) MI (mock infected) are pictured **(A)** MDM (n=6) and **(B)** Wild-type BMDM (n=4) **(A-B)** Cells were challenged with increasing MOIs (10, 50, 100) of NTHi for 24 or 48 hours and macrophages were stained with DAF-FM for nitric oxide production and analysed by flow cytometry. Unstained values were subtracted from all stained data to account for auto-fluorescence. Median fluorescence intensity (MFI) values are plotted as mean ± SEM on a logarithmic axis and fold-change also calculated for each timepoint in the adjacent graph. Significant results were determined using Friedman test with Dunn's post-test. *=p<0.01.

5.2.10. Loss of inner mitochondrial transmembrane potential occurs at 48 and 72 hours

Since the above data shows levels of Mcl-1 decrease overtime and there is evidence of both NO production and features of late apoptosis I next addressed whether there was evidence of mitochondrial events linked to apoptosis (Figure 5-15). NO and reduction in levels of Mcl-1 lead

to a mitochondrial mediated pathway of apoptosis involving MOMP, which is associated with loss of mitochondrial inner transmembrane potential ($\Delta\psi_m$) (Marriott et al., 2004; Maurer et al., 2006a). I decided to assess $\Delta\psi_m$ after 24, 48 or 72 hours of NTHi challenge. Macrophages were challenged with NTHi 49247 for 24, 48 or 72 hours and assessed for $\Delta\psi_m$. Loss of red fluorescence was used as a read-out for loss of $\Delta\psi_m$ indicating a decrease in J-aggregate formation which occurs as the mitochondrial inner transmembrane potential falls. Mock infected samples were used as a negative control, macrophages were challenged with *S. pneumoniae* serotype 1 and analysed at the 24 hour time point as a positive control. Bacterial challenge with NTHi for 24 hours did not cause any loss of $\Delta\psi_m$ in comparison to the mock infected control however at 48 hours there was significant loss of $\Delta\psi_m$ comparable to that following *S. pneumoniae* challenge for 20 hours and an even larger loss in $\Delta\psi_m$ at 72 hours, indicative of cell death and consistent with a delayed pathway of apoptosis.

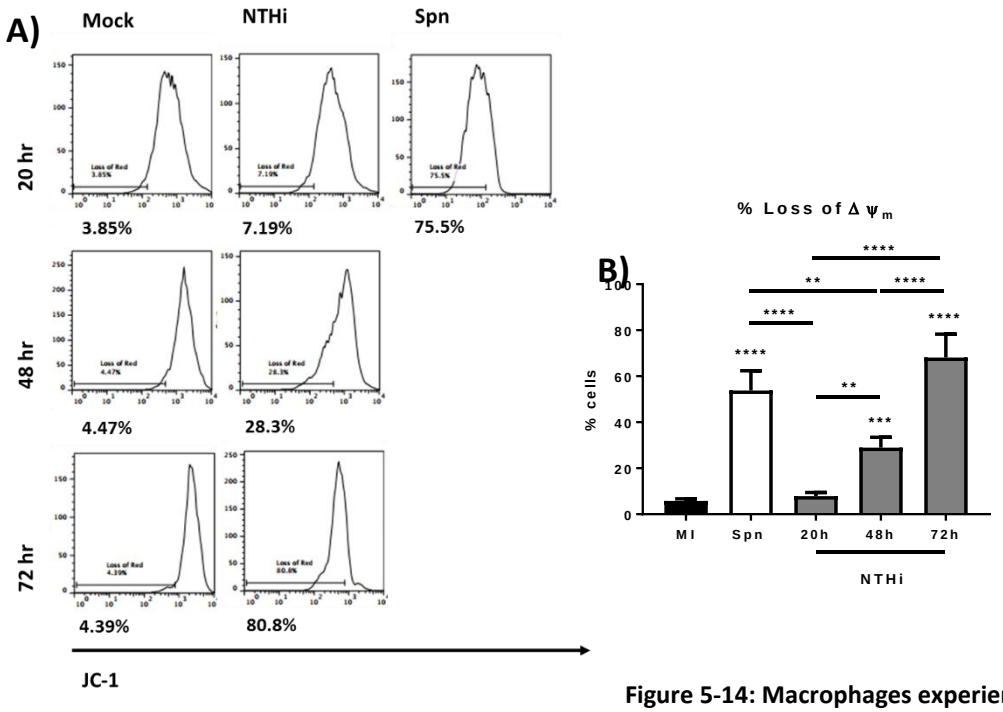


Figure 5-14: Macrophages experience loss of mitochondrial inner transmembrane potential ($\Delta\psi_m$) after 48 and 72 hours of NTHi challenge

MDMs were challenged with NTHi for a duration of 24, 48 or 72 hours. At each time point, mock infected (MI) and macrophages exposed to bacteria were analysed by flow cytometry for loss of $\Delta\psi_m$. Opsonized *S. pneumoniae* (Spn) was used as a positive control for loss of $\Delta\psi_m$. % loss of red fluorescence was deemed as a loss of $\Delta\psi_m$. **(A)** Representative flow plot pictured. **(B)** Data is plotted as mean ± SEM. A one-way ANOVA with Tukey's multiple comparisons test was used to determine statistical significance compared to MI. n=7, **=p<0.01, ***p<0.001, ****=p<0.0001.

5.3. Discussion

NTHi is readily internalised by macrophages due to it being an un-encapsulated bacterium (Clementi and Murphy, 2011). Phagocytosis of NTHi by AMs typically occurs by cytoskeleton rearrangement involving F-actin, the PI3K – Akt pathway and lipid raft generation (Clementi and Murphy, 2011; Martí-Lliteras et al., 2009). Furthermore, NTHi can form biofilms which aggregate on the surface of cells and become internalised by micropinocytosis (Clementi and Murphy, 2011). After internalisation, macrophages then proceed to traffic NTHi by phagosomal maturation terminating in the acidic phagolysosome where NTHi have been shown to localise until clearance (Martí-Lliteras et al., 2009). However, there is a fixed capacity to how many particles can be phagocytosed by macrophages, the limiting factor being the availability of cell surface area (Cannon and Swanson, 1992). The results presented in this chapter demonstrate that there is no significant alteration of internalised NTHi at 20 hours with increasing MOI of 10 – 100. This could be due to the upper limit of phagocytosis being reached with only an MOI of 10 but more likely the extracellular burden of NTHi has increased with bacterial replication to an overwhelming level over the 20 hour incubation period, meaning internalisation capacity is at its maximum by the time the cells are lysed.

If macrophages utilised apoptosis associated killing as a mechanism to clear NTHi challenge, at the time period of 16 – 24 hours observed for pneumococci (Marriott et al., 2005), I would expect to see a decrease in the number of intracellular bacteria in the wild-type mouse compared to the Mcl-1 transgenic mouse. Theoretically apoptosis could have proceeded by a pathway not regulated by the Mcl-1 transgene, or to such a great extent that the effect of the transgene was overwhelmed, however I did not observe significant cell death at this time point after challenge with NTHi. Moreover, as there was no significant difference in bacterial clearance between wild-type and transgenic macrophages, this suggests macrophages are not utilising apoptosis associated killing as a significant mechanism of pathogen clearance at this time point. However, at high MOIs, there is a significantly lower level of apoptosis in transgenic macrophages compared to wild-type macrophages and an increase in MDM apoptosis with increasing MOI, indicating MOI is having some effect on levels of apoptosis, potentially as a stress response due to the increased extracellular burden. This also indicates that the apoptosis associated killing pathway is engaged to a limited extent, albeit at levels lower than seen with *S. pneumoniae*.

There are many possibilities for why NTHi does not appear to engage a host response involving apoptosis associated bacterial killing with comparable kinetics to that observed with *S.*

pneumoniae. It might be that the low levels of apoptosis that are occurring (~10%) result due to cellular stress and involve pathways less dependent on Mcl-1 regulation or overwhelm its subtle regulation. This could result in the apoptosis that is observed not being linked to apoptosis associated killing as a mechanism of host defence, unlike the apoptosis observed when challenge with *S. pneumoniae* occurs. Another possibility could be that macrophages have not yet exhausted their early killing mechanisms therefore they do not need to induce an apoptotic pathway to clear infection, and in keeping with this view there was sustained bacterial killing observed at 14-18 hours. Although the lack of difference in intracellular bacterial colony counts between MOI suggests macrophages could be becoming overwhelmed at 20 hours of challenge the experimental design without antimicrobial treatment and the prolonged incubation means it is likely cells ended up with a comparable MOI later during the experiment. The fact that my extracellular cfu appeared to vary little between 4 and 20 hours and plateaued at a level of approximately 10^8 cfu/ml would support the latter view. Recent publications suggest adding antimicrobials to prevent the growth of extracellular bacteria (Clementi and Murphy, 2011), however as highlighted in the optimisation stages of this project, I was unable to find a 'tipping point' at which apoptosis could be induced with the addition of antimicrobials.

NTHi has also been described as an intracellular as well as an extracellular pathogen and certain strains have been shown to be able to persist inside the macrophage up to 72 hours of infection (Craig et al., 2001). Therefore, it is reasonable to speculate that NTHi might have a mechanism for preventing high levels of apoptosis. *Mycobacterium tuberculosis* has recently been shown to down-regulate EBP50 (NHERF-1). When EBP50 is over expressed in macrophages it significantly increases expression of iNOS and NO dependant apoptosis, involving increased expression of Bax and caspase-3 which engages cell apoptosis and intracellular bacterial killing (Guo et al., 2016). Therefore, it is possible the low levels of apoptosis I see at 24 hours are due to signalling cascades orchestrated by the bacterium. Furthermore, NTHi is a very pro-inflammatory pathogen (Berenson et al., 2005) and has been shown to potentiate TNFα mediated apoptosis in human bronchiole epithelial cells as a mechanism for evading the host response (Gallelli et al., 2010).

Direct comparison of NTHi apoptosis levels to *S. pneumoniae* and an alternative *H. influenzae* serotype; Hib showed a marked reduction of apoptosis in both NTHi and Hib compared to *S. pneumoniae* challenge. These results are in line with previous data which shows 5-10% apoptosis after macrophage challenge with Hib at the 20 hour time point (Marriott, H.M., unpublished data). The NTHi 1479 strain showed a more marked reduction than our NTHi laboratory strain

(49247) (2-4% apoptosis compared to 8-10%). Strain 1479 is a clinical isolate from the sputum of a patient with COPD whereas strain 49247 is a laboratory quality control strain which overtime are known to lose some of their pathological relevance (Fux et al., 2005). Others have reported a high degree of variability between NTHi strains (Craig et al., 2001) it is therefore perhaps not surprising to see differences in induction of macrophage effector functions by different strains and the lower levels of apoptosis with the clinical strain NTHi 1479 were in keeping with a role for pathogen factors inhibiting apoptosis. Interestingly, NTHi strain 1479 has been shown to have increased production of OMV, which contribute to propagation of infection in a number of ways, compared to other strains (Roier et al., 2016), further demonstrating the increased virulence of this strain and providing another factor for the variability seen between strains in induction of host responses.

Analysis of Mcl-1 levels over a 16 – 72 hour time course reveal that after challenge with both strains of NTHi, Mcl-1 protein levels remain high until 20 hours post inoculation (NTHi 49247) or 24 hours (NTHi 1497) MDM or 48 hours in NTHi 49247 challenged BMDM after which levels fall from peak. This implies that the apoptosis observed at 20 hours, as seen with NTHi 49247 is not dependent on Mcl-1 degradation but rather cells are sensitised by an alternative pathway or to an extent which cannot be inhibited by Mcl-1, which results in caspase 3/7 mediated apoptosis, since activation of caspase 3/7 was determined by NucView positive nuceli. It is also possible that both strains cause upregulation of Mcl-1 levels to prevent apoptosis so macrophages cannot effectively engage microbicidal activities triggered by apoptosis. Since NTHi are capable of persisting intercellulary up to 72 hours post infection, increases in Mcl-1 could be due to a pathogen mediated invasion mechanism which persists until induction of apoptosis at later time-points. Interestingly, Morey *et al* showed in bronchiole epithelial cells, that although NTHi associate with late endosomes which become acidified, there is a lack of lysosomal fusion and less than 10% of NTHi containing compartments associate with cathepsin D after 8 hours of bacterial exposure (Morey et al., 2011). As lysosomal fusion and cathepsin D activation provide the trigger for apoptosis associated killing (Bewley et al., 2011a), this could be a conceivable mechanism by which NTHi prevent apoptosis associated killing. It would therefore be interesting to analyse cathepsin D activation and lysosomal membrane permeabilisation, another factor required for induction of apoptosis-associated killing of *S. pneumoniae* (Bewley et al., 2011a) over the extended time course of 24 – 72 hours and interpret whether there are any variations in cathespin D activation and lysosomal membrane permeabilisation between our laboratory and clinical isolate. Other bacteria, such as the Gram-negative intracellular pathogen *Ehrlichia chaffeensis*, have been shown to upregulate anti-apoptotic proteins including Mcl-1 and other

Bcl-2 family members in THP-1 cells to aid formation of a protected intracellular niche (Faherty and Maurelli, 2008; Zhang et al., 2004). Other pathogens such as several Chlamydia species; *Chlamydia muridarum, Chlamydia caviae and Chlamydia psttaci* do this by preventing MOMP and cytochrome c release by inhibition of the pro-apoptotic proteins Bax and Bak (Zhong et al., 2006), (Häcker et al., 2006). Although NTHi is not capable of intracellular replication, it is capable of persisting inside cellular compartments in both epithelial cells and macrophages (Craig et al., 2001; Morey et al., 2011). A more recent study demonstrates increasing levels of airway epithelial cell apoptosis up to 24 hours of NTHi infection, arguing that this process is a bacterial mechanism used for induction of cytotoxicity and NTHi endosomal escape (Goyal et al., 2015). Contrastingly, David Singh and colleagues recently showed COPD patient AMs demonstrate low basal levels of apoptosis in AMs with no significant induction of apoptosis after NTHi infection with MOIs of 10 up to 4000 (Khalaf et al., 2017), however, results were only recorded at 24 hours which may not be a sufficient time course. Further, our group have previously shown COPD AMs have decreased capabilities of undergoing apoptosis in response to pneumococcal infection (Bewley et al., 2017), this apoptosis defect may be the reason there is no positive apoptosis data from NTHi challenged COPD AMs in the Khalaf study, but it would therefore be interesting to see inclusion of apoptosis data in NTHi challenged AMs from healthy subjects at 24 hours and later time points.

NO is an established modulator of apoptosis in immune cells (Brüne et al., 1999; Chung et al., 2001). NO detection by DAF-FM shows increases in NO at 24 hours after NTHi challenge with MOIs of 10 – 100. It is therefore possible that the 10% apoptosis at 24 hours of challenge is linked to NO release. As has been previously shown NO is an essential component of apoptosis associated killing activated by pneumococci (Marriott et al., 2004). While I observed NO release at 24 hours, it is not directly comparable to that in the pathway described for *S. pneumoniae* clearance as Mcl-1 protein levels remain high. It is noteworthy however that my host group have previously suggested that is the fall from peak rather than downregulation compared with baseline which triggers apoptosis (Marriott et al., 2005). Mcl-1 levels decrease from peak from 24-48 hours after NTHi challenge, my data shows NO levels are falling from peak levels by 24 hours. The fact that peak cell death as analysed by DAPI doesn't coincide with peak NO at 24 hours might be indicative of an alternative form of cell death. My demonstration, however, of $\Delta\psi_m$ and more specifically caspase 3/7 activation and nuclear features of apoptosis is more consistent with apoptosis as the cause of late cell death at 48 – 72 hours after bacterial challenge, even though $\Delta\psi_m$ can occur with other forms of cell death (Yu et al., 2014). So, it remains possible that NO still contributes to the cell death but that all the conditions required

to induce apoptosis are not in place during peak NO production and the induction of apoptosis is delayed. In part, this may reflect the competing anti-apoptotic stimuli being induced by the bacteria, as discussed above. Conversely, NO has also been shown to have anti-apoptotic effects under certain circumstances at low-doses through NFκB activation and subsequent cyclooxygenase 2 (COX-2) expression (von Knethen et al., 1999). NFκB activation has been shown to enhance levels of pro-survival Bcl-2 family members, including Mcl-1 in immune tissue (Chen et al., 2000). The opposite scenario is therefore also possible; a delay of apoptosis until later timepoints (i.e. 48 hours) might be mediated by low levels of NO production, which have been shown to be triggered by classical activation stimuli such as LPS and IFN-γ activation (von Knethen et al., 1999) In this regard this might be a host adaptation allowing a sustained period of bacterial killing since intracellular microbicidal mechanisms did not appear to be exhausted.

Interestingly, analysis of DAF-FM by flow cytometry reveals a population of NO "high" producing cells at baseline (mock infected) and with bacterial challenge. Following bacterial exposure, the whole population shifts in fluorescence intensity, including the NO high population, by around 0.5 – 1 log decade. To understand the potential importance of the NO high population, it would be interesting to conduct DAF-FM microscopy to see whether the NO high population is localised within cells with a high amount of intracellular NTHi and, whether NO high cells are undergoing more apoptosis or microbicidal killing than the rest of the population.

In keeping with low levels of apoptosis at 20 hours and maintenance of Mcl-1 protein levels, NTHi challenge did not reveal a significant loss of $\Delta\psi_m$ until 48 hours. This finding is in line with increased cell loss at 48 and 72 hours in comparison to earlier time points and the decrease in Mcl-1 levels from 24-48 hours onwards. Loss of $\Delta\psi_m$ occurs in several different kinds of cell death, as well as apoptosis. It is a possibility that macrophages are undergoing a more pro-inflammatory form of cell death such as pyroptosis and this form of cell death shares many features with apoptosis (Vande Walle and Lamkanfi, 2016) but the caspase 3/7 activation and nuclear morphology would be less consistent with this. Although pyroptosis also involves DNA damage and can alter nuclear morphology it is primarily reliant on caspase 1 activation and results in membrane leakage and IL-1 family cytokine production (Fink and Cookson, 2005). It would however be of interest to check for evidence of caspase 1 activation, mature IL-1β release and membrane permebalisation in my macrophages to more fully exclude this death process as a contributory factor during exposure to NTHi. To determine the cause of loss of $\Delta\psi_m$ and cell loss at later time points, my current work is focused on defining cell death by flow cytometry analysis of caspase 3/7 activation and cell permeabilization using DRAQ7 dye, indicators of cell

apoptosis or general cell death respectively, over a time course of 16 – 72 hours of NTHi challenge. I am also currently determining whether NTHi infection causes cytochrome c release from the mitochondria, a marker of MOMP and an indicator of induction of the apoptosis cascade (Bewley et al., 2011b; Cai et al., 1998) to further validate apoptosis as the cell death mechanism responsible for cell loss at 48 – 72 hours.

The work presented in chapter 4 of this thesis demonstrates a role for IFN-γ in enhanced clearance of *S. pneumoniae* infection. I also wanted to determine whether CAM were capable of enhanced clearance of NTHi bacteria compared to other activation phenotypes. Surprisingly, my results revealed no differences in the clearance NTHi from challenged cells at early time points (2-4 hours) or over a late time course (14 – 18 hours), with the exception of late killing in M(IFN-γ) BMDMs which mostly cleared bacteria by 16 hours. Likewise, there were no significant differences in NO or ROS levels with IFN-γ stimulation. This result was initially unexpected since challenge with NTHi stimulates the release of several pro-inflammatory cytokines and chemokines such as TNFα, IL-1β, IL-6, CXCL8 (Khalaf et al., 2017; Leichtle et al., 2010) and microbicidals such as NO (Lin et al., 2014) through activation of NFκB (Watanabe et al., 2004) and I had predicted this would be further enhanced by IFN-γ stimulation. Furthermore, reduction of classical activation mediators such as TNFα and iNOS attenuates killing of NTHi in murine peritoneal macrophages (Leichtle et al., 2010; Lin et al., 2014). NTHi has previously been shown to create a large pro-inflammatory response from human macrophages (Berenson et al., 2005). The data presented in this chapter shows NTHi instigates a potent pro-inflammatory response within the first few hours of challenge in agreement with other reports (Foxwell et al., 1998b; Khalaf et al., 2017) and in comparison to *S. pneumoniae*. Since macrophages are known to be highly plastic to their local microenvironment, it is reasonable to suggest that the strong inflammatory signatures produced by NTHi challenge override prior macrophage activation forcing the population into a classically activated phenotype. In order to confirm switching of macrophage phenotypes in response to NTHi infection it would be interesting to undertake ELISA analysis of some key pro-inflammatory cytokines and chemokines and mRNA analysis of pro-inflammatory modulators such as those involved in NF-κB pathway to check for production of key classical activation markers in 24 hour activated macrophages after short term exposure to NTHi. It is also noteworthy that apoptosis induction is associated with downregulation of pro-inflammatory cytokine production and effectively reduces its production in pneumococcal infection, (Kobayashi et al., 2003; Marriott et al., 2006). The higher levels of cytokines I detected, and others have described in macrophages exposed to NTHi (Berenson et al., 2005), will I predict

in part be due to failure to engage apoptosis to comparable levels to *S. pneumoniae* at 16-24 hours.

To conclude, the work presented in this chapter delineates novel innate immune responses of human and murine macrophages in response to NTHi infection. Firstly, this work demonstrates low levels of apoptosis at 20 hours post NTHi challenge - in association with sustained upregulation of the anti-apoptotic protein Mcl-1. Secondly, I provide evidence for a later induction of cell death process which occurs after peak NO production and coincides with the fall from peak Mcl-1 production. Although this is not associated with Mcl-1 down-regulation the cell death process has many typical features of apoptosis such as loss of $\Delta\psi_m$, caspase 3/7 activation and nuclear morphology. Finally, this work tests the effects of NTHi on differentially activated human macrophages and indicates that irrespective of macrophage baseline phenotype macrophages are rapidly responsive to NTHi and maintain a marked production of pro-inflammatory cytokines which may in part be accentuated by the low levels of apoptosis. This explains the marked pro-inflammatory response associated with NTHi infections at sites such as the airway (Clemans et al., 2000).

6. Discussion

6.1. M(IFN-γ) show enhanced *S. pneumoniae* clearance and apoptotic responses

The first aim of my thesis was to understand the effect of macrophage activation on macrophage effector functions during *S. pneumoniae* challenge, with special attention to macrophage apoptosis associated killing. I used two models to explore my question; wild-type and CD68 Mcl-1 transgenic BMDMs and human MDMs from healthy donors. I have shown that M(IFN-γ) macrophages in both wild-type and transgenic BMDMs display enhanced clearance of *S. pneumoniae* at early and late time points after bacterial challenge. Furthermore, my results show an enhancement of M(IFN-γ) apoptosis at timepoints key to the late clearance of intracellular pneumococci in both BMDMs and MDMs.

The relationship between macrophage activation and macrophage apoptosis associated killing has not been explored in detail, nor has the possibility of Mcl-1 modulation of macrophage activation. While other members of Mcl-1 signalling pathways, such as Akt and Mammalian Target of Rapamycin (mTOR) have been shown to play key roles in macrophage commitment to classical or alternative activation (Arranz et al., 2012; Byles et al., 2013; Vergadi et al., 2017), my results did not demonstrate any effect of Mcl-1 overexpression on macrophage activation or any effect of macrophage activation on endogenous levels of murine Mcl-1. Furthermore, pathways known to regulate macrophage activation were not altered by the presence of the Mcl-1 transgene following transcriptomic analysis. Therefore, it appears the effect of M(IFN-γ) on late pneumococcal clearance and macrophage apoptosis overwhelms the relatively subtle level of apoptosis inhibition provided by the Mcl-1 transgene (Zhou et al., 1998).

6.1.1. Transcriptomic T-cell signatures are observed following infection in Mcl-1 transgenic mice

My host group has previously described a role for Mcl-1 downregulation and apoptosis in *S. pneumoniae* clearance at 16 hours of challenge and this phenomenon is inhibited in Mcl-1 overexpressing transgenic macrophages (Bewley et al., 2017). Transcriptomic analysis of 16 hour BAL fluid from an *in vivo* model of resolving infection revealed the presence of a T-cell signature in transgenic samples. This finding lead to a hypothesis that, one possible source for IFN-γ during pneumococcal infection *in vivo* is from Th1 cell activation and the production of T-cell IFN-γ aids pro-inflammatory effector functions in transgenic macrophages which are unable to clear infection at late timepoints due to reduction of apoptosis.

Disappointingly, the large inter-experimental variability likely masked many of the significant gene changes in the transcriptomics study which might have given clues to the specific nature of the T-cell response, meaning verification by other methods is now essential to confirm and further investigate this finding. Despite the limitations of the transcriptomics study, which I outline in chapter 4, several reports have revealed the importance of T-cell responses in the resolution of pneumococcal infection, in particular the role of Th1 and Th-17 cells (Olliver et al., 2011; Wang et al., 2017). Interestingly there are also reports of Th17 subsets that can produce IFN-γ (Boniface et al., 2010).

Initial analysis of T-cell populations by flow cytometry are inconclusive due to excessive neutrophil recruitment which demonstrates the inoculation dose was too high for induction of a low dose resolving infection which AMs have sufficient ability to handle alone (Dockrell et al., 2003). However, there are also several other potential cellular sources of IFN-γ which should be considered in future experiments. These include ILC subtypes; NK cells, previously shown to be essential for the resolution of *S. pneumoniae* infection and also non-cytotoxic group 1 ILCs and $CCR6^-$ $T\text{-}bet^+$ group 3 ILCs which do not currently have a defined role in the resolution of pneumococcal infection, but should not be ruled out as contributors (Artis and Spits, 2015; Baranek et al., 2017). My data has shown IFN-γ is helpful to clearance of *S. pneumoniae* in an *in vitro* setting, however, *in vivo* the T-cell signature might not be attributable to just IFN-γ producing cells, for example, the literature suggests IL-17 producing cells are more effective in the clearance of *S. pneumoniae* infection and Th17 cells and IL-17/IL-22 producing group 3 ILCs have been implicated in the immune response against *S. pneumoniae* (Olliver et al., 2011; Van Maele et al., 2014). The role of IL-17 on macrophage effector functions was not explored here but would be interesting to study. One final speculation is that rather than, or in addition to, working to reprogram AMs, a small T-cell presence might be responsible for the observed signature and instrumental in controlling recruitment of phagocytes from the blood to aid the transgenic response, for example; IFN-γ and IL-22 producing Th1 cells have been shown to be essential for recruitment of neutrophils in the resolution of *S. pneumoniae* infection (Yamamoto et al., 2004).

6.1.2. IFN-γ enhance macrophage killing of *S. pneumoniae* and macrophage apoptosis

The second major question still to be answered in this body of work is the mechanism by which macrophages stimulated with IFN-γ are capable of enhanced killing and apoptosis of *S. pneumoniae*. I would like to know whether apoptosis is directly linked to killing activity or

whether macrophages are more susceptible to apoptosis whilst also acting as effective pneumococcal killers. I would hypothesise that IFN-γ stimulation generates the release of mitochondrial ROS in concert with apoptosis as my host group has previously described (Bewley et al., 2017) but overwhelms the subtle capacity of Mcl-1 to prevent apoptosis (Zhou et al., 1998), as outlined above. Previously, it has been shown that IFN-γ can induce apoptosis by Bcl-2 downregulation and increased Bak expression (Zhou et al., 2008) and, IFN-γ mediated production of NO has been shown to increase ROS levels which leads to the production of peroxynitrite, shown to induce cell death in tumour cells (Rakshit et al., 2014). IFN-γ has been used as a therapeutic in chronic granulomatous disease where it has been shown to restore microbicidal activity (Errante et al., 2008) and its use has also been evaluated for other clinical conditions such as atypical mycobacterial infections (Milanés-Virelles et al., 2008). If future exploration continues to reveal a role for IFN-γ in overcoming the apoptosis associated killing defect, treatment with this cytokine could be therapeutic in conditions where apoptosis associated signaling is impaired such as COPD and HIV (Bewley et al., 2017).

6.1.3. Future work

There are several experiments which are currently in progress to directly address some of the open questions remaining in this thesis. The most obvious questions which I have already acknowledged are clarification of the T-cell signature, including the cellular source, and identification of the specific modulators which lead to IFN-γ associated apoptosis and enhanced pneumococcal clearance.

It is firstly essential to re-optimise the *S. pneumoniae* low dose infection model, using a newly obtained serotype 1 strain from Professor Tim Mitchell, which has similar characteristics of the original Statens Serum Instiut (SSI) strain which is no longer available because of changes to the Materials and Transfer Agreement, to mimic the same infection conditions which were used for the transcriptomic analysis. Once completed, the myeloid and T-cell flow experiments can then be repeated for verification of T-cell contribution and exploration of the T-cell phenotype. It would then be necessary to conduct T-cell and macrophage co-culture experiments to determine if specific T cell phenotypes are capable of reprogramming macrophage responses to aid clearance of *S. pneumoniae*. To further our understanding of AM activation phenotypes during *S. pneumoniae* challenge in wild-type and transgenic BMDMs, it might also be useful to include an additional panel for analysis of AM subsets.

To unravel the molecular details of IFN-γ induced apoptosis and enhanced clearance of *S. pneumoniae*, it will be important to determine how apoptosis is initiated. NO is an important

product of IFN-γ activated macrophages and has roles in both induction of apoptosis and bacterial killing (Marriott et al., 2004; Rakshit et al., 2014). It would therefore be interesting to measure levels of NO production and co-localisation of NO with *S. pneumoniae* in intracellular compartments. Although I did not find a role for IFN-γ as a direct modulator of Mcl-1 and it is therefore unlikely IFN-γ modulates apoptosis by Mcl-1 downregulation, it is possible other Bcl-2 family members are involved (Zhou et al., 2008). It would therefore be interesting to look at modulation of other Bcl-2 family members which have been previously implicated in IFN-γ induced cell death.

6.2. NTHi challenge induces different kinetics of apoptosis compared to previous studies with *S. pneumoniae*

Previously, my host group has demonstrated that apoptosis associated killing is induced in response to *E. coli* and *H. influenzae* challenge (Webster et al., 2010). On the other hand, we have also described the activity of pathogens which escape apoptosis associated killing such as *Staphylococcus aureus* and *Neisseria meningitidis* (Jubrail et al., 2016; Tunbridge et al., 2006). Given recent recognition of the apoptosis defect in COPD (Bewley et al., 2017), it is interesting to speculate if part of the success of NTHi as a pathogen in COPD exacerbations is due to its ability to thrive in a host with defective macrophage apoptosis associated killing. My results demonstrate macrophages do undergo apoptosis in response to NTHi challenge, however, whilst sharing some similarities, this process does not directly mimic macrophage apoptosis associated killing described with *S. pneumoniae* challenge.

Firstly, at crucial timepoints when *S. pneumoniae* causes around 30% apoptosis NTHi caused only low levels of 10% and under and this was not associated with decreased Mcl-1 levels. When NTHi challenge was continued to late time points (48 – 72 hours), higher levels of apoptosis and a decrease in Mcl-1 from peak levels with levels approaching close to baseline were observed. The delay of apoptosis is likely due to the fact that NTHi are easily internalised and killed, despite presence of genes that help promote anti-oxidant defence, by comparison to *S. pneumoniae* (Brockman et al., 2017; Jonsson et al., 1985a) and macrophages therefore may not become overwhelmed until much later. The fact that Mcl-1 remains high supports the idea that it is advantageous for macrophages to remain alive and continue canonical (e.g. NOX2 mediated) phagolysosomal killing at earlier time points. In addition they traffic appropriately to phagolysosomes furthermore NTHi (Martí-Lliteras et al., 2009), which will allow both early canonical killing mechanisms but also potentially allow later apoptosis associated killing, which requires phagolysosomal bacteria to trigger it (Ali et al., 2003). Eventually, macrophage

apoptosis is associated with nuclear fragmentation, caspase 3/7 positivity and loss of ΔΨ, these features fulfil the requirements for apoptosis associated killing.

Levels of apoptosis are lower with the clinical isolate 1479 which also shows increased levels of Mcl-1 protein earlier after challenge (i.e. at 16 – 24 hours). Levels of Mcl-1 peak at 24 hours and later decrease whereas with the laboratory reference strain NTHi 49247 levels peak at 20 hours and decrease thereafter. Therefore, it is conceivable that a more virulent clinical strain might be a stimulus stressful enough to cause host upregulation of Mcl-1 levels which prolongs macrophage lifespan to ensure bacteria are properly killed by canonical phagolysosomal killing mechanisms. Mcl-1 may then be downregulated when cells become overwhelmed and apoptosis associated killing ensues to clear bacteria slightly later. Another perspective is that NTHi may benefit from an increase in host Mcl-1 levels for the utilization of macrophages as a protective niche. Although reports have speculated that NTHi has the ability to persist inside macrophages (Craig et al., 2001) and bronchiole epithelial cells (Morey et al., 2011), it is not usually regarded as an intracellular pathogen. To explore this idea, it would be interesting to look at co-localisation of NTHi with markers of acidified lysosomes and mediators of bacterial killing such as ROS and NO to determine if NTHi is associated with microbicidals or resides in a less hostile environment.

My results demonstrate increased early pro-inflammatory cytokine responses induced by NTHi in comparison to *S. pneumoniae* but no significant differences in early killing between macrophage activation conditions. This observation indicates the pro-inflammatory nature of NTHi challenge can override prior macrophage activation stimuli, in keeping with the plastic nature of macrophage activation (Sica and Mantovani, 2012). Furthermore, retainment of inflammatory cytokine production at later time points of NTHi challenge, might be attributable to the delay in macrophage apoptosis, since apoptosis and the efferocytosis of apoptotic cells downregulates pro-inflammatory cytokine production in response to respiratory bacteria (Marriott et al., 2006). Previously published work suggests macrophage apoptosis in response to *S. pneumoniae* challenge is beneficial to resolution of the inflammatory response (Dockrell et al., 2003). Prolonged presence of NTHi in the COPD lung and its potentiation of pro-inflammatory cytokine production are consequences of the reduced macrophage apoptosis and subsequent impairment of bacterial killing (Bewley et al., 2017) and may be one cause of the increased inflammation seen in COPD (Tuder and Petrache, 2012). The pro-inflammatory consequences of impaired NTHi clearance may contribute to the apparent contradictions in observations relating to macrophage polarisation in COPD where some studies suggest

macrophages show predominant alternative activation (Kaku et al., 2014; Shaykhiev et al., 2009b). But other studies suggest classical and alternative activation features may both be observed in the lung and often in the same cells (Hodge et al., 2011). It remains possible that an NTHi stimulus might modify an intrinsic alternative activation stimulus in COPD to produce this mixed phenotype. Certainly, in murine models, NTHi exposure has been shown to contribute to certain aspects of COPD, such as continued pro-inflammatory cytokine production and inflammatory cell infiltration (Moghaddam et al., 2008). It would therefore be interesting from a therapeutic perspective to induce apoptosis at earlier timepoints and see if this leads to enhanced bacterial clearance and resolution of the pro-inflammatory response.

6.2.1. Future work

To round off my current findings there are several remaining experiments which are key to completion of this story. My current work is focused on understanding if the apoptosis cascade also involves release of cytochrome c from the mitochondria by measurement of cytochrome c protein levels from mitochondrial and cytosolic fractions at key timepoints of NTHi challenge.

Although I have analysed the kinetics of NTHi killing from 14 – 18 hours, I did not continue this time course until complete bacterial clearance. It would be interesting to extend this analysis to examine whether macrophages can clear all bacteria or if killing becomes exhausted and a population of NTHi are capable of intracellular survival. This would in part address the alternative hypothesis that NTHi are capable of up-regulating cell survival programs in order to persist inside macrophages.

I have used human MDMs from healthy donors as a model of host response to bacterial challenge, however NTHi infection is most frequent in those who are vulnerable due to insufficient ability to mount an effective immune response (van Wessel et al., 2011). Therefore, it would be beneficial to repeat aspects of my analysis in a more physiologically relevant model, such as human AMs ideally from patients with diseases associated with NTHi such as COPD. Murine models which involve LPS and elastase exposure for 4 weeks prior to infection induce characteristics of the COPD lung (Sajjan et al., 2009). Use of this model in our CD68 Mcl-1 transgenic macrophages would provide an experimental system which includes the apoptosis defect *in vivo* and allows for genetic manipulation of a regulatory factor, to check if it enhances the defect, albeit in a murine not a human disease model, which therefore has drawbacks.

6.3. General limitations

Although useful for analysis of the Mcl-1 apoptosis defect, and appropriate when availability of clinical samples is scarce, it is important to acknowledge that macrophages derived from the CD68 Mcl-1 transgenic mouse are a crude representation of a clinical defect which likely doesn't exist in singularity. In addition, healthy MDMs are not representative of the AM population (Guilliams et al., 2013; van de Laar et al., 2016). A physiological disease setting would allow analysis of macrophages, which are likely impaired in other effector functions, and are subject to the input of and crosstalk with several other cell types, which may also be functionally defective. Furthermore, most of the experiments conducted in this thesis use only blood derived macrophages. It is now widely acknowledged that resident AMs in the steady state are derived from the foetal liver which affects their phenotype and function (Guilliams et al., 2013; van de Laar et al., 2016). Furthermore, this thesis has only fully explored the effects of three macrophage phenotypes and in a physiological setting the macrophage activation profiles would be more specific to the local tissue environment than the *in vitro* models used here. Since COPD AM phenotypes have been shown to be dysregulated (Kaku et al., 2014; Shaykhiev et al., 2009b), and consist of phenotypes which extend beyond classical and alternative macrophage activation (Xue et al., 2014), it would be interesting to sort COPD AMs based on their cell surface expression markers and then extend the killing and apoptosis experiments conducted in this thesis into specific *in vivo* COPD AM phenotypes. A recent report highlights how AMs can be sorted based on their size which is also indicative of macrophage activation state, the group then go on to analyse phagocytotic capacity of healthy and COPD AMs (Dewhurst et al., 2017).

6.4. Concluding remarks

In summary, the data presented in this thesis demonstrates a role for classical macrophage activation in the modulation of the innate immunity of *S. pneumoniae* challenge and a potential role for T-cells in the orchestration of this response. I show increased apoptosis and pneumococcal killing in M(IFN-γ) stimulated murine BMDMs and MDMs from healthy donors. From my analysis, it appears IFN-γ stimulated increases in apoptosis are not associated with alterations in Mcl-1 degradation and further work is needed to delineate the specific signalling pathways involved in this response. I also show NTHi macrophage challenge results in an apoptotic response which is delayed in comparison to that previously described for *S. pneumoniae* infection and the nature of this response varies between NTHi strains. Furthermore, I show this response is associated with enhanced pro-inflammatory cytokine responses to NTHi in comparison to *S. pneumoniae* responses. These findings highlight the important roles of macrophage activation in fine tuning the innate response to pathogenic

bacteria and the potential for altered pathogenicity when consequences of this activation are altered in response to specific pathogens.

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