An Investigation into the Regulatory Capacity of Invariant Natural Killer T (iNKT) Cells during the Innate and Adaptive Immune Response to Influenza Infection



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Influenza A virus (IAV) infection is a highly contagious respiratory disease, which can cause substantial morbidity and occasionally death. Invariant natural killer T (iNKT) cells, a subset of CD1d-restricted T lymphocytes, have been identified as important modulators of immunity, mediating both pro- and anti-inflammatory responses. We show that iNKTs play an important role for the generation of protective innate and adaptive immune responses to IAV, and enhance heterotypic immunity to influenza virus following vaccination with a novel pseudotyped virus, S-FLU, which lacks HA expression.

iNKT-deficient mice (*Jα18*$^{-/-}$) showed increased susceptibility and lung pathology following IAV infection, which correlated with an exaggerated accumulation of inflammatory monocytes and neutrophils in the lung. Consistent with these findings, we demonstrated in IAV-infected CD1d$^{-/-}$:CD1d^{+}/$^{+}$ mixed bone marrow chimeric mice, that the lack of CD1d expression on myeloid cells purified from the lungs of IAV-infected mice significantly increased expression of genes linked to cell activation, survival and polarisation between pro- and anti-inflammatory responses. We extended these results by examining the role of chemokine signalling during IAV infection, and identified a novel role for fractalkine (CX3CL1) and its receptor (CX3CR1) in innate immune cell recruitment. The use of CX3CR1-iNKT cell double knockout mice revealed that, although upregulated in *Jα18*$^{-/-}$ mice, CX3CR1-CX3CL1 signalling is not required for cell migration during exacerbated IAV-responses.

Finally, we showed that iNKT-deficient mice displayed reduced longevity of peripheral IAV-specific CD8^{+} T cells following S-FLU vaccination, compared with wild-type mice. S-FLU vaccination protected mice following 5 day heterotypic challenge, however vaccinated mice exhibited reduced viral clearance, and importantly a significant reduction in IAV specific memory T cell response, suggesting a possible role of iNKT cells during T cell priming in modulating the lifespan of antigen-specific T cell responses. Although additional experiments are warranted, these results suggest that harnessing iNKT cells should be considered to modulate the innate and adaptive immune response to optimise heterotypic vaccine design and for therapeutic intervention against acute influenza infection.

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Declaration of Authorship

I declare that this thesis represents my own work, except where stated below. It has never been submitted for any other degrees.

CD1d and NP monomers were generated by Dawn Shepherd and John-Paul Jukes.

FACS sorting was performed by Craig Waugh, Kevin Clark and Paul Sopp in the WIMM Sorting Facility.

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Abbreviations

α-GalCer	α-Galactosylceramide
AMs	Alveolar Macrophage
APCs	Antigen Presenting Cell
B cell	B Cell
B6	C57BL/6 mice
BAC	Bacterial Artificial Chromosome
BALF	Bronchi-Alveolar Lavage Fluid
BCL	B-cell Lymphoma 2
BCR	B cell Receptor
BSA	Bovine Serum Albumin
CD40L	CD40 Ligand
CLP	Common Lymphoid Precursor
CMP	Common Myeloid Precursor
COPD	Chronic Obstructive Pulmonary Disorder
CRBG	Computation Biology Research Group
CTL	Cytotoxic T Lymphocyte
CX3CL1	Fractalkine Ligand
CX3CR1	Fractalkine Receptor
DAMP	Danger-Associated Molecular Pattern
DCs	Dendritic Cell
DNA	Deoxyribonucleic Acid
DP	Double Positive
ELISA	Enzyme-Linked Immunosorbent Assay
EMCV	Encephalomyocarditis Virus
FACS	Fluorescence Activated Cell Sorting
FDR	False Detection Rate
FPR	Formyl-Peptide Receptor
FSC	Forward Scatter
GFP	Green Fluorescent Protein
G-CSF	Granulocyte-Colony Stimulating Factor
GMCSF	Granulocyte-Macrophage Colony-Stimulating Factor
gMDSC	Granulocytic MDSC
GMP	Granulocyte-Macrophage Precursor
GVHD	Graft Versus Host Disease

H&E	Hematoxylin and Eosin
HA	Haemagglutinin
HCL	Hierachical Clustering
HIV	Human Immunodeficiency Virus
HSP	Heat Shock Protein
HSV	Herpes Simplex Virus
I.N	Intranasal Injection
IBD	Inflammatory Bowel Disease
IDO	Indoleamine 2,3-Dioxygenase
IFN-γ	Interferon-Gamma
IGb3	Isoglobotrihexosylceramide
IgG	Immunoglobulin
IL	Interleukin
IL-1Ra	IL-1 Receptor Antagonist
IMs	Interstitial Macrophage
N	Neutrophil
Neut	Neutrophil
Inf. Mono	Inflammatory Monocyte
iNKT	Invariant Natural Killer T cell
iNOS	Inducible Nitric Oxide Synthase
KSHV	Kaposi sarcoma-Associated Herpes Virus
LAIV	Live Attenuated Influenza Vaccine
LCMV	Lymphocytic Choriomeningitis Virus
LN	Lymph Node
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LTHSC	Long Term Haematopoietic Stem Cell
M	Monocyte
MAPK	Mitogen-Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
MDSCs	Myeloid Derived Suppressor Cells
MEP	Megakaryocyte-Erythrocyte Progenitor
MHC	Major Histocompatibility Complex
mMDSC	Monocytic MDSC
MMP	Matrix Metallopeptidase
MoDC	Monocyte-Derived DC
Mono	Monocyte

NA	Neutraminidase
Neut	Neutrophil
NF-κB	Nuclear Factor-κB
NLR	NOD-like Receptor
NO	Nitric Oxide
NOD	Nucleotide-Binding Oligomerization Domain Receptors
NOS2	Inducible Nitric Oxide Synthase
NP	Nucleoprotein
PAMP	Pathogen-Associated Molecular Pattern
PCA	Principle Component Analysis
pDC	Plasmacytoid Dendritic Cells
PGE2	Prostaglandin E2
PR8	Mouse adapted H1N1 strain, Influenza A/PuertoRico/8/34
PRR	Pattern Recognition Receptor
RBC	Red Blood Cell
RIN	RNA-Integrity Number
RMA	Robust Multi-Array Average Algorithm
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RSV	Respiratory Syncytial Virus
SA	Sialic Acid
SAA	Serum Amyloid A
sCX3CL1	Soluble Fractalkine Ligand
SD	Standard Deviation
SEM	Standard Error of the Mean
SEV	Sendai Virus
S-FLU	Pseudotyped Influenza Virus with Suppressed Hemagglutinin Sequence
SPF	Specific Pathogen Free
SSC	Side Scatter
STHSC	Short Term Haematopoietic Cell
T Cell	T Lymphocyte
TCM	Central Memory T Cell
TCR	T cell Receptor
TECs	Thymic Epithelial Cells
Tem	Effector Memory T cell
TGF	Tumour Growth Factor
Th	T helper Cell

TIPDC	TNF-α/iNOS-Producing Dendritic Cells
TIV	Trivalent Inactivated Influenza Vaccine
TLR	Toll-Like Receptor
TNF α	Tumour Necrosis Factor
T regs	Regulatory T Lymphocyte
Trm	Resident Memory T cell
V(D)J	Variable (V) Diverse (D) and Joining (J)
VGM	Viral Growth Media
VSV	Vesicular Stomatitis Virus
X31	Influenza A/HKx31

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CHAPTER 1.

INTRODUCTION

Chapter 1: Introduction

1.1. An Introduction to Invariant Natural Killer T cells

Natural killer T cells (NKT) cells are a unique population of T lymphocytes. Two subsets of NKT cells have been identified thus far: (i) invariant NKT cells (otherwise known as iNKT or type 1 NKT cells) and (ii) diverse NKT cells (more commonly called type II NKT cells [1-3]. iNKT cells, which are the focus of this study, express an antigen-specific T-cell receptor (TCR) composed of an invariant α-chain (Vα14-*Jα18* in mice and Vα24-*Jα18* in humans) paired with a restricted repertoire of β-chains (Vβ2, Vβ7 and Vβ8.2 in mice, or Vβ11 in humans). Genetic manipulation of the above gene segments has further demonstrated the requirements of these gene segments for NKT cell development, since deletion of the *Jα18* segment in mice results in an iNKT cell-deficient animal, whereas transgenic overexpression of Vα14 leads to an increased frequency of iNKT cells [4]. iNKT cells form around 80% of the total NKT cell population, with the remainder made up of type II NKT cells, or non-invariant, NKT cells [5]. Other than the invariant-TCR which distinguishes iNKTs, the lack of reagents to track type II NKT cells means their functional and phenotypical characterization remains largely uncharacterised. To distinguish the roles of the two NKT populations, many researchers currently compare the phenotypes of *Cd1d*$^{-/-}$ mice (which lack all NKT cells) with *Jα18*$^{-/-}$ mice (which lack only iNKT cells). Notably, recent studies have highlighted that *Jα18*$^{-/-}$ mice exhibit additional defects in the T cell repertoire [6, 7], therefore, the iNKT cell relevance of results obtained using *Jα18*$^{-/-}$ mice should be considered in the context of these findings. The heterogeneity of Vα14^{+} iNKT cells has been further appreciated with the identification of several subsets of iNKT cells with distinct developmental and functional properties [8-10]. Indeed, a distinct Vα50-Jα10 iNKT cell subset was recently identified, which although absent in *Cd1d*$^{-/-}$ mice, was found to be present in *Jα18*$^{-/-}$ mice [11]; it is clear that considering these subsets will be critical in order to accurately interpret forthcoming data.

1.2. iNKT cell recognition of antigen

In contrast to conventional T cells which recognize protein-derived antigens presented by major histocompatibility complex (MHC) class I and class II molecules, the TCRs on iNKT cells recognize either exogenous or endogenous glycolipids presented in the context of the non-polymorphic, MHC class I-like CD1d molecules [12-14]. The importance of CD1d for iNKT activation and development is demonstrated by the finding that *Cd1d*$^{-/-}$ mice are devoid of iNKT cells (as well as type II NKT cells) [15].

Insights into iNKT and type II NKT TCR-binding modes have been greatly advanced in recent years following structural characterisation of CD1d-antigen-TCR complexes (reviewed by [16]). Briefly, iNKT TCRs bind to the surface of the CD1d-antigen in a distinctive orientation; the footprint of the bound TCR essentially aligning with the long axis of the binding groove of CD1d [17]. Identification of the nature and range of lipids that activate iNKT cells and how these can affect iNKT cell activity is continually unfolding [18]. Indeed, increasing studies are successfully identifying and generating molecular structures which could promote more potent iNKT cell activation [19].

The first lipid antigen to be identified, which potently activated iNKT cells, was α-galactosylceramide (α-GalCer), derived from the glycosphingolipid extract of the marine sponge Agelas mauritianus [20]. The α-linked glycan in α-GalCer has since been shown to be a structural motif common to many of the identified self and microbial antigens which activate iNKT cells [21] (**Figure 1**). Importantly, whilst synthetic iNKT cell agonists such as α-GalCer evoke stronger and faster iNKT cell activation than microbial proteins or TLR agonists [22-24], a fine balance of activation needs to be met to ensure iNKT cells do not transcend into an over-active, anergic state [25, 26]. In recent years, several additional synthetic analogues of α-GalCer have been synthesized which are increasingly able to activate iNKT cells both *in vitro* and *in vivo*, and are aimed at optimising activation. Such analogues are able to activate iNKT cells without inducing anergic effects, have greatly

advanced our understanding of iNKT cells, and our ability to modulate these cells effectively *in vivo* [27-29]. Furthermore, there is great interest in the use of α-GalCer derivatives that have the potential to skew NKT cell- mediated responses toward Th1 or Th2 signalling; such derivatives illustrate the possibility of tailored NKT cell-based immunotherapy which would be applicable to many diseases (reviewed by [30]). It is also important to note that iNKT cell arrest can be induced by the injection of the cytokines IL-12 and IL-18, which induce iNKT cell activation, suggesting iNKT cell anergy can result from iNKT cell activation, regardless of whether cognate interaction occurs [31].

The use of α-GalCer has allowed further advancements into the understanding of iNKT cells following the generation of CD1d-tetramers loaded with α-GalCer, which are able to unambiguously label iNKT cells [32]. This tool subsequently revealed that α-GalCer-CD1d tetramer-positive cells are composed of several phenotypically and functionally different subpopulations [8, 33, 34], and highlights a new dimension to understanding this cell population.

1.3. iNKT cells and self-lipids

In addition to direct activation by CD1d-presented microbial or synthetic antigens, iNKT cells can undergo potent activation by antigen-presenting cells (APCs) which have been stimulated by Toll-like receptor (TLR) agonists in the absence of infection, and in the absence of pathogen-associated lipid antigens, for example during auto-immune disorders or stimulation by TLRs [35-38]. Indeed, in the absence of microbial-derived glycolipid, iNKT cell responses are induced, at least in part, as a result of stimulation of TLRs on APCs, which causes the secretion of cytokines, such as IL-12 or type I IFN, which subsequently participate in the iNKT cell activation process [36, 37, 39]. Indeed, such activation requires a signal from a lipid–CD1d complex, suggesting that endogenous, or ‘self’, antigens can also contribute to iNKT cell activation during infection [40]. This function is especially interesting

given the CD1d-dependent activation of iNKT cells by pathogens which do not contain glycolipids, such as during viral infections, cancer and auto-immune diseases, where foreign lipids are not present [41]. In further support of a role for iNKT cells during viral infection, it has been shown that CD1d expression can be upregulated following viral or bacterial infection of antigen presenting cells (APCs), thus enhancing antigen presentation of endogenous lipids to iNKTs [42-44].

Since endogenous lipids have demonstrated a clear role during a number of inflammatory conditions, a vast amount of research has been directed at identifying key self-lipids, both during inflammation, and during iNKT cell development [45]. One such example is the endogenous glycosphingolipid (iGb3), which was shown to be a weak agonist for human and murine iNKT cells [46-48], and is involved in iNKT cell activation in the periphery [49]. Although iGB3 was originally thought to be the only endogenous iNKT cell agonist implicated in iNKT thymic selection, this has since been questioned, however, following the finding that iNKT cell development and function are normal in iGb3 synthase-deficient mice [50], and furthermore, that iGB3 expression was not found in the thymus [51]. Other possible candidates for self-activation of iNKT cells include lysophospholipids, such as lysophosphatidylcholine (LPC), which are less universal, and often stimulate only human or murine iNKT cell subsets [52] [53-55]. Similarly, the endogenous lipid β-D-glucopyranosylceramide (β-GlcCer), which is a potent iNKT cell self-antigen in both mice and humans, is a major determinant of peripheral iNKT cell activation following myeloid cell infection and in response to TLR agonists [40]. Finally, fractionation of lipids from mouse thymocytes led to the identification of peroxisome derived ether-bonded lipids as important iNKT self-antigens [56].

Although type II NKT cells also recognise lipid presented on CD1d molecules, they express a more diverse TCR repertoire, and in contrast to iNKT, type II NKT cells do not react with α-GalCer. The antigenic specificity of type II NKT cells is still largely unknown, with some cells

demonstrating reactivity to self-antigens, such as sulfatide [57, 58] and β-glucosylceramide [59]; it is clear that extensive work is still required to elucidate the roles of self-lipids and iNKT cell (and type II NKT cell) activation in both development and disease.

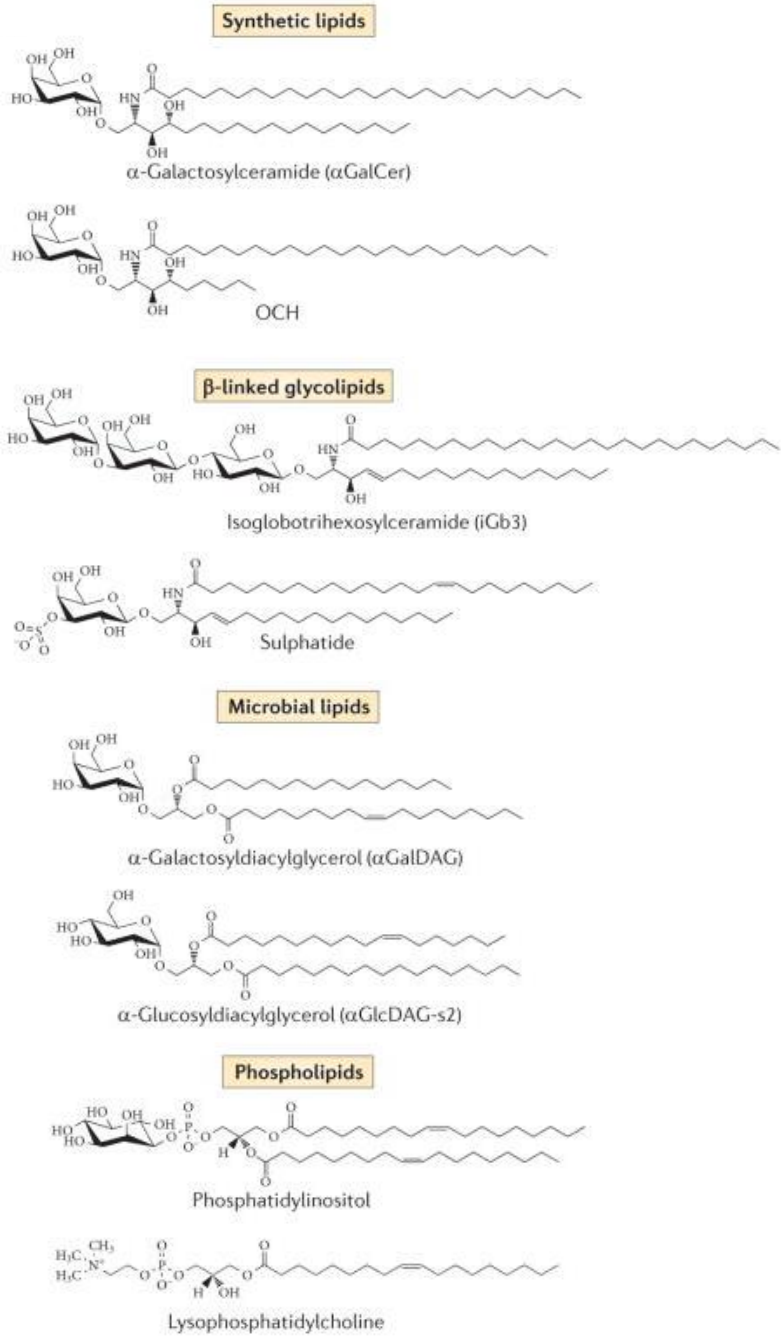


Figure 1**. Examples of iNKT cell agonists.**

Structures of identified iNKT cell agonists: These include examples of synthetic lipids (α-GalCer and OCH), microbial lipids (α-galactosyldiacylglycerol from Borrelia burgdorferi and α-glucosyldiacylcerol from Streptococcus pneumoniae), β-linked glycolipids (isoglobotrihexosylceramide (iGb3) and sulphatide) and phospholipids (phosphatidylinositol

and lysophosphatidylcholine). Abrreviations: β2m, β2-microglobulin. Figure modified from [60].

1.4. iNKT cell development

Early developmental stages of iNKT cells and MHC-restricted $CD4^+$ and $CD8^+$ cells are similar; both develop in the thymus and derive from a common progenitor, however at the $CD4^+$ $CD8^+$ double positive (DP) stage of development, iNKT cells branch away from conventional T cell development (reviewed by [61]). Unlike conventional T cell selection which occurs via thymic epithelial cells (TECs), iNKT cells are positively selected upon recognition of self-lipid presented by $CD1d^+$ DP thymocytes [62]. The resulting cells down-regulate CD8 expression, expand, and once in the periphery, become $NK1.1^+$ (CD161 in humans), and gain further NK cell markers such as NKG2D and Ly49 (reviewed by [63]). iNKT cells reside in the periphery with high surface levels of the activation markers CD44 and CD69, along with low expression of CD62L, thereby resembling effector memory T cells [64, 65]. In humans, iNKT cells may be $CD4^+$, $CD8^+$ or double negative, whereas mice have only $CD4^+$ and DN, and lack the $CD8^+$ subset (reviewed by [61]). It has been suggested that circulation of iNKT cells in this semi-activated state is one of the fundamental features which allows these cells to respond quickly to infection, and to elicit functions contributing to both the innate and the adaptive immune response. In light of the evolutionary conservation of these cells, where mouse NKT cells recognize human CD1d-glycolipid antigen complexes, and vice versa [66], this suggests that these cells play a critical, and probably unique, role in the immune system.

1.5 Distribution and Dynamics of iNKT cells

The use of CD1d-tetramers loaded with α-GalCer has allowed investigators to study the distribution of iNKT cells *in vivo* [32, 67, 68]. In mice, iNKT cells account for approximately 20-30% of the total T lymphocyte population in the liver [69, 70], however they represent only between 0.5-2%, in the spleen, lymph nodes, bone marrow and blood. Using a variety of techniques, it has since been shown that iNKT cells exhibit a wide distribution, and can have distinct, environment-dependent subpopulations. For example, by exploiting intra-vital microscopy of *cxcr6*-gfp reporter mice, it was shown that iNKT cells are situated inside the vasculature of the lungs, and following activation, contribute to intra-parenchymal inflammation and adaptive immune responses [71]. iNKT cells are also highly enriched in mouse adipose tissue, where they have a role in metabolic regulation [72-74], and in the lymph nodes, and where they are enriched for a subpopulation of cells that have a $NK1.1^+$ $CD103^+$ $CCR6^+$ phenotype and produces interleukin 17A (IL-17A) [75]. In humans, iNKT cells are found in relatively comparable locations as with mice, however, unlike mice where iNKT cells are enriched in the liver, in humans, iNKT cells are enriched in the omentum, where they represent ~10% of T cells [76]. Notably, in humans, iNKT cells are observed at lower frequencies (0.1- 0.2% of T cells on average), and much person-to-person variation has been reported [70]. It is important to bear in mind that despite their somewhat low frequencies, iNKT cells have a semi-invariant TCR and are therefore found magnitudes higher in frequency than naïve major histocompatibility complex (MHC) class-I (MHC cI) or MHC class-II (MHC cII)-restricted T cells [77].

1.6. iNKT cell activation and host immune functions

During an infection, iNKT cells are actioned earlier than cells of the adaptive immune system but later than the innate effectors [78]. This occurrence is thought to stem from the specialised development of iNKT cells, where the iNKT cells emerge and circulate with a distinct memory-activated phenotype [79]. The ‘activated’ phenotype of iNKT cells allows

rapid mobilisation in large numbers during inflammatory conditions (reviewed by [16]), and the ability to promptly secrete large amounts of cytokines in response to TCR engagement [80, 81]. As well as direct contact via CD1d, pathways independent of CD1d permit iNKT cells to respond to diverse infectious agents, including viruses. Indeed, iNKT cells can be activated during viral infection via soluble factors released by TLR-activated dendritic cells (DCs), such as type I interferon (IFN) and interleukin (IL)-12 [37, 82, 83], or by co-stimulatory molecules like OX40/OX40L [84]. Such mechanisms augment iNKT cells to elicit a bi-directional relationship with APCs, such as DCs. A further hallmark of iNKT cells is their ability to rapidly produce both IFN-γ and IL-4 in response to stimulation, such as with α-GalCer. This rapid release mechanism has been attributed to the constitutive presence of pre-formed mRNA in resting cells [85, 86]. iNKT cells have since demonstrated a diverse repertoire of cytokine production including IL-2, IL-5, IL-6, IL-10, IL-17, IL-21, TNF-α, TGF-β and GM-CSF [61, 87-90], as well as chemokines such as RANTES, Eotaxin, MIP-1α and MIP-1β [91]. Both murine and human iNKT cells can exhibit potent cytotoxic function following activation. This is mediated through the expression of FasL and TRAIL, as well as the up-regulation of granzyme B and perforin, which have shown to be essential iNKT functions for mediating the killing of tumour cells [92, 93]. Interestingly, distinct subsets of iNKT cells have different functional profiles; human $CD8^+$ and DN iNKT cells are highly cytolytic and produce Th1-type cytokines, whereas $CD4^+$ iNKT cells produce both Th1 and Th2 cytokines [94, 95]; understanding the contribution of each subset will be important if these cells are to be targeted in therapeutic design.

1.7. The dual roles of iNKT cells during the immune response

During pathological processes, the aspects of iNKT cell biology are intricately interconnected, and the relative contribution of specific factors, and the subsequent effect on disease outcome, is often context-dependent. Indeed, iNKT cells and their CD1d-expressing cognate partners orchestrate the immune response through production of cytokines,

chemokines, and surface molecules **(Figure 2)**. Consistent with their ability to produce both type 1 (Th1) and type 2 (Th2) cytokines upon activation, iNKT cells have been associated with both pro- and anti-inflammatory functions *in vivo*. The mechanism/s accountable for this ability remains unclear; however, reports have indicated that the ability of iNKT cells to modulate the immune response is dependent on their cytokine secretion and, as discussed above, by their ability to crosstalk with multiple different cell types.

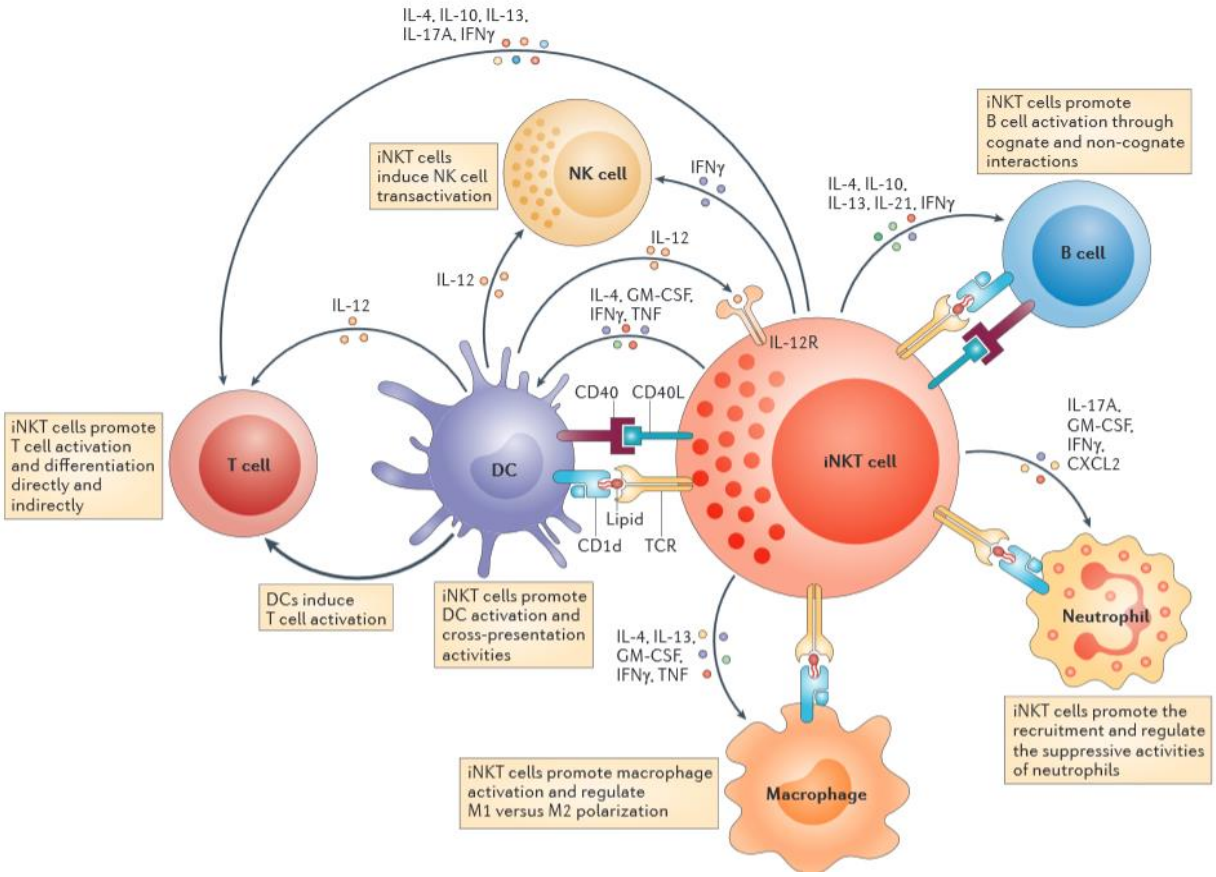


Figure 2. iNKT cells interact with arms of both the innate and adaptive immune system.

iNKT signalling is bidirectional, whereby they both send and receive signals from antigen-presenting cells (APCs) leading to downstream activation of the immune response. Signalling occurs through cell-surface receptors, such as T-cell receptors (TCRs), which recognise glycolipid–CD1d complexes, co-stimulatory receptors, such as CD40, CD27 and

OX40 following the recognition of their ligands CD40L, CD70 and OX40L, respectively, as well as through soluble mediators, such as IL-12, IFN-γ and IL-4. iNKT cells can also interact with influenza-induced MDSCs via CD1d and CD40/CD40L signalling, resulting in the inhibition of their production of arginase and iNOS (not depicted). Figure modified from [96].

1.7.a. Polarisation of iNKT cells

In addition to the different roles played by phenotypically distinct iNKT subsets (reviewed by [96]), polarisation of iNKT cell activation, and subsequent downstream signalling, can play an important role in regulating the host immune response. Mechanisms which can modulate iNKT cell polarisation include the strength of the antigenic signal delivered to the NKT cell, as determined by antigen abundance and/or T-cell receptor (TCR) affinity, the subcellular localisation of lipid-loaded CD1d molecules, and differential targeting of antigens to APCs [97-100]. Indeed, McCarthy et al. demonstrated that the length of lipid bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation [18].

iNKT activation can additionally be modulated by the presence or absence of cytokines that co-stimulate NKT cells, for example interleukin (IL)-12, IL-18 and interferon (IFN)-alpha, and finally by APC-intrinsic factors such as differentiation state, for example monocyte presentation versus DC presentation, and toll-like receptor (TLR) stimulation (reviewed by [101]). As such, activation of iNKT cells with potent lipid antigen leads to the production of both Th1 and Th2 type cytokines, whereas activation with IL-12 or TLR agonists results in IFN-γ, but not Th2 cytokines [35, 38]. Importantly, different lipid antigens can influence both the magnitude and the quality of iNKT cell activation; lipid antigens have been generated with the ability to skew iNKT cells to produce either Th1 or Th2 type cytokines [29, 102-104], and

it is hypothesised that different endogenous or exogenous pathogenic antigens may possess the same ability.

1.7.b. Pro-inflammatory roles for iNKT cells during innate and adaptive immunity

Due to the ability of iNKT cells to recognise both endogenous and exogenous lipid, they have been increasingly implicated in host responses to many bacterial, viral and parasitic infections. The presence of microbial-derived glycolipids during infection is especially significant since these can be loaded onto CD1d, and thus, will directly activate iNKT cells. iNKT cells have shown to play a protective role during inflammation in a number of models; infection with *Borrelia burgdoferi*, the causative agent of Lyme's disease, which was shown to express two CD1d-compatable glycolipids (BbGL-1 and BbGL-2), induces iNKT cell activation, via upregulation of CD25 and CD69, in wild-type animals [24]. Likewise, infection of $CD1d^{-/-}$ mice results in increased bacterial burden and the development of severe arthritis of the knee, supporting the hypothesised protective role for iNKT cells in this model. *Sphingomonas* species possess various cell wall-derived glycolipids which can activate iNKT cells [49], and in line with this, infection of $J\alpha18^{-/-}$ mice with *Sphingomonas* leads to significantly decreased clearance from the liver and lungs. Similar reports have recently been published describing the role of iNKT cell recognition of antigens derived from Streptococcus pneumoniae [105] and Helicobacter pylori [106]. Interestingly, it is not only direct activation via CD1d presentation which can activate iNKT cells during pathogenic infection; LPS-positive bacteria which are not directly recognised by iNKT cells, such as Salmonella and Pseudomonas aeruginosa, can also be controlled by iNKT cells *in vivo*. The responses of iNKT cells was found to be dependent on TLR- signalling and IL-12, which synergised with the recognition of a self-lipid, thus connecting the role APC-derived soluble factors in iNKT cell activation, and the subsequent immunity, to infection by these pathogens [35, 37].

In further support of a protective role for iNKT cells, iNKT cells have increasingly been implicated in the enhancement of the adaptive immune response, a characteristic which can be harnessed in the development of novel vaccine strategies (reviewed by [107]). iNKT cells can signal directly with other cells via co-stimulatory factors; CD40 ligand (CD40L) is upregulated on activated iNKTs and following signalling with CD40 on APCs, has shown to stimulate up-regulation of surface MHC Class II and co-stimulatory molecules. This then drives DCs to produce IL-12, thereby controlling subsequent polarisation, for example towards T helper 1 (Th1) responses [108-110]. Such signalling was also shown to be important in enhancing both $CD4^+$ and $CD8^+$ T cell responses following co-administration of ovalbumin with the iNKT cells adjuvant, α-GalCer [111, 112].

The effect of iNKT cells on the overall immune response, however, extends beyond their cytokine release and their interactions with DCs; for example, iNKT cells can provide cognate help for B cells, in a CD40-dependent manner. Recognition of a lipid antigen presented by CD1d on B cells promotes humoral immunity via inducing B cell maturation, higher antibody titers and the production of antigen-specific, class-switched antibody responses and expansion of the B cell memory pool [107, 113, 114]. This mechanism has been harnessed by targeting α-GalCer presentation on CD1d to B cells, resulting in B cell-iNKT cell crosstalk, as well as B cell proliferation and differentiation into plasma cells, which produce large amounts of immunoglobulin [114]. In addition to influencing T and B cell responses, iNKT cells also contribute to NK cell activation through IFN-γ and IL-2 secretion, either directly by iNKT cells themselves, or indirectly via iNKT-stimulated APCs [115-117]. Such observations strongly suggest that the precise kinetics of iNKT cell signalling are fundamental in optimising DC, and consequently, T cell responses.

1.7.c. Tolerogenic roles for iNKT cells during innate and adaptive immunity

Despite the reported ability of iNKT cells to enhance immunity, experiments using animal models for autoimmunity have suggested that iNKT cells also play an anti-inflammatory role *in vivo*. Indeed, Zeng and colleagues published that iNKT cells can suppress graft-versus-host disease (GVHD) induced by T cells in bone-marrow grafts. This suppression was attributed to iNKT cell-derived IL-4 [118]. Studies in type 1 diabetes have revealed that activation of iNKT cells with α-GalCer or Complete Freund's Adjuvant is able to prevent the onset of disease [119, 120]. This was also supported by work from Lehuen et al. who showed protection from type 1 diabetes in Valpha14-Jalpha281 transgenic non-obese diabetic (NOD) mice, which have an increased number iNKT cells [121].

It has since been documented that iNKT cells have the ability to crosstalk with plasmacytoid DCs (pDCs) via the OX40-OX40L pathway; such interactions elicit type I IFN release in the pancreas and subsequently dampens inflammation [84]. This crucial cooperation between iNKT cells, pDCs, and T reg cells has been shown for prevention of type-1 diabetes by viral infection; using both virus-induced and spontaneous mouse models of type 1 diabetes, Diana et al. demonstrated that upon viral infection, iNKT cells in the pancreatic lymph nodes (LNs) induce TGFβ-producing pDCs. These tolerogenic pDCs converted naïve anti-islet T cells into $Foxp3^+$ $CD4^+$ regulatory T cells (T-regs) in pancreatic LNs, which are then recruited to the pancreatic islets. Here the T-regs produce TGF-β, thus dampening the activity of viral- and islet-specific $CD8^+$ T cells, thereby preventing type-1 diabetes development [122]. Notably, iNKT cells have shown to be beneficial in an increasing number of other autoimmune conditions; these include collagen-induced arthritis [123], colitis [124], and EAE [125], whereby transgenic enrichment of iNKT cells (in TCR Valpha14-Jalpha281 transgenic NOD mice) delayed onset and reduced severity of EAE [126].

An anti-inflammatory, yet protect role has also been observed during pathogenic infection; Kok et al. showed that iNKT cell-deficiency resulted in elevated immunopathology and

mortality due to increased influx of inflammatory myeloid cells into the lungs [127]. Infection with *Legionella pneumophila* yielded similar results, whereby iNKT cells were shown to reduce the inflammatory infiltrate [128] which was associated with reduced pulmonary tissue damage.

Importantly, there are some controversial reports regarding iNKT cell plasticity (reviewed in [129, 130]). Genetic background is thought to be an important contributor, and should be taken into consideration when reporting the pro- and anti-inflammatory ability of iNKT cells; indeed, in a cerebral malaria model system, NKT cells promoted pathogenesis in C57BL/6 mice, but they inhibited pathogenesis in BALB/c mice [131]. Similarly, the effect of NKT cells on immunity to respiratory syncytial virus (RSV) also differs between C57BL/6 and BALB/c mice [132].

Moving forward, it will be important to gain a more comprehensive understanding of how the microenvironment modulates iNKT cell activation, how the strength of signalling of iNKT cells mediates their regulatory effect, and whether these immunogenic or tolerogenic features can be targeted to determine the final outcome of disease.

1.8. Influenza virus:

Influenza viruses are a genetically diverse group of viruses of the family *Orthomyxoviridae*, which contain a negative sense, single stranded, segmented RNA genome [133]. Influenza viruses are classified into three subtypes: influenza A, B, and C viruses, which differ in host range and pathogenicity. The most pathogenic of these subtypes are influenza A viruses (IAV), which are highly contagious, and can cause acute respiratory infection, afflicting humans and animals with significant morbidity and mortality. Influenza A viruses can further be divided into different subtypes of HA and NA- there are 17 HA subtypes (although a possible 18th has more recently been identified in fruit bats [134]), and 9 subtypes of NA of influenza known at present (reviewed by [135, 136]). There have been reports of a tenth NA subtype in fruit bats [137, 138], however lack of NA enzymatic activity of this protein suggests this subtype may function differently to the NA of influenza A virus, and further characterisation is required [139].

Typical of many RNA viruses, influenza virus undergoes high mutation rates and frequent genetic re-assortment leading to variability in HA and NA antigens. Minor changes in the protein structure in influenza A strains, denoted “antigenic drift", occur frequently, enabling the virus to cause repetitive seasonal influenza outbreaks by evading immune recognition. Major changes in the influenza type A HA antigen, denoted "antigenic shift", are caused by re-assortment of proteins from different influenza A subtypes, such as between human and animal subtypes. These antigenic drift viruses can produce strains capable of causing global pandemic outbreaks. Such pandemics, which as a result of their un-predictability, are more difficult to control, often cause hospitalisations, and have been shown to cause increased fatalities in immune-compromised individuals, the very young, and the elderly (reviewed by [140-142]). Novel strategies for both vaccination and treatment following infection need to be investigated; this will not only help combat seasonal epidemics and reduce the likelihood of future pandemics, but further understanding of the immune response to infection will also

benefit in the development of treatments for highly susceptible patients who would otherwise succumb to viral infection.

Whilst studies addressing influenza-mediated immune response in humans are the most informative, they are somewhat limited by the accessibility of patients during the initial stages of the disease, and data is often obtained at later time points, following a severe or lethal outcome. To gain a better insight into the true mechanisms of influenza infection and the host response, mouse models (as well as models of common ferrets and macaques) are widely accepted, and commonly used. Although mouse models are inexpensive, their genetics are well characterised and knockouts are available, it is important to consider that optimised rodent models will not be entirely representative of human infection, and findings should be validated in human infection where possible.

1.9. The innate immune response:

The major challenge remaining in this field is to elucidate the beneficial or unfavourable effects of individual components of the host immune response, and subsequently the mechanisms regulating these responses to IAV infection.

The first line of defence is formed by the innate immune system; this response is rapid but lacks specificity and memory. The primary cellular targets for influenza viruses are the epithelial cells that line the respiratory tract; these cells express surface receptors containing sialic acid (SA), to which influenza virus is able to bind. Upon infecting the host cell, virus then destroys host cell pre-mRNAs, inhibit translational of cellular mRNAs, and kills host cells either by cytolytic or apoptotic mechanisms (reviewed by [143, 144]). It is now accepted that influenza viruses may differ in their preference for a certain type of sialic acid, a characteristic which has been attributed to the linkage type that connects the terminal neuraminic acid residue of a sialic acid to the penultimate galactose. Commonly, α2,6-linked sialic acids are

present on oligosaccharides that are recognized by human influenza viruses, whereas most avian influenza viruses have a preference for receptors that contain the α2,3-linkage [145, 146]. More recently it has become clear that animals may express both sialic acid linkages [146, 147], and additionally that several avian influenza viruses have been shown to recognize α2,6-linked sialic acids with reasonable efficiently [148, 149] .

Epithelial cells are able to recognise influenza A virus infection via a number of pattern recognition pathways. These include the cytoplasmic RNA recognition protein, RIG-1, and the toll-like receptors (TLRs), TLR3, TLR7 and TLR8, and possibly TLR10, which has recently been shown to be implicated in human influenza infection [150], which recognize viral RNA at the cell surface or in endosomal compartments. Finally nucleotide oligomerization domain (NOD)-like receptors (NLR), can detect IAV and subsequently trigger the inflammasome pathway, leading to caspase-1 activation and IL-1β and IL-18 production. This infection and activation of epithelial cells as well as lung resident or recruited leukocytes triggers a complex pathway of immune signalling, including release of the key factors type I interferons (IFN), TNFα, IL-6, IL-8, RANTES and MCP, which recruit and activate further immune cells to clear virus and repair local damage **(Table 1)**. Notably, neutrophils are a key player in the innate immune response, infiltrating the lung by day three post infection, and exhibiting a range of functions for viral control, such as reactive oxygen species (ROS) release and phagocytosis. Additionally, via chemoattractants such as CCL2, monocytes are recruited, peaking around day 5 post infection. Monocytes subsequently upregulate CD11c and MHC class II, before differentiating into either macrophages or monocyte-derived DC [151]. In combination, these innate cells target virally infected cells, and induce recruitment and activation of further innate cells and cells of the adaptive immune response (reviewed by [152].

Chemo/ cytokine	Function	Produced by	References
IFN-γ	Inhibits viral replication Stimulates CTL mediated killing Increases MHC I expression Activates macrophages and neutrophils Neutrophil recruitment Promotes T-cell proliferation	T cells, NK cells	[153-159].
TNF-α	Direct antiviral effects Neutrophil chemoattractant Stimulates macrophage phagocytosis and production of IL-1 Increases vascular permeability	T Cells Monocytes/ macrophages Dendritic cells Neutrophils	[155, 158-167]
IL-1α /1β	Increases expression of adhesion factors on endothelium Increases vascular permeability Stimulates IL-6 production	Monocytes/ macrophage Dendritic cells	[158, 168-170]
IL-6	Pro-inflammatory cytokine Activates T cells	Respiratory epithelium T cells Monocytes/ macrophage Dendritic cells	[153, 154, 158, 168, 171-174]
CXCL8 (IL-8)	Neutrophil and T-cell chemoattractant Activates neutrophils	Respiratory epithelium Monocytes/ macrophages Neutrophils	[154]
CCL4 (MIP-1β)	Monocyte, T cell chemoattractant Activates neutrophils	Monocytes/ macrophages Neutrophils T cells Dendritic cells	[159, 168, 175]
CCL5 (RANTES)	Monocyte, T cell, dendritic cell and neutrophil chemoattractant Activates T cells	T cells Respiratory epithelium	[159, 171, 175, 176]
CXCL2 (MIP-2)	Neutrophil recruitment, lymphocyte recruitment	Respiratory epithelium	[177]
CXCL10 (IP-10)	Monocyte and T-cell chemoattractant	Respiratory epithelium T cells Monocytes/ macrophages	[154, 167, 171, 176,

			178]
MCP-1	Monocyte recruitment	Respiratory epithelium Monocytes/ macrophages	[127]

Table 1. Links between influenza infection and cytokine- or chemokine- mediated lung pathology.

A summary of the reported cytokines and chemokines which have been implicated in the host response to IAV infection.

1.10. IAV-induced lung pathology

The aim of the innate immune system is containment of a pathogen at the site of infection prior to the commencement of an effective adaptive immune response. However, effector mechanisms must control the pathogen while simultaneously limiting undesirable destruction of pulmonary tissue. As such, the atypical innate immune response resulting from infection with highly pathogenic IAV is, in fact, thought to exacerbate pulmonary inflammation and contribute to lethality [154, 179, 180]. Unlike most sub-lethal influenza infections, lethal influenza infections are not restricted to the larger airways, but can penetrate into the alveoli and lung parenchyma, resulting in increased viral spread through infection of host epithelial cells, pneumocytes and alveolar macrophages. In the majority of cases of influenza infection, severe complications are limited to the lung; viremia is uncommon, and is not considered a contributor to IAV-associated mortality [141]. In contrast, the highly pathogenic avian influenza, H5N1, causes pneumonia, which is further complicated by viremia and extra-pulmonary symptoms, and can result in multi-organ failure [181]. Highly pathogenic influenza A virus (IAV) infection is also associated with exaggerated and prolonged release of pro-inflammatory cytokines and chemo-attractants from epithelial cells, type II pneumocytes and alveolar macrophages, called a ‘cytokine storm’, and the subsequent

excessive infiltration and activation of immune cells [154]. The observed pathological changes in the lungs, as a result of infection with influenza virus, include destruction of the tracheal and bronchial epithelium, sub-mucosal edema, vascular thrombosis, recruitment of inflammatory cells into the airways and throughout the lung parenchyma, alveolar damage and hyaline membrane formation [140, 180, 182-184].

1.10.a. Neutrophils, monocytes and cytokines in IAV-induced lung pathology

As previously discussed, IAV-induced lung pathology has been associated with exacerbated release of several specific immune regulators; indeed mice with NOS2 deficiency or mice depleted with anti-TNFα displayed decreased mortality when infected with lethal mouse-adapted influenza virus [185-187]. Although TNFα and nitric oxide (NO) are very useful for bacteria killing, this increased survival in influenza infection was thought to result from NO and TNFα inducing significant lung damage, but contributing little to anti-influenza immune responses [185, 188]. Other cytokines and chemokines released during influenza infection, including IFN-γ, IL-1, IL-6, and MIP-1α, also contribute to virus-induced immune pathology, but provide positive effects on viral clearance at the same time [169, 174, 189-191] **(Table 1)**. The source of pathogenic cytokines during influenza infection has not been conclusively determined; however, development of pulmonary immune pathology during sepsis has been associated with NOS2 production by a bone marrow-derived cell type, and is reduced by macrophage depletion [192]. Furthermore, lung damage during many cases of acute lung injury and ARDS has been attributed to neutrophils and, to a lesser extent, macrophages [193]. Accordingly, following influenza infection, myeloid infiltrates predominate in lungs [194], and macrophages infected *in vitro* are a major source of inflammatory cytokines [175, 195, 196].

The protective or detrimental capacity of infiltrating neutrophils is particularly controversial; cytokines and growth factors found during exacerbated IAV infection are major regulators of neutrophil survival, however, the ensuing neutrophilia frequently results in a disproportionate release of toxic metabolites causing tissue injury and subsequent organ. A protective role for neutrophils has been suggested following antibody-mediated neutrophil depletion prior to sub-lethal IAV infection in mice; neutrophil depletion resulted in increased viral replication, intensified pulmonary pathology, greater weight loss, and premature death, compared to wild-type animals [155, 197, 198]. Conversely, IAV infection models utilising protease-activated receptor 2 $(PAR2)^{-/-}$ mice [199] or $IL\text{-}17RA^{-/-}$ mice [200], demonstrated that reduced neutrophil infiltration during IAV infection correlated with milder lung pathology and reduced mortality. Currently, the most notable chemokine receptor implicated in IAV-induced neutrophil recruitment is CXCR2, whereby *CXCR2*$^{-/-}$ mice demonstrated abrogated neutrophil infiltration, compared to wild-type mice, following a low dose infection (100 plaque forming units (PFU)) with the mouse adapted H1N1 strain, influenza A/PuertoRico/8/34 (PR8) virus [201]. Interestingly, CXCR2 was shown to assist in migration of neutrophils, but not contribute to viral clearance or lung pathology [201].

The accumulation of monocytes, and monocyte derived cells have also been associated with IAV-induced lung pathology; mice lacking CCR2 have not only decreased accumulation of monocytes after influenza infection but also exhibited decreased mortality and lung damage [175, 202] (reviewed by [203]). Conversely, mice lacking CCR5 display increased pulmonary infiltrates and increased lung damage [175]. Notably, neutrophilia is also observed in CCR2 deficient mice, which demonstrates a key interaction between monocytes and neutrophils in the defence toward influenza infection [195]. In line with this, Kok et al. report a correlation between inflammatory monocyte accumulation and lung pathology [127]. Similarly, $CCR2^{+}$ monocyte-derived DCs and exudate macrophages have been shown to enhance influenza-induced pulmonary immune pathology and mortality [151]. Finally, although TNF/iNOS-producing dendritic cells (TipDCs) enhance the frequency of virus-specific T cells, they may

also enhance the survival of virally-infected T cells, and contribute to lung damage via the release of soluble factors. While we will predominantly focus on the roles of neutrophils and monocytes during influenza-induced lung pathology and disease resolution, it is important to note that other immune cells, such as $CD8^+$ T cells infiltrate influenza-infected lungs, and these also secrete the implicated pathogenic cytokines such as TNFα and IFN-γ [204].

Although studies in animal models have been useful in elucidating the role/s of the specific cytokines and chemokines implicated in cellular infiltration and lung pathology during IAV infection **(Table 1)**, the use of varying viral doses, strains and differing neutrophil and monocyte depletion models makes comparisons between models challenging. Importantly, identification and depletion of neutrophils or monocytes in many of the earlier studies is complicated by the use of the anti-Gr-1 for 'specific' neutrophil depletion [155, 197]. Anti-Gr-1 binds to both Ly6G and Ly6C antigens, and can therefore deplete additional leukocyte populations, including $CD8^+$ T cells, monocytes, macrophages, NK cells and specific dendritic cell subsets [205], thus leading to doubts of the accuracy of any conclusions draw from these studies. More recently anti Ly6G (neutrophils) and anti-Ly6C (monocyte) antibodies have been utilised, which has greatly enhanced analysis of these populations *in vivo*. Notably, the kinetics of cell depletion have shown to be vital for an altered outcome; Tumpey et al. demonstrated that the depletion of neutrophils or alveolar macrophages at later stages of infection has little impact, whereas depletion before infection converted a sub-lethal influenza virus infection into a lethal infection [155].

Influenza infection induces a highly complex and interactive host response; although focusing on single aspects, such as cell infiltration of immune cells, is useful to determine the function of individual component or cells, it is important to consider reciprocal signalling and simultaneous functions of host components in order to gain a comprehensive understanding of disease etiology **(**Figure **3)**.

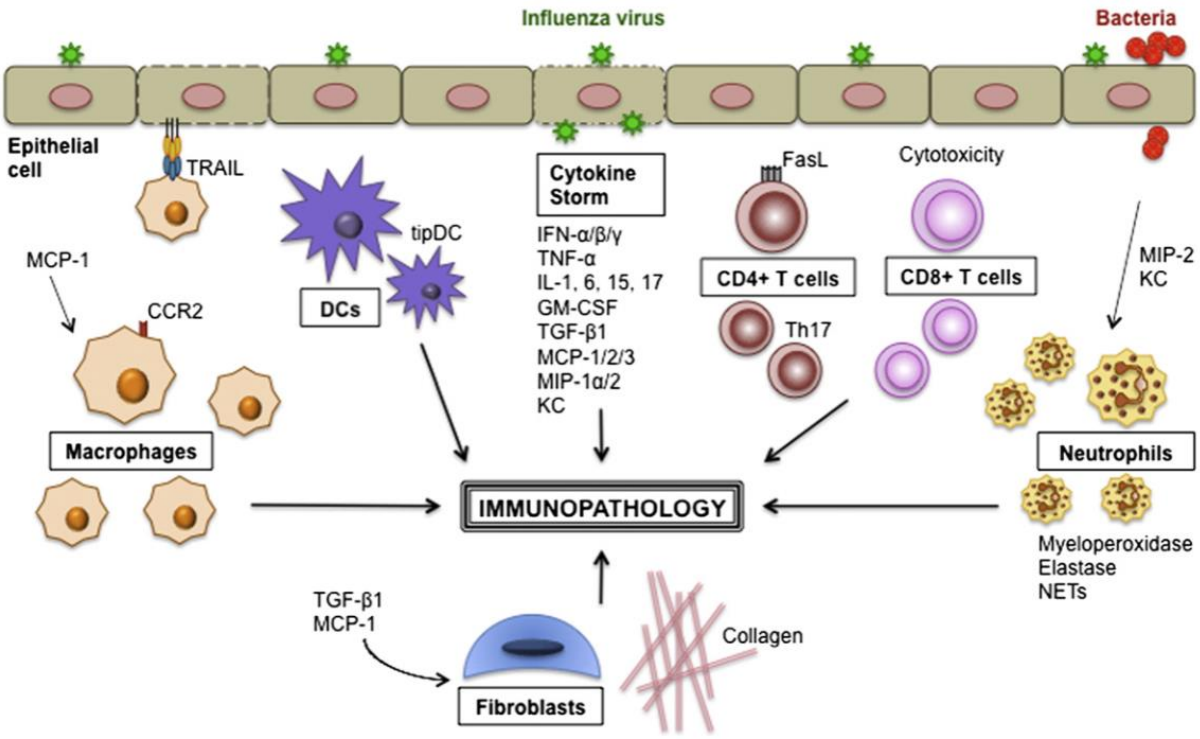


Figure 3: Influenza immunopathology is caused by excessive host responses to infection.

Excessive acute inflammatory responses (macrophages, neutrophils, DCs, and the 'cytokine storm'), CD4 and CD8 T cells, lung remodelling, and bacterial superinfection all contribute to severe lung immunopathology following influenza infection (Figure modified from [142]).

1.11. Adaptive Immunity

The adaptive immune system forms the second line of defence against invading pathogens; although this highly specific response is relatively slow on first encounter with a pathogen, as a result of the formation of immunological memory, the response to secondary infection with the same pathogen is rapid and strong. The adaptive response consists of humoral (virus-specific antibodies) and cellular (virus-specific $CD4^+$ and $CD8^+$ T cells) immunity **(Figure 4)**.

1.11.a. Humoral Immunity

B cells are the main population responsible for mediating the humoral response, and function by producing antibodies, also known as immunoglobins (Ig). The influenza virus surface glycoproteins, predominantly haemagglutinin (HA) and neuraminidase (NA), are the targets of the humoral immune response [206]. Many HA-specific antibodies bind to the trimeric globular head of the HA, which blocks virus attachment to host cells, and/or blocks receptor-mediated endocytosis [207, 208]. The surface proteins of viruses, such as HA, are under constant selection pressure, and as such, rapidly and continuously accumulate mutations to escape recognition by the immune system. Since most antibodies directed against HA are influenza virus strain-specific, they subsequently they fail to neutralize viruses which have undergone genetic drift, or viruses of other subtypes [209-211]. Recently, broadly reacting antibodies directed against the conserved stem region of HA have been identified [212-217]. The generation of high-affinity heterotypic antibodies, or antibodies against a conserved viral epitope could provide immunity to the diverse influenza subtypes and protection against future pandemic viruses, and thus would greatly enhance vaccine design [212, 218].

1.11.b. Cellular Immunity

T lymphocytes are the key cell population in cellular immunity. T cells express highly diverse antigen-specific receptors, which are divided into subsets according to co-receptor expression; firstly $CD4^+$ cells recognize Ag in the context of MHC class II molecules (only expressed on professional APC such as B cells, macrophages and DCs), and modulate the immune response via functions such as releasing cytokines and stimulating the production of antibodies. Secondly, $CD8^+$ lymphocytes are activated by antigenic peptides presented by MHC class I molecules (expressed on all nucleated cells) and form effector cytotoxic T lymphocytes (CTL) which kill virus-infected cells. T and B cells detect the presence of

antigen through T and B cell receptors (TCRs and BCRs), respectively. TCRs and BCRs are generated by genetic rearrangement of Variable (V) Diverse (D) and Joining (J) gene fragments encoding TCR and BCR chains during differentiation, in a process called V(D)J recombination; this ensures the generation of a highly varied repertoire of antigen-specific T and B cells.

$CD8^+$ cells are particularly important during influenza infection; influenza-specific $CD8^+$ T cells recognize multiple viral epitopes on target cells and antigen-presenting cells. A small proportion of $CD8^+$ T cells are able to recognise the basic polymerase subunit 1 ($PB1_{703-711}$, $H2K^b$), non-structural protein 2 ($NS2_{114-121}$, $H2K^d$), matrix protein 1 ($M1_{128-135}$, $H2K^b$), and a protein derived from an alternative open reading frame within the PB1 gene ($PB1\text{-}F2_{62-70}$, $H2D^b$) [219], however, predominantly, $CD8^+$ responses are mediated by the recognition of another two viral determinants: the nucleoprotein ($NP_{366-374}$, $H2D^b$) [220], and the acid polymerase ($PA_{224-233}$, $H2D^b$) [221]. $NP_{366-374}$ $CD8^+$ T cells account for up to 80% of CD8 response, and additionally represent the predominant population found in the memory pool [222], although $CD8^+$ T cells specific for the other viral determinants can also be found, albeit at lower frequency.

The kinetics influenza-specific $CD8^+$-responses are complex, and likely context-dependent; three to four days after infection, priming, activation and expansion of naïve influenza-specific $CD8^+$ T cells occurs in the draining mediastinal lymph node [223], where APCs, such as DCs and macrophages traffic from inflamed lung tissue, present viral antigens to precursor T lymphocytes and cause their clonal expansion. Importantly, the antiviral capacity of influenza-specific T cells during the acute infection is strongly dependent on their ability to migrate and localise to the infected airways, where they appear approximately five to seven days post-infection [224, 225]. It is here, in the lungs, where the CTLs exert their effector functions, producing antiviral cytokines, and promoting granzyme and perforin-mediated lysis of any cells which present the viral determinant for which the $CD8^+$ cells bear a specific T cell

receptor [226]. Upon clearance of the virus, T cell memory is gradually generated, and subsequently maintained in the absence of antigen. Adaptive immune cells are responsible for establishing immunological memory, which is essential for producing a rapid and robust anamnestic response upon re-encountering pathogens, and can additionally limit reactivation of existing latent infections [227]. Indeed, following secondary infection, it is generally thought influenza-specific CTL arise approximately 2 days earlier in the secondary response, as compared with the primary response, with an enhanced level of activity [228].

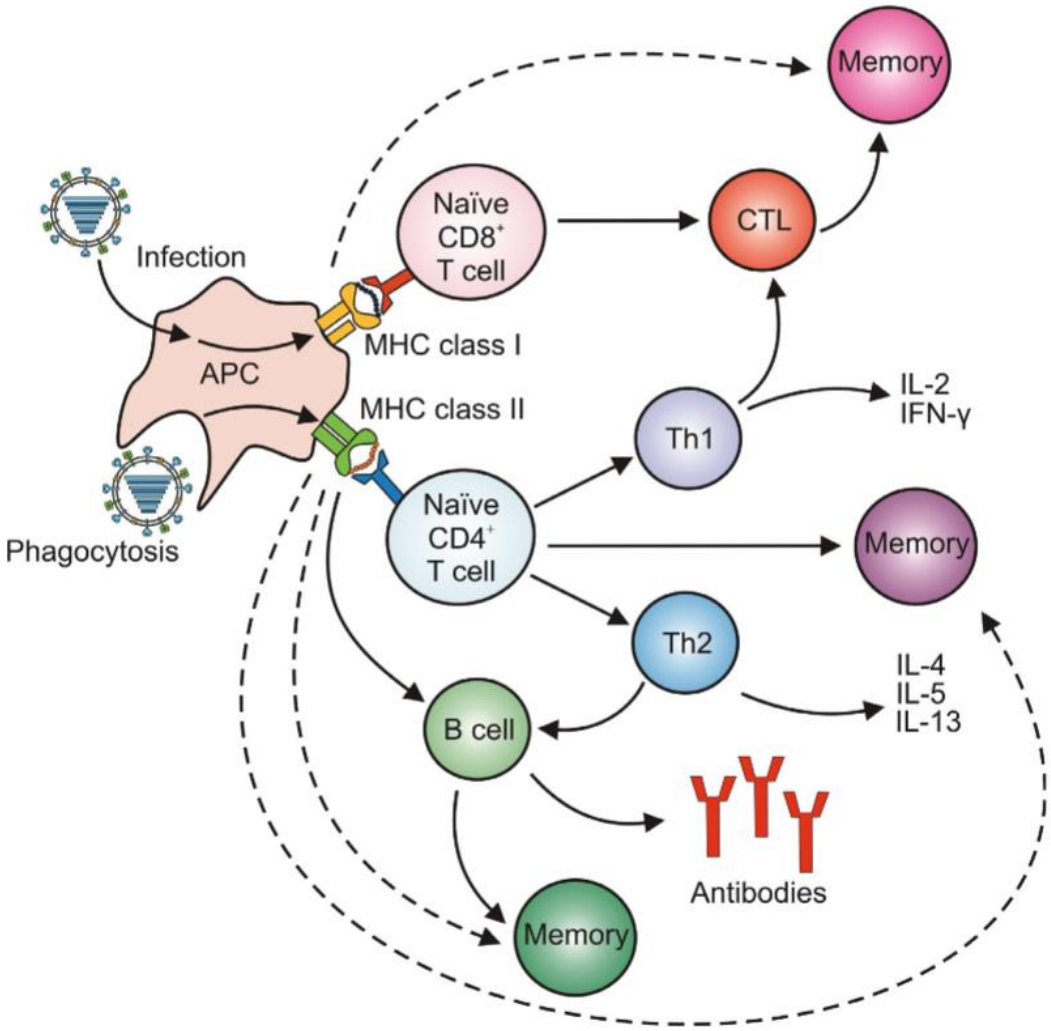


Figure 4: Mechanisms of humoral and cellular immunity.

Immune responses following a primary infection are shown with solid arrows, and the activation of virus specific, memory responses following secondary infection are indicated with dashed arrows. Figure modified from [229].

1.12. Vaccination against seasonal and pandemic influenza

Vaccination, as discovered by Edward Jenner in the 18th century and Louis Pasteur in the early 19th century, describes the administration of antigenic material to stimulate an

individual's immune system, thus promoting the development of adaptive immunity to a specific pathogen. Vaccination has successfully eradicated or reduced a number of diseases, such as smallpox; however, continued generation and development of vaccines is required to prevent the return of such diseases, and to prevent or ameliorate morbidity of currently circulating infections. In line with this, over the past century, human influenza A pandemics such as the 1918 H1N1 Spanish influenza, 1957 H2N2 Asian influenza, and 1968 H3N2 Hong Kong influenza, as well as the more recent 2009 H1N1 swine pandemics have killed ~50–100 million people worldwide, and continually pose a threat to humans and livestock alike. Each pandemic virus is associated with direct interspecies transmission or exchange of genetic material between avian and human viruses, namely, where a novel HA envelope glycoprotein was acquired that was antigenically distinct from the HA of the human viruses in circulation at that time [230-233].

Antigenic drift of circulating seasonal influenza virus leads to a requirement for frequent changes in vaccine composition, since current vaccination strategies predominantly generate strain-specific antibody response against the surface viral HA and NA proteins, which have limited cross-neutralization of drifted strains. At present, two types of vaccines for seasonal influenza are licensed for use: trivalent inactivated influenza vaccine (TIV), given by intramuscular injection; and live attenuated influenza vaccine (LAIV), administered intranasally. TIV and LAIV contain three strains of influenza viruses that are usually changed annually following the results of global influenza surveillance data. It is estimated that the process of receiving the reference strains for use in seasonal vaccination, through virus propagation, purification, finally inactivation can take up to six months. The success of influenza vaccination, therefore, depends largely on the antigenic match between the influenza vaccine strain, and the viral strains currently circulating [234]. Importantly, in the event of a pandemic, where a novel, or mutated viral strain begins circulation, or with inaccurate predictions of circulating seasonal strains, it is likely that current vaccines will be inadequate, as specific humoral immunity against the ‘new’ viral surface proteins will be

lacking [235]. Recent efforts at applying systems-level approaches to vaccine development have shown promise, particularly in defining molecular signatures induced early after vaccination that correlate with and predict the later adaptive immune responses in humans (reviewed by [236]). This field of research requires much further study; the successful generation of an influenza vaccine which elicit heterotypic immunity could remove the requirements and associated costs for yearly re-evaluation and generation of seasonal vaccines, and reduce the risks of further influenza pandemics occurring.

1.13 An introduction to heterotypic Immunity

Heterotypic immunity is defined as immunity generated by a given IAV subtype, or its' antigens, which subsequently protects against challenge with other IAV subtypes. The prospects of therapeutics which can enhance heterotypic immunity is highly appealing, and following the seminal discovery by Schulman and Kilbourne in 1965, where it was demonstrated that infecting mice with one IAV strain reduced pathology and decreased viral pulmonary titers upon challenge with a heterotypic IAV [237], numerous studies have attempted to further understand heterotypic immunity in additional animal models. Heterotypic immunity has now been shown in mice [238, 239], ferrets [240-242], guinea pigs [243], pigs [244] and non-human primates [245] using various combinations of influenza A virus subtypes for priming and challenge [246]. Importantly it was shown that infection with seasonal influenza virus afforded protection against the pandemic influenza A/H1N1 (2009) virus. Additionally since it is thought that the induction of cross-reactive virus-specific $CD8^+$ and $CD4^+$ T cells is key for an efficient heterotypic response, these populations represent a realistic target population for improving vaccine design [242, 247, 248]. Although such heterotypic priming has not yet shown to induce sterilizing immunity, it can greatly improve the clinical signs of infections upon reinfection with a seasonal or pandemic virus of another subtype, and limit dissemination of infection in the lower respiratory tract. Heterotypic

immunity could additionally limit secondary bacterial infection occurring, as is often observed with severe or pandemic infection. Importantly, this phenomena is specific for influenza A virus, since infection of animals with influenza B virus, or other respiratory viruses, does not induce heterotypic immunity against influenza A virus infection, indicating that heterotypic immunity is dependent on virus-specific adaptive immune responses [241, 249]. One final note, it is suggested the method and site of infection can also affect the outcome upon heterotypic infection [250, 251], and thus may determine whether future vaccines are designed for enteral or parenteral administration.

In contrast to the findings of the above animal studies, there is only limited and circumstantial evidence that heterotypic immunity also exists in humans [252-255]. Analysis of epidemiological data collected before and during the 1957 pandemic suggests heterotypic immunity was observed only in adults, but not found in children [246, 256]. Furthermore, unlike findings in animals, where heterotypic immunity has been shown to last for up to 18 months [240], in humans, heterotypic immunity is thought to be shorter-lived than strain-specific immunity [257]. Definitive data characterising the role that heterotypic immunity plays in resistance to influenza virus infection is lacking, however, an understanding of the immune mechanisms responsible for this broad, long-lasting cross-protection [239], would be valuable in vaccine development.

1.13.a. Host factors responsible for heterotypic immunity

Various arms of the immune system contribute to heterotypic immunity [246]. Virus neutralising antibodies can play a minor role, and accordingly, it has been demonstrated that cross-reacting and cross-protecting antibodies against the stem region of the HA, and not the head region, afford heterotypic immunity, particularly against infection with an influenza A virus with a HA of the same influenza group [218, 258, 259]. In the same way, antibodies

against the ectodomain of internal and conserved M2 protein can assist immunity [260], however antibodies against the internal proteins nucleoprotein (NP) and matrix protein (M) 1 do not appear to contribute to protection (reviewed by [250].

As discussed above, a vital correlate of protection in heterotypic infection is the presence of virus-specific $CD4^+$ and $CD8^+$ T cells; T cells have specificity to viral peptide epitopes derived from internal proteins, such as nucleoprotein (NP) and M1, which are less subjected to antibody-mediated selection pressure, and are thus relatively conserved between different influenza A viruses [255, 261, 262]. $CD8^+$ T cells, in particular, have a well-established role in mediating protective immunity against influenza, with a significantly delayed viral clearance in $CD8^+$ T cell-deficient mice [263]. Furthermore, $CD8^+$ T cells have been shown to promote recovery in mice deficient of antibodies or B cells [264, 265], and finally cloned influenza-specific CTLs can passively transfer protection [266]. Although in humans the role of cytotoxic T lymphocytes (CTL) in heterotypic immunity is still under investigation [267, 268], in mice, virus-specific $CD8^+$ CTLs play a critical role in terminating IAV infections [263, 269]. In line with this, adoptive transfer of T cells induced by H3N2 influenza afforded recipient mice protection against a pandemic strain of H1N1 influenza [248]. $CD4^+$ cells specific for NP or M have shown to be fundamental in subsequent antibody responses to HA [270], and depletion of either $CD4^+$ or $CD8^+$ leads to partial abrogation of the heterotypic response upon viral re-challenge in mice [239, 271]. Importantly, in contrast to anti-HA antibodies, virus-specific $CD4^+$ and $CD8^+$ T cells are able to afford protection against all subtypes of influenza A virus, regardless of the HA group. As such, infection with IAV strains comprising different HA or NA proteins, still allows priming, and generation of a broad range of subtype-specific CTLs, which is not possible with current antibody-mediated vaccination strategies.

1.14. iNKT cells, vaccination and heterotypic immunity

1.14.a. A role for iNKT cells in viral immunity

A role for iNKT cells in viral immunity was suggested following the observations that many viruses have developed iNKT cell specific immune-evasion strategies, whereby viral infection impaires CD1d-mediated antigen presentation, and depletes iNKT cells numbers in the periphery (reviewed by [272]). Such examples of this include type 1 HIV (HIV-1), which has been show to downregulate CD1d expression by increasing CD1d internalisation from the cell surface, and retaining it in the trans-Golgi network. This downregulation was shown to be dependent on a tyrosine-based motif present in the CD1d intra-cytoplasmic tail, which interacts with the Nef protein of HIV [273, 274]. Similarly, the lytic replication cycle of the Kaposi sarcoma-associated herpes virus (KSHV) promotes downregulation of cell-surface CD1d in BCBL-1 lymphoma cells [275]. This is caused by expression of the 2 modulator of immune recognition (MIR) proteins of the virus, and strongly decreased subsequent iNKT cell activation by these cells. Detrimental effects on CD1d expression or function have also been shown with vesicular stomatitis virus (VSV) and vaccinia virus [276, 277], and finally HSV type 1 (HSV-1) infection [278]. Interestingly, human iNKT cells have been found to be strongly activated during severe dengue infection, however the role for iNKT cells in in the host immune response, or viral clearance is yet to be determined [279].

In further support of a role for iNKT during viral infection, and although viruses do not contain lipid antigens as antigens for iNKT cells, studies performed with *Cd1d*$^{-/-}$ mice or *Jα18*$^{-/-}$ mice have shown that iNKT cells are critical for the clearance of various pathogens. iNKT cell-deficiency during encephalomyocarditis virus (EMCV) infection [280], and during herpes simplex virus (HSV) infection [281] resulted in an increased viral load. Raftery et al. expanded on these findings showing that in HSV-1- infected iNKT cell-deficient mice, the amount of specific IgM and IgG Abs were significantly reduced as compared with wild-type mice. Moreover, iNKT cell-deficient mice were unable to upregulate CD1d on B cells and

failed to establish an IFN-γ-driven subtype profile of HSV-1–specific IgG Abs [282]. This could be consistent with the finding that iNKT cells produce large amounts of IFN-γ following infection with genital HSV type 2 [283]. Interestingly, infection with lymphocytic choriomeningitis virus (LCMV) infection showed that iNKT cells exerted different antiviral functions in different infected tissues; iNKT cells inhibited early viral replication in the pancreas and liver, however exhibited no influence on early splenic viral load. In the pancreas, iNKT cells promoted type I interferon (IFN) production by pDCs through OX40-OX40 ligand (OX40L) interactions. Furthermore, this iNKT cell-pDC cooperation attenuated the antiviral adaptive immune response in the pancreas, which prevented tissue damage [84].

Of particular interest to this study, iNKT cells have been increasingly implicated as regulators during influenza infection; work published by our group has previously demonstrated a significant regulatory effect of iNKT cells on myeloid derived suppressor cells (MDSCs) in the lung, following influenza infection [41, 127]. The lack of iNKT regulation of MDSCs promoted a suppressive phenotype of these cells, which could diminish the percentage of influenza-specific $CD8^+$ T cells Similarly, Kok et al. demonstrated a direct lysis function of iNKT cells on inflammatory monocytes [127]; this regulation of both infiltrating myeloid numbers and of cell polarization during the innate immune response was shown to be essential in preventing lung pathology and reducing mortality. In line with this, Paget el al. have shown that IL-22 release by DC-activated iNKT cells is essential in repair of the epithelial following IAV-induced damage [284]. Finally, it has been proposed that IFN-γ production by iNKT cells during influenza promotes cytolytic activity of NK cells, and enhances CTL responses, thus reducing viral load and enhancing survival [285]. It is clear viruses can adopt a multitude of evasive strategies to prevent iNKT cell- mediated viral clearance, however, there is still much unknown of the specific mechanisms responsible for host-virus responses during different viral models, and infection with viruses of different pathogenicity.

1.14.b. α-GalCer-activated iNKT cells can enhance heterotypic immunity to influenza infection

The efficient generation of appropriate antigen-specific T and B cell responses is fundamental in the success of vaccination strategies. In line with this, the unique position of invariant natural killer T (iNKT) cells at the interface of the innate and adaptive immune systems, and their ability to direct the maturation of DCs and B cells suggests that iNKTs could be harnessed to boost the antigen-specific immune response against both microbial pathogen and tumour antigens alike (reviewed by [286]). Increasingly, adjuvants—mediators which enhance the natural immune response—have been utilised to promote influenza-specific antibody, and CTL-based immunity. Current adjuvants which are approved for human use, such as Aluminum salts, the most world-wide and historically used adjuvant, MF59 ® from Novartis and AS03 from GlaxoSmithKline, however, are inefficient inducers of $CD8^+$ T cell responses (reviewed by [287]). In line with this, the seminal discovery by Gonzalez-Aseguinolaza et al. in 2002 demonstrated that co-administration of α-GalCer with suboptimal doses of irradiated sporozoites or recombinant viruses expressing a malaria antigen greatly enhances the level of protective anti-malaria immunity in mice [288]. Numerous groups, including our own, have since shown that iNKT cell activation is an efficient adjuvant, enhancing protective efficacy, against a range of immune challenges [112, 289-291].

In the mouse system, exogenous activation of lung iNKT cells by administering α-GalCer alongside influenza infection ameliorated lethal H1N1 and H3N2 influenza via enhanced innate immunity, and reduced lung pathogenicity [127, 292]. Agreeably, when combined with inactivated virus or IAV antigens, α-GalCer also affords potent adjuvant properties, amplifying both antibody, and $CD8^+$ T cell responses, and which can neutralise IAV in both homotypic and heterotypic infection [290, 293-295]. In addition, Youn et al. demonstrated that intranasal immunization of α-GalCer together with inactivated PR8 is effective in

promoting long-term clearance of influenza virus [296]. Lastly, an insightful paper by Guillonneau et al., showed that co-administration of α-GalCer with inactivated influenza A, increased the survival of long-lived $CD8^+$ memory T cells by influencing the upregulation of the pro-survival gene *Bcl2* [297].

It is not just the cellular response to influenza or antigenic peptides which appears to be modulated by iNKT cells, a role for iNKT cells in modulating the humoral response has also been suggested; mice immunized with OVA protein and α-GalCer develop antibody titers 1–2 logs higher than those induced by proteins alone [111, 298], and the decay of circulating antibodies following administration of replication-deficient adenovirus is faster in mice lacking iNKT cells [290]. α-GalCer co-administration with PR8-HA induced high levels of systemic IgG and mucosal secretory IgA antibodies in both BalbC and C57BL/6 mice [295]. Finally, Kamijuki et al. further demonstrated that coadministration of α-GalCer with a H1N1 HA vaccine effectively enhanced both humoral immunity, namely mucosal IgA production, and antigen-specific cellular immunity in an NKT cell-dependent manner [299]. Notably, they show that enhanced antigen-specific nasal IgA and serum IgG production were only observed when α-GalCer was administered intranasally with the vaccine.

Importantly, all the publications addressing the role of iNKT cells in the heterotypic response have focused on using potent doses of α-GalCer to activate iNKT cells, and thus enhance the immune response. There are some limitations with such adjuvant models; multiple injections of α-GalCer, or systemic administration, have been shown to polarize immune responses and to induce iNKT anergy, and thus this method may not be optimal for all vaccination strategies [22, 25]. Indeed, Guillonneau et al. demonstrated that subcutaneous administration of α-GalCer along with an inactivated influenza virus, although promoting the memory response, actually diminished the acute CTL response, via a NKT cell-dependent expression of the suppression mediator, indoleamine 2,3-dioxygenase (IDO) [297]. Only one paper has addressed the role of iNKT cells during influenza infection without adjuvant;

Benton et al, used CD1d$^{-/-}$ mice primed and challenged with influenza H1N1 or H3N2, and subsequently suggested that iNKT cells are not required for heterotypic immunity. This study only assessed disease associated weight-loss was discussed, and no analysis of lung pathology or viral load was investigated [300]. Additionally, it is not possible to conclude and difference was down to iNKT cells alone, since these mice also lack type II NKT cells. Furthermore, whether endogenous lipids are implicated in iNKT cell-mediated heterotypic immunity to influenza is yet to be addressed.

These reported iNKT-mediated enhancements of B cell responses, and influenza-specific CD8^{+} cells following activation with α-GalCer are highly thought-provoking; importantly, however, no publications to our knowledge have been able to pin-point the precise mechanism, direct or indirect, utilised by iNKT cells to control this response. We aim to further characterise the interactions of iNKT cells with the innate and adaptive immune response, and suggest that harnessing iNKT cells should certainly be considered to modulate the innate and adaptive immune response to optimise heterotypic vaccine design and for therapeutic intervention against acute influenza infection.

Thesis Objectives

Over the last few years, it has become clear that activation of the innate and adaptive immune systems is crucial for successful viral clearance and disease resolution following influenza A virus (IAV) infection; ability of the adaptive antigen specific immune response to clear influenza virus infection is modulated by the recruitment, activation and interactions with innate cells, such as inflammatory monocytes and neutrophils, in the lungs. Minor alterations to the induction or prevalence of host signalling can substantially impact upon whether innate and adaptive immune cells adopt a pro- or anti-inflammatory phenotype, and thus can determine the outcome of the disease. Indeed, numerous cytokines and chemokines, and IAV-induced cell populations have been implicated in lethal IAV-induced lung pathology. Although we and others have previously shown the central role of iNKT cells in modulating IAV infections, it remains unclear whether the lack of iNKT cells could modulate the gene profiling of monocytes and neutrophils during IAV infection and the frequency and activity of IAV specific T cells.

To address these questions, we performed a series of experiments, whose results are presented in this thesis, and divided into three complementary aims:

(i) To characterise the role of direct iNKT cell interactions on myeloid cell expansion and activation during influenza infection. We address whether CD1d-dependent interactions with iNKT cells can modulate the exacerbated cell infiltration, activation and polarisation of myeloid cells observed during lethal influenza infection.

(ii) To assess whether the chemokine, fractalkine (CX3CL1) and its receptor (CX3CR1), which are widely expressed on a number of APCs and lymphocytes, and have previously been implicated in leukocyte cell recruitment and survival, could contribute towards IAV-induced lung pathology in wild-type or iNKT cell deficient mice (*Jα18*$^{-/-}$ mice).

(iii) Finally, to determine the importance of iNKT cells in regulating the adaptive immune response to influenza infection, and to elucidate impact of iNKT cells in vaccination-induced heterotypic immunity.

The results of these experiments have provided important insights into the mechanisms by which iNKT cells modulate the signalling components and cellular interactions present during the IAV-induced immune response, and thus, how iNKT cells can regulate the balance between host-protection and tissue pathology. These results are of importance for the design of therapeutics not only against influenza infection, but also for other sterile or non-sterile diseases.

Chapter 2.

Materials and Methods

Chapter 2: Materials and Methods

2. Materials:

2.1. Mice

All mice were maintained in the Biological Services Unit, John Radcliffe Hospital, University of Oxford. Approval of care and use was obtained from the Clinical Medicine Ethical Review Committee, University of Oxford, and procedures were carried out following the Home Office Animals (Scientific Procedures) Act 1986 (PIL: 30/9614: PPL:40/3636). Mice were used between 6-8 weeks of age, and housed under specific pathogen free (SPF) conditions in accordance with guidelines of the Biological Services Unit, John Radcliffe Hospital, University of Oxford. Euthanasia was performed by exposure to carbon dioxide and confirmed using cervical dislocation, in accordance with the Home Office Animals (Scientific Procedures) Act 1986.

Strain	Description
C57BL/6 (B6)	These animals are of the H-2b haplotype
B6.NKT$^{-/-}$ (*Jα18*$^{-/-}$)	These mice lack the *Jα18* TCR gene segment, and are devoid of Vα14 iNKT cells, while having other lymphoid cell lineages intact.
B6.SJL-PtprcaPep3b/ BoyJ (B6-SJL)	These congenic mice express the lymphocyte surface antigen CD45.1 (also referred to as Ly5.1). This marker allows the discrimination between lymphocytes from these mice and C57BL/6 leukocytes, which express CD45.2.
Cd1d$^{-/-}$	These mice lack CD1d expression, and are devoid of iNKT cells, as well as type II NKT cells, while having other lymphoid cell lineages intact.
Cx3cr1-$^{gfp/gfp}$	The cx3cr1 gene in these mice is replaced by a green fluorescent protein (GFP) reporter gene. These mice are on the C57BL/6 background[301].
Cx3cl1-mcherry	CX3CL1-mCherry mice were generated using bacterial artificial chromosomes (BACs) engineering. The artificial CX3CL1 gene encoding mCherry is randomly integrated into chromosomes but not specifically into endogenous CX3CL1 loci. These mice are on the C57BL/6 background [302].

2.2. Tissue culture and cellular assay reagents

Reagent	Catalogue number	Supplier
1x Phosphate Buffered Saline (PBS)	BE17-516F	Lonza
RPMI- 1640	8758	Sigma
Fetal Calf Serum (FCS)(Lot A15211-0229)	A15-152	PAA
L-Glutamine (200mM in 0.85% Azide)	G7513	Sigma
PEN-STREP (5000U Penicillin/ml, 5000U Streptomycin/ml)	P11-010	PAA
Non-essential Amino-acids (NEAA) (x100)	11140-035	Gibco
Sodium Pyruvate (100mM)	11360-39	Gibco
HEPES (1M)	15630-056	Gibco
Trypan Blue	15250-061	Gibco
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA)	E5134	Sigma
β-mercaptoethanol (50mM)	31350-010	Gibco
RBC lysis buffer	158904	Qiagen
Collagenase type-2 filtered	4202	Worthington
Nuclease free water	AM993	Ambion
BSA (30% v/v)	A0336	Sigma
DNase	D4263-5VL	Sigma

2.3. Composition of tissue culture medium:

Buffer/medium	Composition
R10	RPMI-1640 with L-Glutamine and $NaHCO_3$ (Sigma) supplemented with 10% (v/v) heat-inactivated FCS, Penicillin/Streptomycin (1x), L-Glutamine (1x), Non-essential Amino-acids (1x), 1mM Sodium Pyruvate (1x), HEPES (1x) and 0.05µM β-mercaptoethanol.
R0	Same as R10, minus FCS.
VGM	DMEM with pen/strep (1x), glutamine (1x), 0.1% (v/v) BSA (Sigma #A0336) and HEPES (1x).
D10	DMEM with 10% FCS, pen/strep (1x) and glutamine (1x).
1x Collagenase	For a 3X stock: Collagenase (Worthington #4202) is dissolved in 16.5ml RO, plus 1 vial DNase (Sigma # D4263). Dilute to 1X stock in R0.

2.4. Flow cytometry, FACS sorting and MACS sorting reagents

Buffer	Description
FACS buffer	1x PBS containing 10% (v/v) FCS.
MACS buffer	1x PBS containing 2 mM EDTA and 10% (v/v) FCS.
MACS LS columns	Miltenyi, for positive selection
Anti-Ly6G MACS beads	Miltenyi, #130-092-332

2.5. Flow cytometry and cell sorting antibodies

Antibody	Isotype	Conjugate	Cat. #	Supplier
Mouse Fc block	Rat	Purified	553142	BD Pharmingen
Anti-mouse CD11b	Rat IgG2b, k	APC	17-0112-81	eBioscience
Anti-mouse Ly6G(1A8)	Rat IgG2a, k	FITC	551460	BD Biosciences
Anti-mouse Ly6G (1A8)	Rat IgG2a, k	PE	551461	BD Biosciences
Anti-mouse Ly6G (1A8)	Rat IgG2a, k	APC	560599	BD Biosciences
Anti-mouse Ly6C (HK1.4)	Rat IgG2c, k	PEcy7	25-5932-80	eBioscience
Anti-mouse Ly6C (HK1.4)	Rat IgG2c, k	PB	48-5932-80	eBioscience
Anti-mouse Ly6C (HK1.4)	Rat IgG2c, k	PE	12-5932-80	eBioscience
Anti-mouse CD8 (53-6.7)	Rat IgG2a, k	PB	48-0081-80	eBioscience
Anti-mouse CD3 (17A2)	Rat IgG2a, k	APCcy7	47-0032-80	eBioscience
Anti-mouse CD3 (17A2)	Rat IgG2a, k	FITC	11-0032-82	eBioscience
Anti-mouse B220 (RA3-6B2)	Rat IgG2a, k	PE	12-0452-81	eBioscience
Anti-mouse B220 (RA3-6B2)	Rat IgG2a, k	FITC	11-0452-81	eBioscience
Anti-mouse NK1.1 (PK136)	Mouse IgG2a, k	PEcy7	25-5941-81	eBioscience
Anti-mouse NK1.1 (PK136)	Mouse IgG2a, k	FITC	11-5941-81	eBioscience
Anti-mouse CD11b (M1/70)	Rat IgG2a, k	PE	12-0112-81	eBioscience
Anti-mouse CD11c (M1/70)	Rat IgG2a, k	FITC	11-0112-81	eBioscience
Anti-mouse F4/80 (BM8)	Rat IgG2a, k	PB	48-4801-80	eBioscience
Anti-mouse CCR2	Rat IgG2b	PE	FAB5538P	R&D systems
α-GalCer/CD1d tetramer	-	APC	-	-
NP-tetramer	-	APC	-	-
Anti-mouse CX3CL1	Rabbit IgG	Purified	TP233	Torrey Pines
Anti-rabbit	Goat	FITC	4010-02	Southern Biotech
Anti-rabbit	Goat	PE	A10543	Invitrogen
Anti-rabbit	Chicken	APC	A21443	Invitrogen
Rabbit IgG	Rabbit IgG	Purified	AB-105-C	R&D Systems

2.6. Cellular assay and *in vivo* reagents

Cytokine	Concentrations	Supplier
Pam3Cys	10µg/ml	Invivogen
Recombinant Human Apo-SAA	2µM	Peprotech
Influenza virus: A/PuertoRico/8/34 (PR8) (#0702181v) (MtSinai)	10^5- 10^8 PFU	Health protection agency
Influenza virus: A/PuertoRico/8/34 (PR8) (Cambridge)	32 HAU	Prof Alain Townsend
Influenza virus: A/Aichi/2/68 (X31)	32 HAU	Prof Alain Townsend
S-FLU	32 HAU	Prof Alain Townsend

Please Note: Chapter 3 and Chapter 4 use PR8 (MtSinai). Chapter 5 uses PR8 (Cambridge).

2.7. qPCR reagents/kits

Material	Supplier
RNeasy Mini Kit	Qiagen
Superscript III Reverse Transcriptase Kit	Invitrogen
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode	Applied Biosystems
MicroAmp Optical Adhesive Film	Applied Biosystems
Taqman Fast Advance Master Mix	Applied Biosystems

2.8. qPCR - Taqman primers

Primer	Catalogue no.	Supplier
Murine CX3CR1	Mm02620111_s1	Invitrogen Life Technologies
Murine CX3CL1	Mm00436454_m1	Invitrogen Life Technologies
Murine HPRT	Mm01545399_m1	Invitrogen Life Technologies
Murine IL-10	Mm00439614_m1	Invitrogen Life Technologies
Murine Adam10	Mm00545742_m1	Invitrogen Life Technologies
Murine Adam17	Mm00456428_m1	Invitrogen Life Technologies
Murine SAA1	Mm00656927_g1	Invitrogen Life Technologies
Murine SAA3	Mm00441203_m1	Invitrogen Life Technologies

2.9. Antibodies and reagents for ELISA

Antibody/Reagent	Catalogue no.	Supplier
96 Well Plate (Half Volume)	3690	Costar
Anti-Mouse CX3CL1 Duo Set	DY472	R&D Systems
Anti-Mouse IL-10 Capture	MAB417	R&D Systems
Anti-Mouse IL-10 Detection	BAF417	R&D Systems
Mouse SAA Immunoassay Kit	KMA0012	Invitrogen

2.10. Reagents for TCID50 and Haemagglutination assays

Antibody/Reagent	Catalogue no.	Supplier
Glass beads	G8772	Sigma
96 v bottom plates	A0336	Greiner Bio-one
Alsevers solution	A3551	Sigma
TPCK Trypsin	T1426	Sigma
Nunc ELISA plates F96 maxisorp	442404	Nunc
Goat- anti-mouse immunoglobins	P0447	DAKO

2. Methods

2.11. Inoculation of mice with PR8

2.11.a. Primary infection with PR8

Female mice (aged 6–8 weeks) were anesthetised, and inoculated intranasally with PR8 virus (MtSinai) (Health protection agency). Mice were weighed and scored daily to assess for signs of sickness.

2.11.b. S-FLU vaccination and challenge

C57BL/6 or *Jα18*$^{-/-}$ females were used at 6 to 8 weeks of age. Mice were immunized intranasally (i.n.) twice with 32 HAU S-FLU or viral growth media (VGM) in 50µl under isoflo anesthesia at two weekly intervals, followed by 21 day wait prior to challenge. The weights of mice were monitored across the vaccination period, and mice were bled at specific intervals via tail vein bleeds; peripheral populations were assessed by FACS analysis, and serum was stored at -20^{o}C for later analysis. At the end of the vaccination period (42 days),

mice were challenged with 32 HAU PR8 (Cambridge, H1N1), 32 HAU X31 (H3N2), or VGM alone for 5 days. A humane endpoint of weight loss and clinical score was used for mice that would otherwise have succumbed to infection. Animals were assessed for clinical score in terms of mobility, appearance, and breathing intensity. Mice reaching 20% weight loss and/or a morbid clinical score were euthanized.

2.12. Preparation of murine cell samples for flow cytometry and functional assays

2.12.a. Blood

For acquisition of cells for FACS: Blood was collected from the tail vein into Sardett Microvette 100ul tubes (#MCVT100-EDTA). Notably, for experimental end-point acquisition, 200μl of blood was removed via tail vein bleeds. For sequential analysis of peripheral blood, 50μl of blood was taken via the tail vein, from alternating experimental groups, to keep within home-office guidelines (<10% of blood volume in any two weeks, and 15% in any one month period). 1ml of RBC lysis buffer was added to each samples, mixed well and left for 15 minutes at room temperature. Following RBC lysis, samples were transferred to clean 1.5ml eppendorfs to be spun at 6000rpm for 5 minutes at 4^{o}C in a tabletop centrifuge. Supernatant was carefully aspirated and cells were re-suspended in 100μl FACS buffer and stored at 4^{o}C prior to FACS staining.

For acquisition of sera: Blood was collected from the tail vein into eppendorfs, in the absence of anti-coagulant. Samples were allowed to stand for 90-120 minutes to enable coagulation and sedimentation. Samples were spun at 13,000 rpm for 15 minutes at 4^{o}C; serum settling above the cells was aspirated off, and was frozen at -80^{o}C until required.

2.12.b. Bone marrow

Following schedule 1 killing, hind legs were dissected from each mouse and bone marrow cells flushed from each hind limb using FACS buffer and a 21G needle. Cell suspensions were pelleted, and erythrocytes removed by re-suspending pellets in 5ml of 1x red blood cell (RBC) lysis buffer for 3-5 minutes. Cells were then washed in FACS buffer, filtered through a 70µm cell strainer (BD Falcon) and re-suspended in R10 medium.

2.12.c. Lung

Following schedule 1 killing, lungs were dissected from the thoraces of the mice and collected in FACS buffer. Once harvested, lungs were subjected to mechanical disruption and digested by incubating each in 2ml of 1x type II collagenase for 40 minutes at 37^{o}C (re-suspending every 15-20 minutes to aid disruption). Following incubation, cells were filtered through a 70µm cell strainer (BD Falcon) and immediately washed in FACS buffer. Cell suspensions were pelleted (1500 rpm, 5 minutes), and erythrocytes removed by re-suspending pellets in 5ml of RBC lysis buffer for 5 minutes. Cells were then washed in FACS buffer, filtered through a 70µm cell strainer (BD Falcon) and re-suspended in FACS buffer and stored at 4^{0}C ready for antibody staining. To determine absolute cell numbers, cells were stained with Trypan Blue and manually counted using a haemocytometer

2.12.d. Bronchioalveolar lavage (BALF)

Immediately after sacrifice, lungs were inflated via the trachea and following insertion of tubing (# 800/100/260) (attached to a 2ml syringe) into the trachea. 1.0 mL of FACS buffer was used to inflate the lungs 3 times and the recovered fluid was transferred into clean 1.5ml

eppendorfs and stored on ice. Cell suspensions were pelleted (1500 rpm, 5 minutes); BALF fluid was removed and stored at -80ºC, and erythrocytes removed from cell pellets by re-suspending pellets in 1ml of RBC lysis buffer for 5 minutes. Cells were then washed in FACS buffer, filtered through a 70µm cell strainer (BD Falcon) and re-suspended in FACS buffer. To determine absolute cell numbers, cells were stained with Trypan Blue and manually counted using a haemocytometer.

2.13 Measurement of virus titer in infected mouse lungs

2.13.a. Homogenisation of mouse lung

The inferior lobe of the right lung of each mouse was removed, snap frozen, and stored at -80ºC until required. Approximately 200µl of glass beads (Sigma #G8772) were added to 2 ml screw-cap tube (Starlab, RNase-free), and one lung sample was transferred to each tube. 1ml of viral growth media (VGM) was added per tube and the sample was homogenised by spinning for 2 x 30s in a Fast-Prep tissue homogeniser at 4ºC (Oncology cold room). Samples were then centrifuged at 500 x g 5 min at 4ºC to pellet beads, and supernatant was transferred to fresh 1.5 ml eppendorfs and used immediately, or stored at -80˚C until required.

2.13.b. TCID50 assay

MDCK SIATI cells were maintained in D10 media, and were plated the night before required at $3x10^4$/well, $3x10^6$/96 well plate, in 100µl/well of D10 media. On the day of the experiment, supernatant was removed from MDCK cells, and cells were washed with 200µl PBS before being stored in PBS until required. Homogenised lungs **(2.13.a)** were thawed on ice, and diluted 1/100 in VGM. 500µl of diluted viral homogenates were filtered and were then further

diluted 1/3 in VGM to make the top plate concentration, prior to further 1/3 serial dilutions down a 96 well U-bottomed plate, leaving 100µl of virus-containing media per well. Two columns were left at the end of the plate for positive controls (PR8 or X31 virus) and negative controls (VGM alone). PBS was aspirated off the washed MDCK cells and 50µl of diluted virus-containing media was transferred onto the cells. The PR8 and X31 positive controls were used with a top dilution of 1/500 in VGM. Plates were incubated at 37^{o}C for 1 hour, before 150µl of VGM media containing TPCK trypsin was added; the final TPCK trypsin concentration was 1µg/ml. Plates were then incubated at 37^{o}C for 2-3 days.

2.13.c. Haemagglutination assay

Following incubation, supernatants from viral-infected MDCK plates were removed, and either used immediately, or stored at $^{-}$80^{o}C. Red blood cells (RBCs) were prepared as follows: Fresh human blood was processed using a lymphoprep gradient; the PBMC layer was removed leaving only the RBCs which were washed with 50ml PBS (1500rpm for 5 minutes). RBCs were re-suspended in PBS to which an equal volume of Alseivers solution (Sigma #A3551) was added to make the final stock solution (50:50, RBC: Alseivers solution). 0.83ml of this 50% RBC stock was diluted in 50mL PBS, prior to centrifugation at 1400 rpm for 5 minutes. After carefully aspirating the supernatant, the RBC pellet was resuspended in 50 mL PBS, and diluted a further 1:2 with PBS. The absorbance of the resulting solution was ~1.8 at 600nm on a spectrophotometer. 50µL of viral supernatant was added into new 96 well V bottom plates (Greiner Bio-one #651101). 50µL of RBC solution prepared previously was then added to each well, mixed gently, and left to incubate at room temperature for 60 minutes. Following incubation, plates were tipped to 45^{o} angle, and assessed for ‘tear-drop’ formation, indicating the presence or absence of virus-mediated agglutination. Wells were marked as positive or negative, and TCID50 was calculated according to Reed and Muench formula [303].

2.13.d. ELISA for detection of anti-HA antibodies

Mouse serum was heat inactivated for 30 minutes at 50^{o}C. Nunc ELISA plates F96 maxisorp (#442404) were coated with 50µL of 20µg/mL HA protein (generated and kindly provided by Professor Alain Townsend and Dr Timothy Powell), and incubated for 2 hours at room temperature. Following incubation, plates were washed with PBS, and blocked for 1 hour, at room temperature, with 200µL PBS containing 5% milk (Marvel milk powder w/v) with 0.02% sodium azide. In the mean-time, serum and BAL were diluted in a separate plate as follows: serum, was diluted 1/20 for the top dilution, and then serially diluted 1/2 across the 96 well plate (a total of 6 dilutions per sample). Following blocking, HA-coated plated were washed 3x with PBS, and following the final aspiration, 50µl of the diluted serum of BALF above was transferred into the plate, and incubated for 2 hours at room temperature. Plates were washed 4x with PBS, prior to addition of 50µl goat anti-mouse immunoglobins secondary antibody (DAKO P0447) at 1/2000, for 2 hours at room temperature. Plates were washed once again using 4 x PBS washes before 50µl TMB Roche substrate was added for 5-15 minutes. The reaction was halted using 50µl of 2M H_2SO_4 and the absorbance was read at 450nm on plate reader. The antibody titer was denoted as the dilution of serum where the OD measured is above that of the 50% max value, using the equation below:

= ((Max OD – background OD) / 2) $^{+}$ background OD

2.14. Generation of Bone Marrow Chimeric Mice

B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (SJL) mice were irradiated with two doses of 450rads (266 seconds), 4 hours apart at the Biomedical Services Unit, John Radcliffe Hospital, University of Oxford. 3 hours following the second irradiation, mice were reconstituted with bone marrow cells from donor mice by intravenous injection of 6x10^{6} bone marrow cells in an inoculum of 100µl PBS using a 30G needle. Sample mice were reconstituted to have a 50:50

ratio of $CD1d^{-/-}$CD45.2: $CD1d^{+/+}$CD45.1 hematopoietic cells (shown in **Figure 9**) while control mice were reconstituted to have a 50:50 ratio of $CD1d^{+/+}$CD45.2: $CD1d^{+/+}$CD45.1 hematopoietic cells. Mice were kept in micro-isolator cages with sterile sawdust, food and water containing 10mg/ml of the paediatric antibiotic Septrin (#23192680) for the initial 3 weeks post-irradiation, and were then transferred to water-alone for the remaining 5 weeks (total engraftment period of 8 weeks). The blood of all animals was extracted using tail bleeds, and was thereafter assessed for 50:50 reconstitution by FACS, prior to influenza infection.

2.15. Magnetic-Activated Cell Sorting (MACS) for $Ly6G^+$ neutrophils

Neutrophils were positively selected using anti-Ly6G mAb–conjugated magnetic micro-beads. The recovered cells were 95-99% $CD11b^+Ly6G^+Ly6C^+$, as determined by flow cytometry.

2.16. *In vitro* stimulation of isolated neutrophils with SAA

One million MACs bead-isolated $Ly6G^+$ neutrophils (purity 95-99% $CD11b^+Ly6C^+Ly6G^+$, as shown by FACs) were plated into a 96 well flat-bottomed plate in 100µl R10 medium, containing the desired stimulant**,** and incubated for between 4-24h at 37^{o}C, 5% CO2.

2.17. Staining of cells for flow cytometry analysis

Cells for staining were made up to suspensions of $3x10^6$ cells/50µl FACS buffer. 50µl of cell suspension was then transferred to each well of a round bottom 96-well plate and pelleted (1500 rpm, 5 minutes at RT). Supernatant was removed by inversion of the plate; 50µl of the pre-prepared, relevant antibody mixture was added to each well of cells and the cells were

stained for 20min at 4ºC. Cells were then washed using 200µl of FACS buffer and plates were centrifuged (1500 rpm, 5 minutes at RT), prior to re-suspension in 200µl fresh FACS buffer. Non-stained cells, single colour-stained cells, and fluorescence-minus-one (FMOs) were included as controls. To allow for exclusion of dead cells, immediately prior to running, 1:20 propidium Iodide (PI), a cell-impermeable nucleic acid dye, was added to the sample. Alternatively, Aqua live-dead stain was used (Invitrogen #L34957); after the final antibody stain, cells were washed twice in PBS, and incubated in 100µl PBS containing a 1/200 dilution of Aqua for 15 minutes at room temperature. Cells were washed in PBS, before re-suspending in 200µl FACS buffer. Samples were run on Dako Cyan machines or a BD LSRFortessa™ cell analyzer, unless otherwise stated. Data analysis and gating were performed using FlowJo 7.6.1 and FlowJo v10.

2.18. Fluorescence-Activated Cell Sorting (FACS)

Cells to be sorted were stained with fluorochrome-conjugated antibodies as for flow cytometry. After the final wash, cells were resuspended in FACS buffer and passed through a cell strainer to remove cell clumps, before being sorted in the WIMM FACS facility. Cells were sorted using a MoFlo sorter (Beckman Coulter) or FACS Aria II sorter (BD Biosciences), with the expert help of Craig Waugh, Kevin Clark and Paul Sopp.

2.19. Reverse transcription and qPCR

For qPCR analysis, cells were lysed in 350-700ul RLT buffer, and frozen at -80 until required. RNA was extracted using a Qiagen RNeasy mini kit. Samples were treated with Turbo DNase (Applied Biosystems) to remove genomic DNA, as per kit instructions. cDNA was obtained by reverse transcription using the Superscript III reverse transcriptase kit

(Invitrogen). The sample and master mix were prepared as per kit instructions. The mixture was pipetted gently up and down and then incubated at 50°C for 30-60 minutes prior to inactivation by heating at 70°C for 15 minutes. The cDNA obtained at this point was then used as a template for amplification. qPCR was performed in a MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 ml (Applied Biosystems). The qPCR reaction mix contained:

10µl TaqMan Gene Expression Master Mix (Applied Biosystems),

1) 9µl cDNA (10-100ng)
2) 1µl of the relevant 20X TaqMan probe

Plates were covered with MicroAmp Optical Adhesive Film (Applied Biosystems), and reactions were run in 'fast mode' in a 7500 fast real-time PCR system (Applied Biosystems. The cycling conditions used were:

1. 95°C, 20 seconds
2. 95°C, 3 seconds
3. 60°C, 30 seconds
4. Back to step 2 for 40 cycles.

Fold induction was analysed using the ΔΔCt method of comparison, where:

$\Delta\Delta CT = (CT - CT_{\text{ref gene}}) - (CT - CT_{\text{ref gene}})_{\text{ref sample}}$.

The reference gene (ref gene) was HPRT, and the reference sample (ref sample) was the naïve group of each strain (C57BL/6 or *Jα18*$^{-/-}$) analysed for all of the *in vivo* experiments, or the 'untreated' group for in all of the *in vitro* experiments, as indicated.

2.20. Enzyme-Linked Immunosorbent Assay (ELISA)

For measurement of soluble cytokine or chemokine production, supernatants, serum (1:200 dilution) or BAL were collected, and stored at -20°C until required. 25ul of sample was then assessed in 96 well plates (Costar) for desired chemokine/cytokine, according to manufacturer's protocol.

2.21. Luminex analysis

For measurement of soluble cytokine or chemokine production BAL was collected, and stored at -20°C until required. Samples were run according to the standard R&D systems protocol (#LXSAMSM).

2.22. RNA processing, cDNA biotin labelling and Affymetrix microarray analysis

Cells were sorted as previously described, with the exception that cells were sorted directly into 1.5ml eppendorf tubes containing 800µl of Trizol® with a final concentration of 250µg glycogen (#10814-010). Trizol®-containing eppendorfs were maintained at 4°C until required. Immediately after sorting, cell/Trizol® mixes were vortexed for 30 seconds, and placed on dry ice, prior to short term storage at -80°C.

2.22.a. RNA extraction

Isolation of RNA was carried out using an adapted Trizol® (#15596-018) protocol for isolation of RNA from a low number of cells: Samples were removed from storage at -80°C,thawed on ice, and were vigorously mixed using a vortex before being placed at room temperature.

Genomic DNA was sheared by passing the samples twice through a 26G needle connected to a 1ml syringe, and transferred to a sterile 1.5 ml microcentrifuge tube. 160µl of chloroform (or 49:1 chlorform:isoamyl alcohol) was added to each sample and vortexed for 30 seconds. Samples were spun at maximum speed in the microcentrifuge for 5 minutes to separate the phases, and the upper aqueous phase of the eppendorf was then transferred to a fresh tube containing 400µl ice-cold isopropanol. Samples were then left to precipitate at -20^{o}C overnight.

Following precipitation, RNA was pelleted by centrifugation at maximum speed (13,000rpm) in the microcentrifuge for 15 minutes at RT, and supernatant was subsequently decanted. RNA pellet was washed in 200µl of 70% ethanol, before spinning for 10 minutes at 13,000rpm. Supernatant was again removed entirely without disturbing the pellet, and pellets were allowed to dry at RT for 30 minutes to remove any residual ethanol. Pellets were resolubilised in 20µl deionized water and centrifuged for 1 minute at 13,000rpm in a microcentrifuge.

RNA samples were purified using the Qiagen RNeasy MinElute Cleanup Kit (#74204) following the kit protocol, and samples were resuspended in 14 µl deionised water. The quantity and quality (RIN number) of RNA was measured using Agilent RNA 6000 Pico Kit (#5067-1513), and analysed using the Agilent bioanalyser, as per manufacture instructions. Samples were stored at -80^{o}C until ready for processing

2.22.b. cDNA conversion and biotin labelling

Purified, quantified RNA was converted to cDNA using of the NuGEN Ovation pico WTA system V2 (#3302), as per manufacturers' instructions. Primary cDNA clean-up steps were carried out using the Agencourt ® RNAClean® XP Purification Beads, included in the NuGEN kit, as per kit instructions. Secondary clean-up steps were carried out using the

Qiagen MinElute Reaction Cleanup Kit (#28204), as per kit instructions. Control samples without glycogen carrier were run in parallel with experimental samples to ensure no inhibitory effect of glycogen on the amplification process. Resulting cDNA was quantified, and the purity checked using a nanodrop reader

Biotin labelling of cDNA was carried out using the NuGEN Encore® Biotin Module (#4200), and samples were stored at -20˚C until ready for hybridisation stages.

2.22.c. Affymetrix gene chip hybrisation and sample analysis

Samples were run according to the Affymetrix protocol- basic protocol is as follows: 24 hours prior to running affymetrix gene chips, the GeneChip® Fluidics Station 450 was cleaned using the bleach program (Bleachv_450). 2 hours prior to chip hybridisation, the GeneChip® Hybridization Oven 645 was switched on and set to 45^{o}C, and 60 RPM.

Samples were hybridised to Mouse ST 2.0 ST gene array chips as per manufacturer instructions detailed below, with minor modifications (shown by underlining) as suggested by NuGEN. Hybrisation cocktail (containing biotin-labelled cDNA) was prepared as outline in the affymetrix protocol, following the 'MIDI ARRAY- 100 FORMAT'. NB: Chips were hybridised and washed in randomised batches of 4, with staggered starting times (2 hours apart) so as to keep consistency of hybridisation, washing and storage time before chips could be scanned. Affymetrix chips were pre-hybridised with 130µl hybridisation mix for 10 minutes. In the meantime, the hybridisation cocktail was denatured for 2 minutes at 99^{o}C, and then transferred to 45^{o}C for 5 minutes, followed by a centrifuge spin for 5 minutes at 13K rpm. Pre-hybridisation mix was removed from affymetrix chips (to be stored at -20^{o}C in case required again), and replaced with 130 µl of hybridisation cocktail described above. Chips were hybridised for 16 hours at 45^{o}C.

1.5 hours prior to the end of the chip-incubation period, the fluidics station was switched on, and primed using the program Prime_450. Following 16 hour hybridisation, hybridisation

mixes were removed from the affymetrix chips (and stored at -20^{o}C in case require further). Chips were then re-loaded with 130ul of array buffer at room temperature, and run on the fluidics system using the program FS450_0002 as per the manufacturer instructions. Washed chips were stored in the dark, and at room temperature, before running on the GeneChip® Scanner 3000, using the Affymetrix expression console software.

2.22.d. Micro-array data processing and analysis

Data pre-processing was performed by Dr Stephen Taylor of CBRG. The Robust Multi-array Average (RMA) algorithm was used to process and normalise raw array data. This 'probe summarization algorithm' performs the following 3 key tasks:

(i) Background correction to identify and account for background signal, caused by optical noise and non-specific binding.

(ii) Quantile normalization: aimed at making the intensity distributions identical across arrays which is necessary to compare and analyse multiple chips with each other

(iii) Probe summarization: conversion of probe level values to probe-set expression values in a robust, and outlier resistant manner) [304].

Although we utilised a detailed RNA preparation strategy and randomised microarray sample order to reduce the probability of sample errors occurring, and wherever possible the reagents kits were of the same batch number, and samples were processed, hybridised and run in an entirely randomised order, it is possible some results could be biased by running-batch variability. Specific quality control analysis was then performed which examines the natural groupings, similarities, and differences between the samples, to identify unintentional bias which could stem from unavoidable experimental or technical factors, such as batch differences, poor hybridisation, or chip anomalies.

Microarray analysis was performed in R using the package Limma [305]. For each analysis, the two conditions (SJL (CD45.1) and Target (CD45.2)) were compared using a linear model

and adjusting for mutation status (WT/MUT), and sibship relations (to model for where the cells originate from within the same mice). The moderated t-statistics, moderated F-statistic, and log-odds of differential expression were computed using an empirical Bayes moderation of the sample variances, as explained in the paper of Smyth et al. [306]. The p-values were adjusted for multiple hypothesis testing using the Benjamini–Hochberg procedure. Significance of a gene upregulation or downregulation was determined by a p value <0.05, and a log2 fold change (Log2FC) ≥1.5.

2.23. Statistical analysis

All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad, La Jolla, CA). Normality of data distribution was first evaluated, and normally distributed data sets were compared using two-tailed student t test. For non-normal data distributed, a student's t test with Welch's correction was performed. For comparisons of multiple, normally distributed data sets simultaneously, one-way ANOVA was used. A p value <0.05 was considered significant. Mean values were reported for normally distributed data. Error bars on graphs represent standard deviation (SD) unless otherwise stated.

Chapter 3.

Investigating a role for iNKT cells in the intrinsic development and expansion of myeloid cells

Chapter 3. Investigating a role for iNKT cells in the intrinsic development and expansion of myeloid cells

3.1. Introduction

3.1.a. iNKT cells modulate the immune response to influenza infection

It is widely accepted that neutrophils and monocytes provide the first line of defence against invading pathogens, rapidly producing of a wide range of effector molecules essential for pathogen killing, and releasing signalling molecules such as cytokines and chemokines, to recruit and activate further cells of the innate and adaptive immune response [307, 308]. As such, the rapid differentiation and expansion of myeloid progenitors into effector neutrophils and monocytes during an infection is an indispensable part of the immune response.

The lungs are the primary location of influenza infection, and represent the organ where much of the IAV-induced response and IAV-associated pathology occurs. It has been postulated that iNKT cells are long-term residents of the lung microvascular network [71]; blood obtained from the right and left ventricles of the heart revealed enrichment of NKT cells by ~20-fold compared with peripheral blood [309], suggesting a continuous accumulation and release of iNKT cells in the microvascular compartments of the lungs. As discussed previously, iNKT cells are recruited to the lung during influenza, where they have demonstrated a variety of roles in disease resolution via interaction with innate and epithelial cells [127, 284, 285]. Infiltrating APCs such as monocytes, neutrophils, DCs and myeloid-derived suppressor cells (MDSCs) constitutively express CD1d; thus, NKT cells are able to interact with these cells not only during times of immune activation, as has been shown previously in influenza, but also in immunologically quiescent periods via auto-reactivity to self-antigens [101]. Indeed, the seminal finding by De Santo et al. showed elevated accumulation and immunosuppressive activity of $CD11b^{+}Gr\text{-}1^{+}$ myeloid derived suppressor cells (MDSCs) isolated from the lungs of influenza-infected iNKT-deficient mice. The authors

extended these findings showing that adoptive transfer of iNKT cells abolished the suppressive activity of MDSCs, restored IAV-specific immune responses, reduced IAV titers, and increased survival rate, and that the crosstalk between iNKT and MDSCs was found to be CD1d- and CD40-dependent [41].

3.1.b. iNKT cells and the regulation of myelopoiesis

iNKT cells have been localised to numerous organs across the human and rodent anatomy, thus, it is plausible that the frequencies and localisation of iNKT cells denote where this subset of cells affords its regulatory control over other cells of the immune system. Indeed, other than the lung, the bone marrow, lymph nodes and spleen are other possible sites of interaction for iNKT cells and monocytes. Barral et al. showed that iNKT cells were positioned within the interfollicular zones and the medulla of the lymph node and could actively scan and communicate with macrophages [310]. The same group demonstrated later that the majority of iNKT cells slowly patrol (speeds of ~ 6 μm/min^{-1}) around the marginal zone of spleen where they may form a net for receipt of circulating monocytes [311]. Of particular interest, iNKT cells have been shown to reside in the bone marrow [32, 312], with total NK1.1^{+}TCRβ^{+} cell percentages ranging between 20-30% of total T cells in the bone marrow, and iNKT cells accounting for approximately 30% of this population [313-315]. This accumulation of iNKT cells in the bone marrow could allow for a prolonged and intimate interaction of iNKT cells with myeloid cells during haematopoiesis and emergency myelopoiesis in the bone marrow. **Figure 5** depicts the currently accepted hierarchy of myeloid precursors in the bone marrow, which highlights the potential for iNKT-myeloid cell interactions at multiple stages during development.

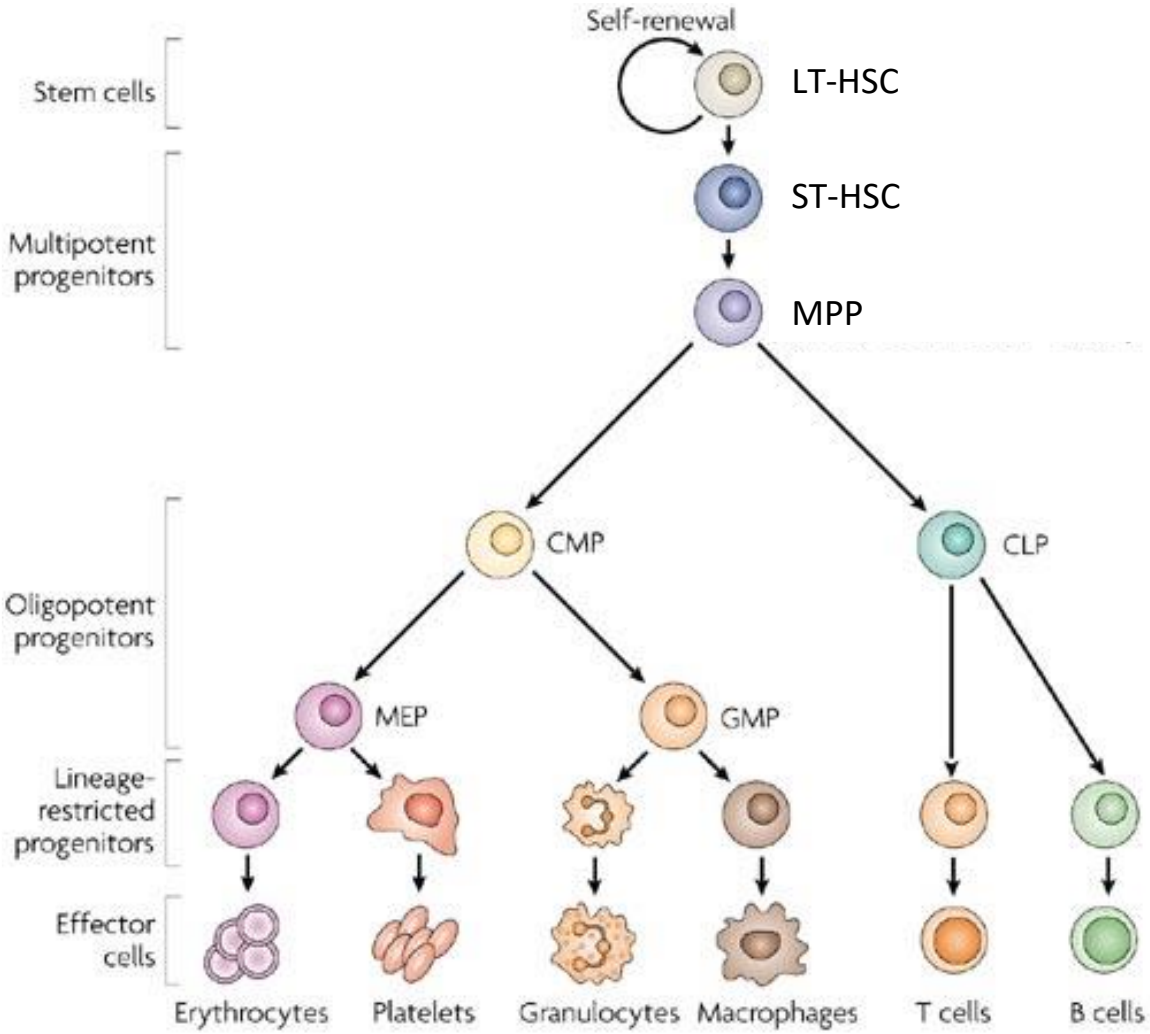


Figure 5. Proposed hierarchy of haematopoiesis, in mice.

Indicated cell populations have been prospectively defined by phenotypic, functional, and for the more mature cells, gene expression profile homogeneity, however many relationships between populations are still being characterised. Long-term hematopoietic stem cells (LT-HSC) self-renew for life, whereas downstream short-term (ST)-HSC self-renew for between six to eight weeks, and multi-potent progenitors (MPP) self-renew for two weeks. Cellular populations further down-stream represent lineage restricted progenitors, these include common lymphoid progenitor (CLPs), common myeloid progenitor (CMPs, megakaryocyte-erythrocyte progenitor (MEPs) and granulocyte-macrophage progenitor (GMPs) (Figure modified from [316].

It is certainly plausible that lymphocytes can afford a role in the bone marrow during haematopoiesis; T cells in general have long been proposed to contribute to the regulation of hematopoietic activity [317-319]. T-cell deficient mice have been show to exhibit severely defective terminal differentiation of myeloid progenitors, leading to immature cells accumulating in the bone marrow, and leukopenia in the periphery, a phenomena which was restored by re-introducing activated $CD4^+$ but not $CD8^+$ T cells into the bone marrow [318]. Cytokines are fundamental in hematopoietic and immune regulation [320], and indeed, restoring the defect in myelopoiesis of myeloid progenitors was suggested to be dependent on cytokines produced in response to TCR engagement [318].

Besides localisation, a number of functional aspects of iNKT cells conceivably link iNKT cells to regulating haematopoiesis; activated iNKT cells release key haematopoietic growth factors, namely granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-3 [94, 95, 321]. Likewise, stimulation of $CD1d^+$ cells with the adjuvant α-GalCer enhanced production of both Th1 and Th2 cytokines by NKT cells [61, 322], which can directly and indirectly influence the proliferation of myeloid progenitor cells [317, 323-325]. It was shown by Leite-de-Moraes and collegues that intravenous injection of α-GalCer into mice led to increased committed haematopoietic progenitor activity, as measured by colony forming units in the bone marrow, spleen and peripheral blood, as well as an efflux of granulocytes from the bone marrow [321]. More recent work by our group demonstrated that the activity of short-term as well as long-term progenitors, is reduced in the absence of NKT cells [326]. This observed reduction in progenitors correlated with reduced marrow cellularity, a lower frequency of hematopoietic stem cells (HSCs), and finally, peripheral blood cytopenia [326]. Similarly, Broxmeyer et al. reported reduced proliferation of bone marrow precursor cells in $CD1d^{-/-}$ mice. However, they propose that not iNKT cells, but CD1d signalling itself, via the C terminal SAYQDIR sequence, was responsible for this phenotype [327]. Although the exact

mechanism/s accountable for this regulation remains under investigation, taken together, these data suggest that iNKT cells can enhance myelopoiesis. This is consistent with the work of Hu et al, who showed that α-GalCer-activated iNKT cells modulate osteoclastogenesis, and subsequent macrophage egress from the bone marrow [328].

As pointed out previously, there are few and differing reports on how iNKT cells could regulate myeloid cell accumulation and lung pathology, and only a limited number of direct iNKT-mediated mechanisms have been identified in the lung [41, 127, 285, 329]. While the above reports indicate a role for iNKT cells in enhancing myelopoiesis in the steady state [321, 326], mechanisms through which iNKT cells regulate emergency myelopoiesis, and also the functional polarisation of myeloid cells from an immunogenic phenotype, to a suppressive or hyper-responsive phenotype, in response to either non-sterile, pathogenic infections or sterile inflammation, remain to be established. This is an important consideration since our group have shown that systemic injection of the pro-inflammatory stimulant, serum amyloid A (SAA), caused neutrophilia to a greater extent in *Jα18*$^{-/-}$, as compared with wild-type mice; this expansion was dependent on CD1d-interactions, and accordingly, CD1d$^{-/-}$ neutrophils isolated from an influenza infected CD1d$^{-/-}$:CD1d$^{+/+}$ chimera were more suppressive than their CD1d$^{+/+}$ counterpart [330]. Because of the relative scarcity of iNKT cells in the periphery, as compared with the vast numbers myeloid and neutrophil frequency found in the lung during infection, we question whether these local iNKT cell responses were solely accountable for the disproportionate response observed in the absence of iNKT cells, or whether iNKT cells can function elsewhere in the periphery to curtail the differentiation of myeloid populations. We suggest that further characterisation of the CD1d-dependent interactions of iNKT cells with myeloid cells may further elucidate the regulatory capacity of iNKT cells over myeloid cells, and assist in identifying developmental stage, phase of infection, or components of the innate immune response. To address these

questions we utilised a range of *in vivo* infection models of iNKT cells deficient mice (*Jα18*$^{-/-}$), and also in CD1d$^{+/+}$: CD1d$^{-/-}$ chimeras.

3.2. Results

3.2. NKT cell-deficient mice exhibit a higher susceptibility to influenza virus infection

We infected both wild-type (C57BL/6), and *Jα18*$^{-/-}$ mice, which are deficient in iNKT cells [331], with 1.8x10^{6} PFU of PR8; consistent with previous findings [41, 127], we demonstrated that following PR8 infection, *Jα18*$^{-/-}$ mice lost a significant percentage of body weight by day 4-post infection (**Figure 6a**), and exhibited increased morbidity and decreased survival rates (data not shown), compared with C57BL/6 mice. Additionally, *Jα18*$^{-/-}$ mice displayed both increased cellular infiltration into PR8-infected lungs (**Figure 6b**), and increased lung pathology, including lung enlargement, extensive haemorrhaging, and hepatisation, compared with the naïve-like morphology observed in C57BL/6 mice (data not shown).

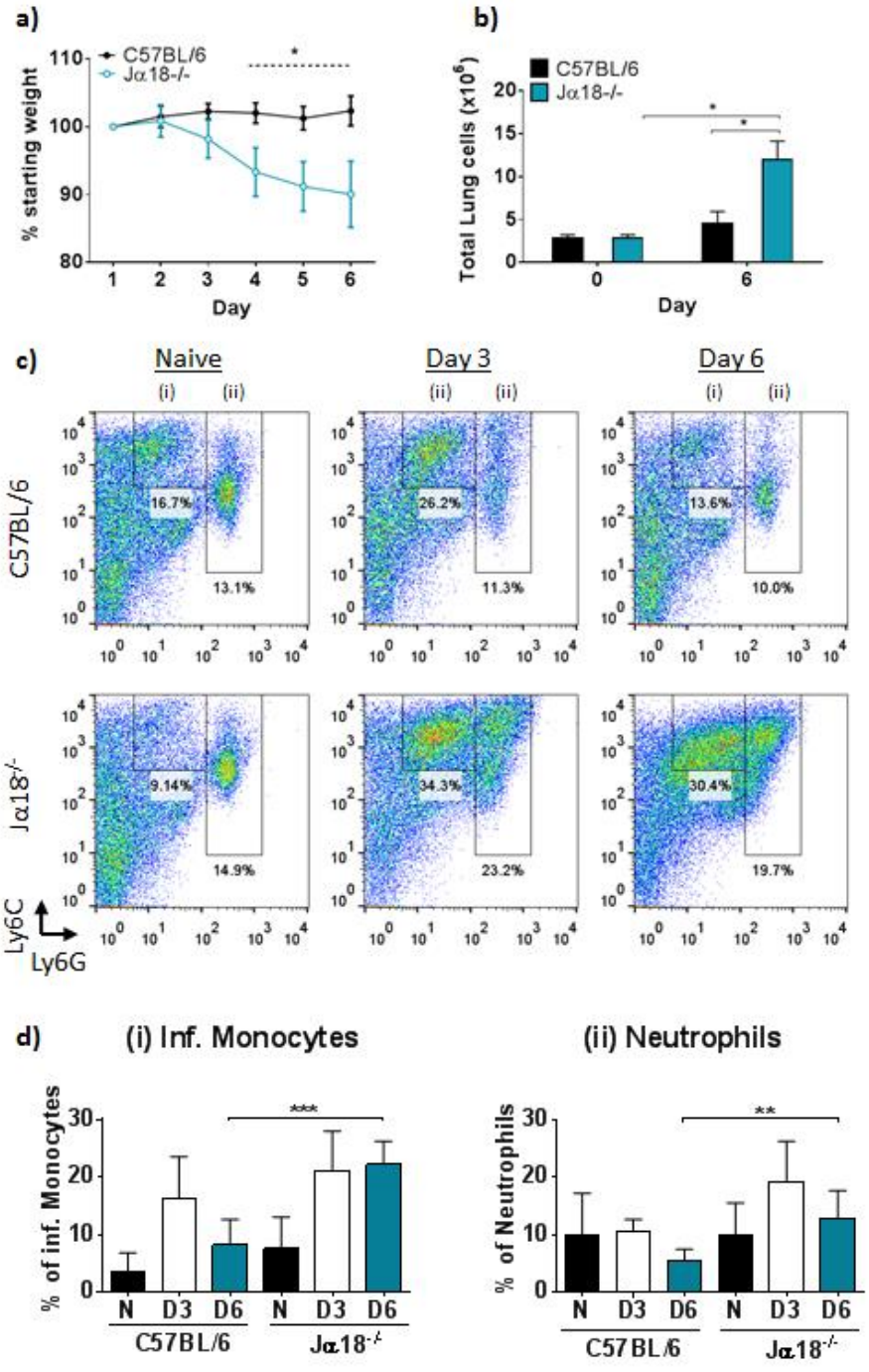


Figure 6. Increased mortality of PR8-infected *Jα18*$^{-/-}$ mice corresponds with infiltration of inflammatory monocytes and neutrophils

a) Jα18$^{-/-}$ *mice exhibit a significant decrease in weight as compared with C57BL/6 mice following PR8-infection. Weights were recorded daily, and are shown as a percentage of starting weight (5 mice per group). b) Total number of lung cells were elevated in Jα18*$^{-/-}$

*mice compared to C57BL/6 mice following PR8-infection. Cells were extracted from homogenised lung, and manually counted using a haemocytometer. c) The percentage of inflammatory monocytes and neutrophils in the lungs of naïve (N), or day 3 (D3), or day 6 (D6) of PR8-infected C57BL/6 or Jα18$^{-/-}$ mice was analysed by flow cytometry. The average cellular infiltration (±standard deviation (SD)) observed by flow cytometry is summarised in (d). Data are representative of three independent experiments, with 3 mice per group. Statistical significance was analysed by two-tailed student's t-test; *p<0.05, **p<0.01, ***p<0.001.*

3.4. Greater susceptibility of *Jα18*$^{-/-}$ mice to PR8 infection corresponds with an expansion of inflammatory monocytes and neutrophils in infected lungs

Monocytes and neutrophils have been reported as key players in the immune response to influenza; to discriminate accurately between monocytic and granulocytic populations during PR8 infection in C57BL/6 and *Jα18*$^{-/-}$, we utilised specific antibodies against CD11b, Ly6C, CCR2 and Ly6G **(Figure 6c)**. Inflammatory monocytes **(Figure 6c(i))** are classed as Ly6C^{+}Ly6G^{-} (also CD11b^{+}CCR2^{high} (data not shown)), whereas neutrophils **(Figure 6c(ii))** are classed as Ly6C^{int}Ly6G^{+} (also CD11b^{+}CCR2^{-} (data not shown)). At six days post-infection, *Jα18*$^{-/-}$ mice demonstrated significant expansion of inflammatory monocyte and neutrophils, as compared to PR8-infected C57BL/6 mice **(**Figure 6**d)**. It may be important to note that when the inflammatory monocytes population was sorted from the lung and analysed for morphology using Hematoxylin and eosin (H&E) staining and light microscopy (data not shown), this population demonstrated a high degree of heterogeneity; morphological analysis indicated the presence of monocytic-like cells, but additionally, many cells containing nuclei indicative of immature granulocytes, for instance metamyelocytes or band cells. In light of the reports of myeloid derived suppressor cells (MDSCs) in the lungs

of influenza infected mice, which have markers and morphological features of immature myeloid cells [41, 332], further in depth characterisation of this population will be essential.

3.5. Influenza-infected *Jα18*$^{-/-}$ mice exhibit differential cytokine and chemokine profiles in the lung

The exacerbated infiltration of immune cells into the lungs of *Jα18*$^{-/-}$ mice suggests that there is differential signalling occurring in the absence of iNKT cells. We therefore performed luminex analysis to detect cytokines or chemokines released into the BALF during PR8 infection in either strain **(Figure 7)**. We observed a striking elevation in IL-10, IL-1β and CCL2 release into the BALF of *Jα18*$^{-/-}$ mice at day 5 post-infection, which was significantly greater than that observed in C57BL/6 mice **(Figure 7a-c)**. We observed comparable release of CXCL2, CCL3, CCL4, TNF-α and IFN-γ between the two strains at this time point (**Figure 7d-h**).

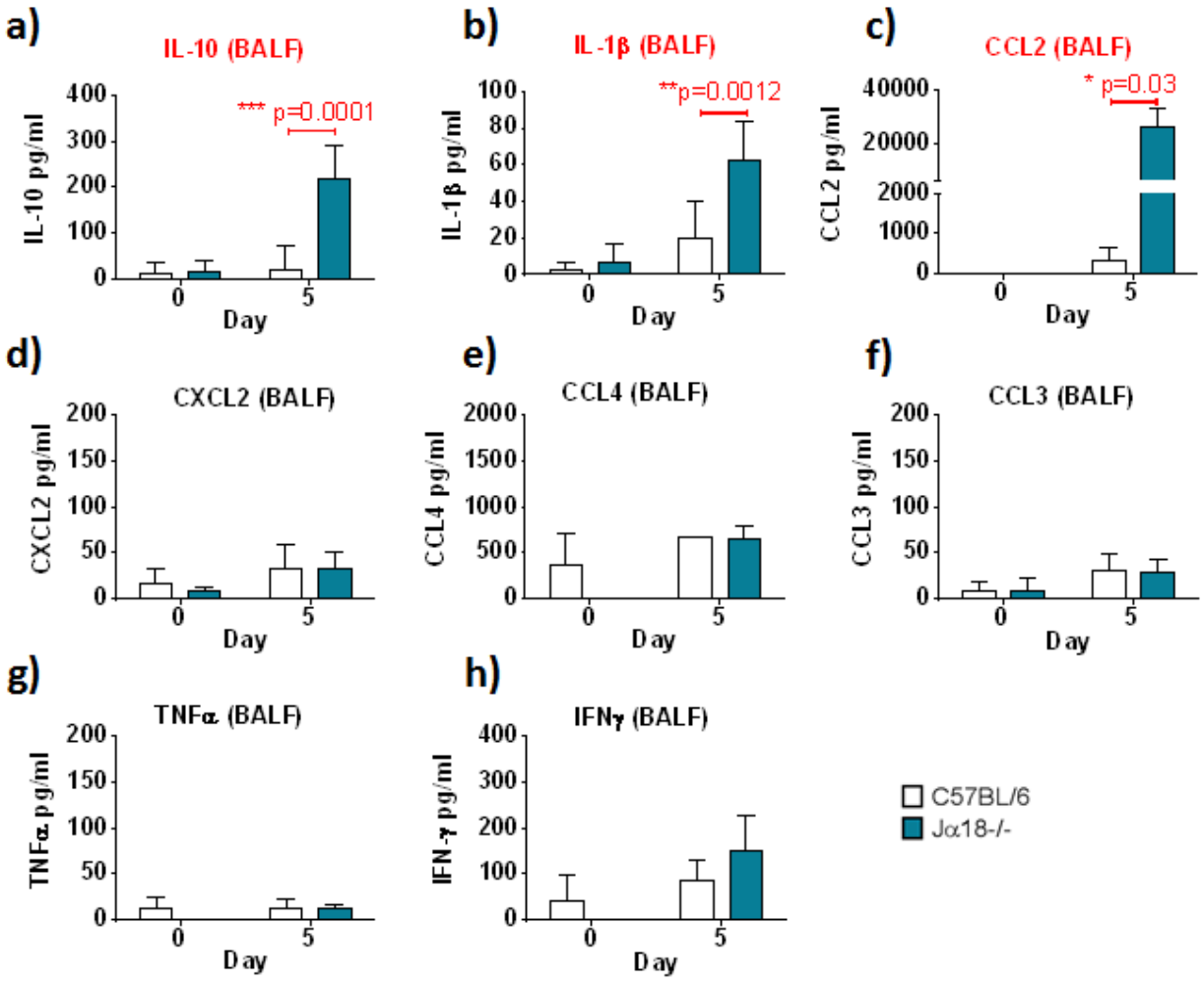


Figure 7. iNKT cells modulate cytokine and chemokine release into the BALF following influenza infection

*Mice were infected with 32 HAU of PR8 for 5 days, and the BALF was analysed using a luminex to assess cytokine and chemokine levels. N= 3-5 mice per group. Statistical significance was analysed by two-tailed student's t-test; *p<0.05, **p<0.01, ***p<0.001. Error bars represent standard deviation.*

In an attempt to identify whether inflammatory monocytes or neutrophils which we observed in greater numbers in PR8-infected *Jα18*$^{-/-}$ mice **(**Figure 7**)** were responsible for the differential cytokine or chemokine profiles in *Jα18*$^{-/-}$ mice, we sorted both populations from

naïve or infected mice, and carried out qPCR analysis for IL-10 **(Figure 8**). We identified a clear upregulation of IL-10 message in both inflammatory monocytes (**Figure 8a**) and neutrophils (**Figure 8b**), indicating that these cells may contribute to the IL-10 protein released into the BALF following influenza infection.

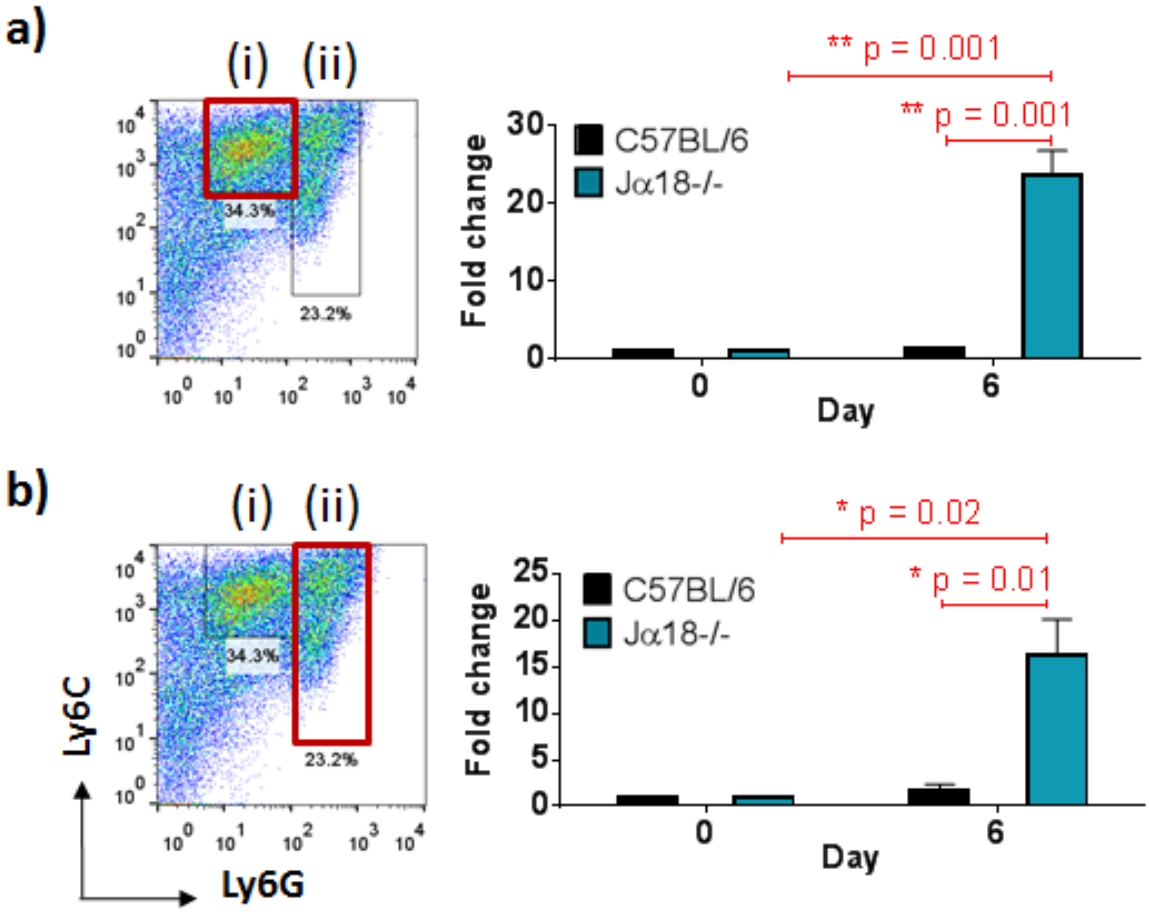


Figure 8. iNKT cells modulate IL-10 message in both inflammatory monocytes and neutrophils following influenza infection.

*Mice were infected with 1.0x10^5 PFU of PR8 for 6 days, inflammatory monocytes (a) and neutrophils (b) were sorted by FACS, and analysed for IL-10 expression by qPCR. N= 3-5 mice per group. Statistical significance was analysed by two-tailed student's t-test; *p<0.05, **p<0.01, ***p<0.001. Error bars represent SEM.*

3.6. Generation of $CD1d^{-/-}$: $CD1d^{+/+}$ bone marrow chimeras

In an attempt to address whether iNKT cells directly affect the expansion of myeloid precursor cells into mature monocytes and neutrophils during IAV infection, via CD1d-mediated interactions, we generated a 50:50 mixed bone marrow chimera in lethally radiated mice, whereby half the hematopoietic cells were $CD1d^{+/+}$ $CD45.1^{+/+}$ (from B6.SJL-$Ptprc^{a}Pep3^{b}$/BoyJ, herein referred to as SJL) donors, while the other 50% of hematopoietic cells were $CD1d^{-/-}$ $CD45.2^{+/+}$ (obtained from CD1d-deficient donors on a B6 background). The recipient mouse would thus possess iNKT cells selected via $CD1d^{+/+}$ compartment, however donor cells from $CD1d^{-/-}$ mice are incapable of interacting these cells directly via CD1d interactions **(Figure 9a**). Notably, although the CD45.1 and CD45.2 isoforms are reported to exhibit no functional differences, to ensure that any differential responses of the $CD1d^{-/-}$ cells was only due to the expression of CD1d on the hematopoietic cells, and not the CD45 congenic marker, control 50:50, SJL: $CD1d^{+/+}$ mixed bone marrow chimera mice were generated and infected in parallel with the SJL: $CD1d^{-/-}$ chimeric mice. The advantage of using the chimera model to address the role of CD1d-mediated NKT interactions during influenza infection is that both $CD1d^{+/+}$ and $CD1d^{-/-}$ cells are contained within one animal, and thus are under identical environmental conditions; this excludes experimental variations which may be present when analysing separate wild-type and knockout mice. We can thus be more confident that the differences observed in the phenotypes and genetic profiling of the cells is due to CD1d- interactions during infection. Genotyping of donor cells confirmed the donor cells were of the correct genotype (**Figure 9b**), and FACS analysis of excess donor cell mixture confirmed that an equal ratio (50:50) of $CD45.1^{+}$ and $CD45.2^{+}$ was injected (**Figure 9c**).

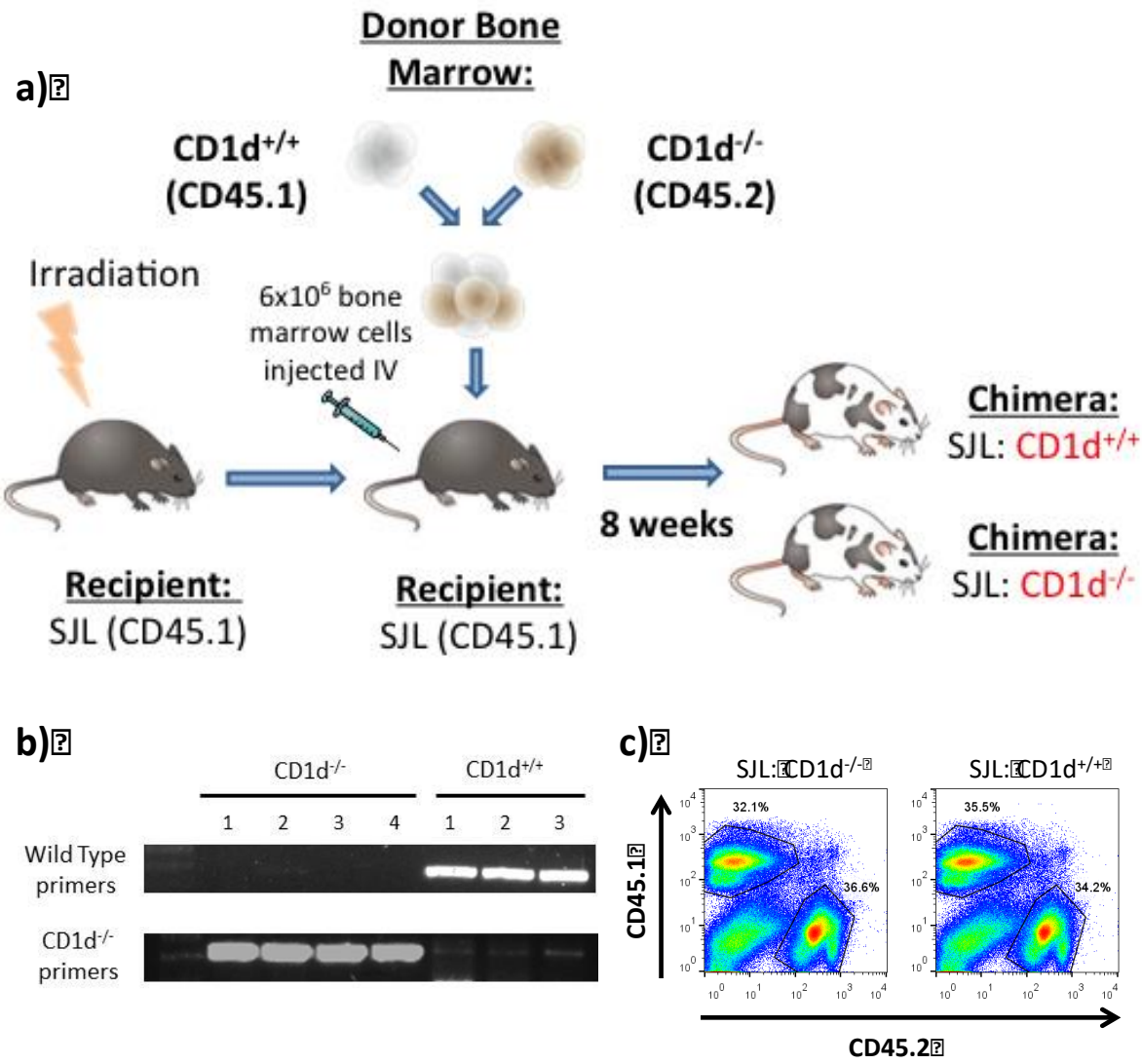


Figure 9. Schematic diagram illustrating generation of CD1d-chimeric mice.

(a) Female SJL (CD45.1) mice, aged between 8 and 10 weeks, were irradiated twice, 4 hours apart, with 450Rad. 4 hours later, 6x10^6 bone marrow cells were injected intravenously. Mice were monitored closely for deterioration of health, and at 8 weeks post irradiation, were bled for confirmation of cell engraftment. (b) Genotyping of donor wild-type and CD1d$^{-/-}$ confirms correct knockout- genotype. (c) Flow cytometry analysis of equal injection of CD45.1$^+$:CD45.2$^+$ bone marrow cells- populations are shown as a percentage of live cells.

Following the 8 week period to allow engraftment of donor bone marrow, and prior to influenza infection, both sample and control animals were bled and examined for a 50:50 reconstitution of $CD45.1^+$ and $CD45.2^+$ cells (**Figure 10**). Analysis of the peripheral blood demonstrated a clear bias of cell engraftment towards $CD1d^{-/-}$ cells, both myeloid and lymphoid, as compared with their $CD1d^{+/+}$ controls. On the contrary, $CD45.1^+$ $CD4^+$ T cells dominated in the periphery, and we observed no bias towards $CD45.2^+CD1d^{-/-}$ $CD4^+$ populations (**Figure 10b**).

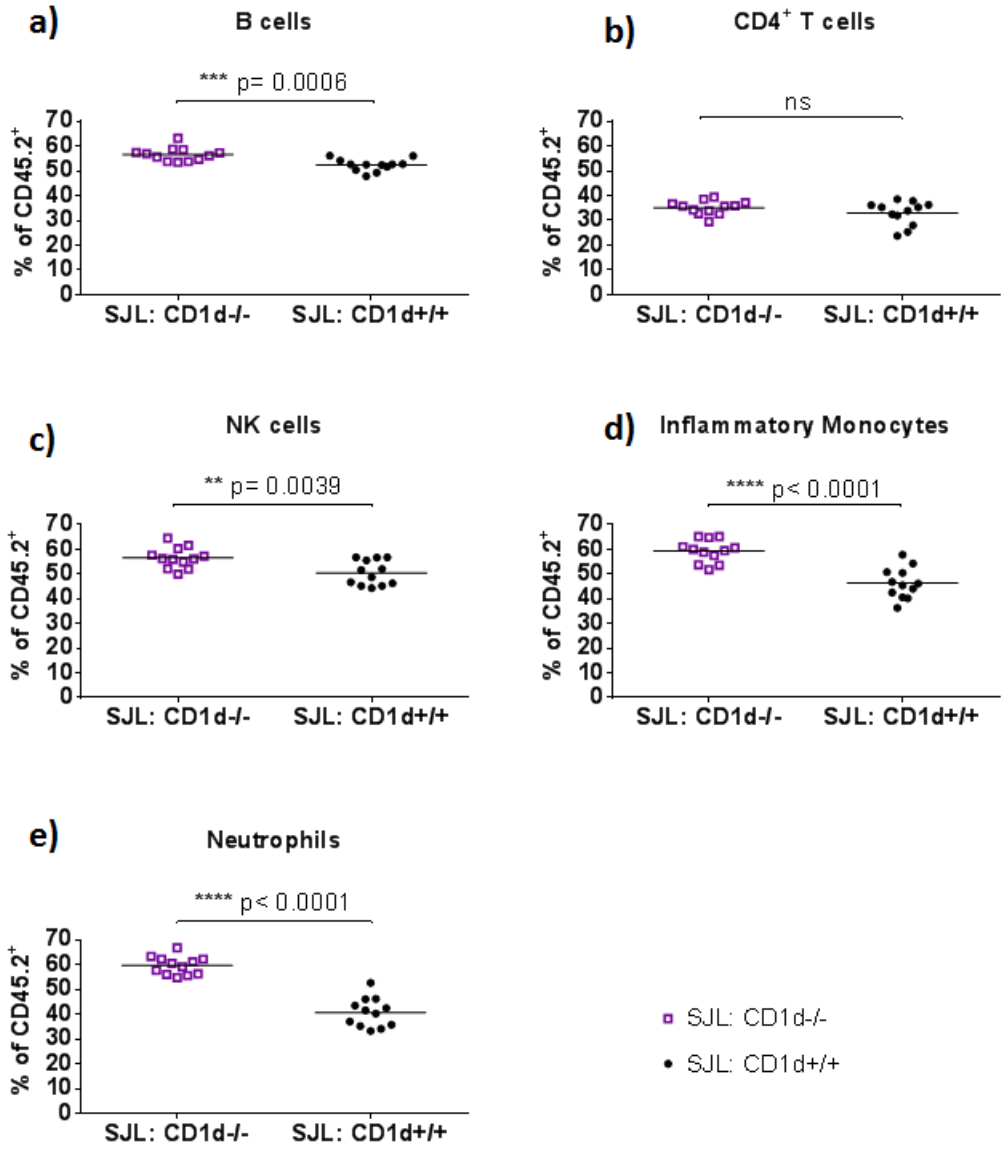


Figure 10. Greater engraftment of CD45.2^{+} cells in CD1d$^{-/-}$: SJL chimeric mice.

8 weeks post-irradiation and bone marrow transfer, mice were bled and assessed for reconstitution of haematopoietic cells. Graphs show percentages of CD45.2 cells (either CD1d$^{-/-}$ or CD1d$^{+/+}$) in the periphery of recipient SJL mice. Statistical difference was evaluated using the student's t test. Graphs are representative of two separate experimental repeats, with 12 and 7 (data not shown) mice per group.

3.7. CD1d-deficiency in chimeric mice does not influence IAV-induced weight-loss

The aim of this series of experiments was to assess how influenza infection could affect the expansion and functional status of myeloid cells, in the presence or the absence of the ability to cross-talk, in a CD1d-dependent manner, with iNKT cells. As such, by utilising a sub-lethal influenza-infection model, we assessed cell infiltration and lung pathology at day 7 following infection with $1x10^5$ PFU PR8. Analysis of weight loss, which represents an accurate indicator of disease progression, demonstrated a comparable weight loss with both SJL: $CD1d^{+/+}$ and SJL: $CD1d^{-/-}$ bone marrow chimeras (**Figure 11a**). This comparable effect on weight-loss was also seen in additional experiments using a higher dose ($1x10^6$) of virus (data not shown).

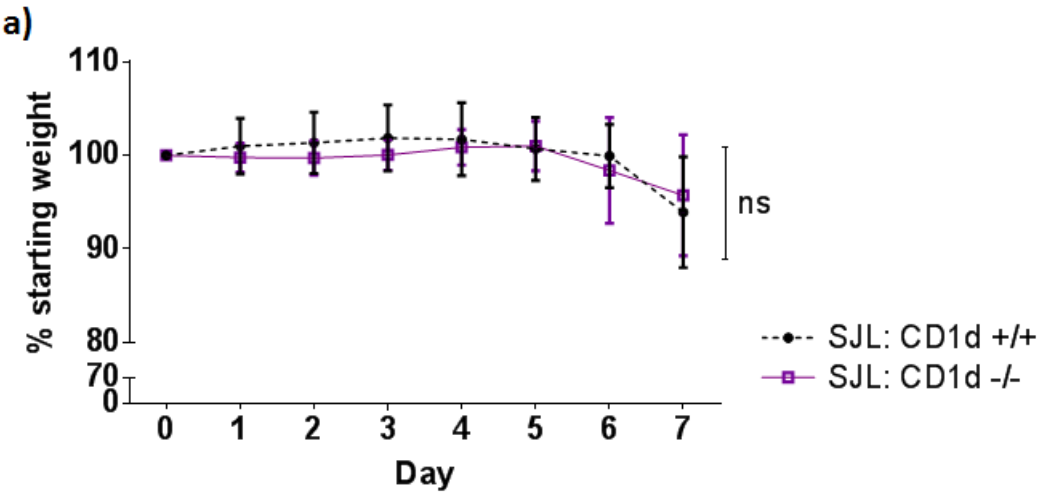


Figure 11. Comparable survival of SJL: CD1d$^{-/-}$ and SJL: CD1d$^{+/+}$ chimeric mice following PR8 infection.

Mice were weighed every day following intranasal infection with 50µl 1x10^5 PFU of PR8 virus. Values are given as a percentage of the starting weight. Error bars represent standard deviation.

3.8. CD1d-deficiency is dispensable for the migration of myeloid cells following influenza infection of chimeric mice

In light of the observation of the greater infiltration of inflammatory monocytes and neutrophils in *Jα18*$^{-/-}$ mice, we addressed whether CD1d-mediated interactions with iNKT cells were implicated in this accumulation. Preliminary analysis of the homogenised lungs demonstrated significant infiltration of both inflammatory monocytes (**Figure 12a**) and neutrophils (**Figure 12b**) following 7 day influenza infection; this confirmed successful induction of an immune response to influenza infection in our experimental model. Interestingly, we did not observe a significant difference in the recruitment of total cells (irrespective of CD45.1 or CD45.2 staining) between influenza infected SJL: CD1d$^{-/-}$ and SJL: CD1d$^{+/+}$ chimeric mice.

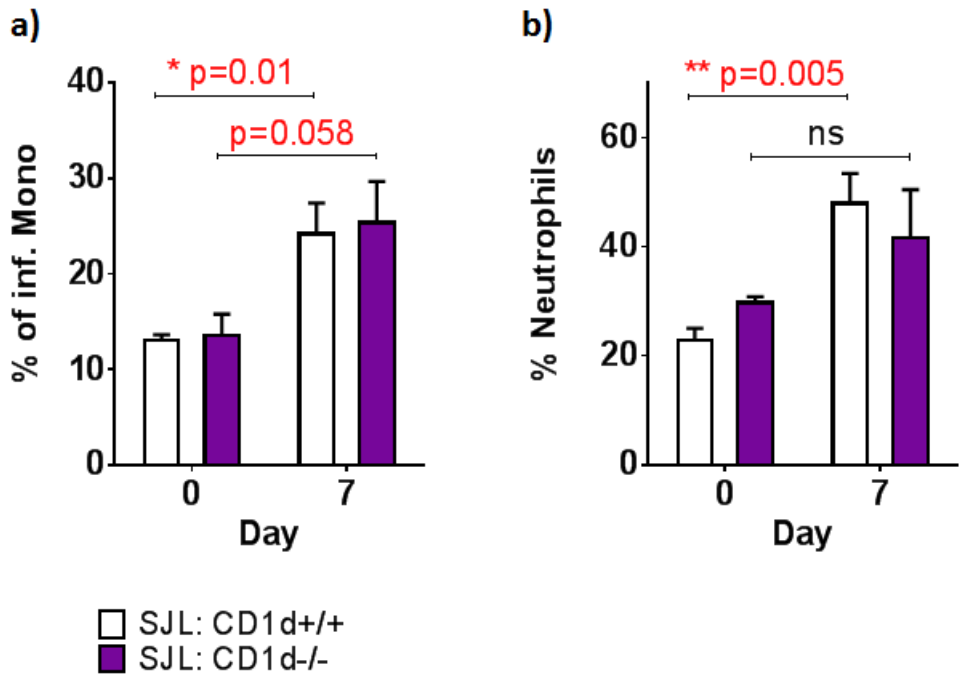


Figure 12. Expansion of inflammatory monocytes and neutrophils in the lungs of influenza-infected mice.

*Bar graph showing the expansion of (a) inflammatory monocytes (inf. Mono) and (b) neutrophils in the lungs of PR8 infected mice, at day 7 post infection. Statistical significance was analysed by two-tailed student's t-test; *p<0.05, **p<0.01, ***p<0.001. Data representative of two experimental repeats, with 3- 5 mice per group. Error bars represent standard deviation.*

To address for differential responses of $CD1d^{-/-}$ versus $CD1d^{+/+}$ cells during infection, we analysed contribution of $CD45.1^{+}$ and $CD45.2^{+}$ cells towards the expanded inflammatory monocyte and neutrophil populations in the lungs of influenza- infected mice (**Figure 13**). We observed two key findings: firstly, in line with the results in the periphery at 8 weeks following engraftment (**Figure 10**), in the naïve lungs we observe a greater baseline reconstitution of $CD1d^{-/-}$ cells, as compared with $CD1d^{+/+}$ cells. Secondly, although we see

an infiltration of both inflammatory monocytes and neutrophils following 7 day influenza infection (**Figure 13**), and we continue to see the bias towards expansion of $CD1d^{-}$ cells, there is no change in the ratio between $CD1d^{-/-}$ and $CD1d^{+/+}$ cells at day 7, as compared with the ratio observed at day 0. These data suggest that direct interactions with iNKT cells via CD1d are redundant in the recruitment of inflammatory monocytes and neutrophils to the lung following infection, and alternative functions of iNKT cells, such as cytokine or chemokine release may be accountable for the differential recruitment of innate cells which we observed in IAV-infected *Jα18*$^{-/-}$ mice.

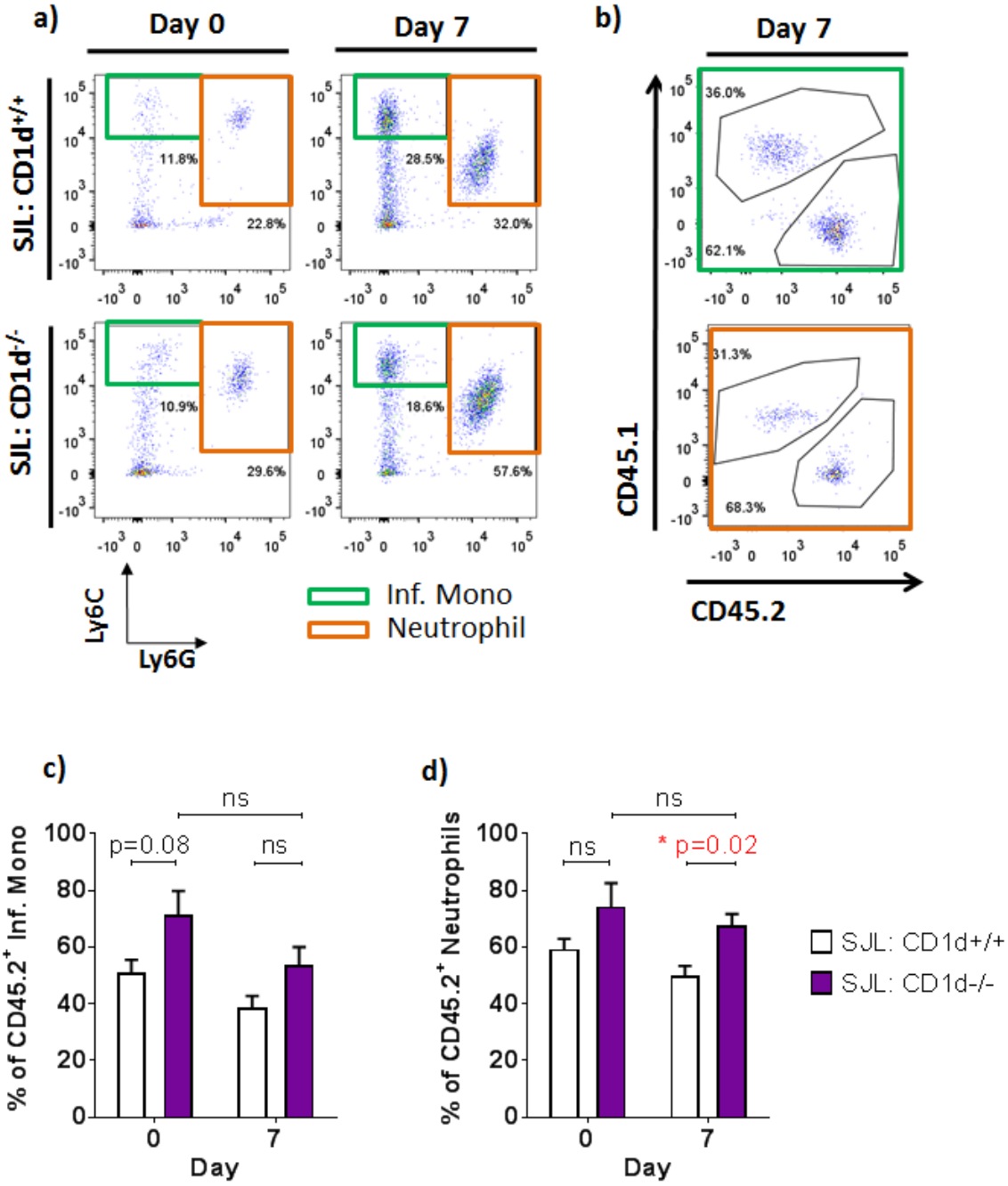


Figure 13. Comparison of the expansion of CD1d$^{-/+}$ compared with CD1d$^{+/+}$ inflammatory monocytes and neutrophils in the lungs of PR8-infected mice.

(a) FACS analysis showing the expansion of inflammatory monocytes (inf. Mono) (green) and neutrophils (orange) in PR8-infected mice. (b) Example plot (taken from Day 7 of SJL: CD1d$^{-/-}$ chimeras) of the relative expression of CD45.1 (SJL CD1d$^{+/+}$ derived) versus CD45.2

(B6 CD1d$^{-/-}$ derived) cells within the inf. Mono (top) and neutrophil (bottom) populations. (c) Graphs summarising the relative expansion of the CD45.1^{+} and CD45.2^{+} (either CD1d^{-} or CD1d^{+} as indicated) within the lungs of PR8-infected mice. Error bars represent standard deviation.

To our knowledge, no previous studies have published a CD1d-chimeric mouse model comparable to our model to study CD1d-mediated iNKT cell interactions in influenza infection; it was important, therefore, to assess localisation of myeloid cell populations in other compartments following influenza infection to compare local and systemic effects of CD1d-mediated signalling. In view of the previous observations of elevated engraftment of CD1d$^{-/-}$ cells in the blood **(Figure 10)**, and the known localisation of iNKT cells in the bone marrow and spleen we analysed peripheral myeloid cells in the spleen and the bone marrow during influenza infection **(Figure 14)**.

Firstly, analysis of total cellular infiltration in the spleen demonstrated no changes in total inflammatory monocyte or neutrophil infiltration in naïve or infected SJL: CD1d$^{-/-}$ or SJL: CD1d$^{+/+}$ chimeric mice **(Appendix 1c, d).** On the other hand, in the bone marrow at day 7 post-PR8 infection, we did observe greater number of both inflammatory monocytes and neutrophils in influenza infected SJL: CD1d$^{-/-}$ chimeras, as compared with PR8-infected SJL: CD1d$^{+/+}$ chimeras **(Appendix 1a, b)**. As with the lung, we progressed to assess the contribution of CD1d$^{-/-}$ and CD1d$^{+/+}$ cells, by analysing the CD45.2^{+} cells in either the SJL: CD1d$^{-/-}$ or the SJL: CD1d$^{+/+}$ chimeras, respectively. In the naïve spleen **(Figure 14c, d)**, we observed the same bias, although not quite significant, towards CD1d$^{-/-}$ cells, as observed in the peripheral blood **(Figure 10)** and the lungs **(Figure 13)**. Notably, as observed in all other organs, following 7 day PR8 infection, CD1d$^{-/-}$ inflammatory monocytes and neutrophils showed a significant expansion, as compared to their CD1d$^{+/+}$ counterparts. Intriguingly, in the bone marrow under naïve conditions, we do not observe the same bias towards CD1d$^{-/-}$

cells as observed in the periphery **(Figure 14a, b)**. Following 7 days infection, however, we observe a significantly greater proportion of $CD1d^{-/-}$ cells, as compared with $CD1d^{+/+}$ cells. This data is somewhat perplexing, however, as although at day 7 post-infection the difference in the contribution of $CD1d^{-/-}$ and $CD1d^{+/+}$ inflammatory monocytes and neutrophils becomes significant, this is not always found to be significantly greater than the bias towards $CD1d^{-/-}$ cells observed at day 0. It remains to be determined whether this minimal, yet statistically significant difference in the bias towards $CD1d^{-/-}$ cells, as compared with $CD1d^{+/+}$ cells found in the bone marrow, spleen, and for $CD1d^{-/-}$ neutrophils in the lung, can significantly effect the overall contribution of these cells during PR8 infection.

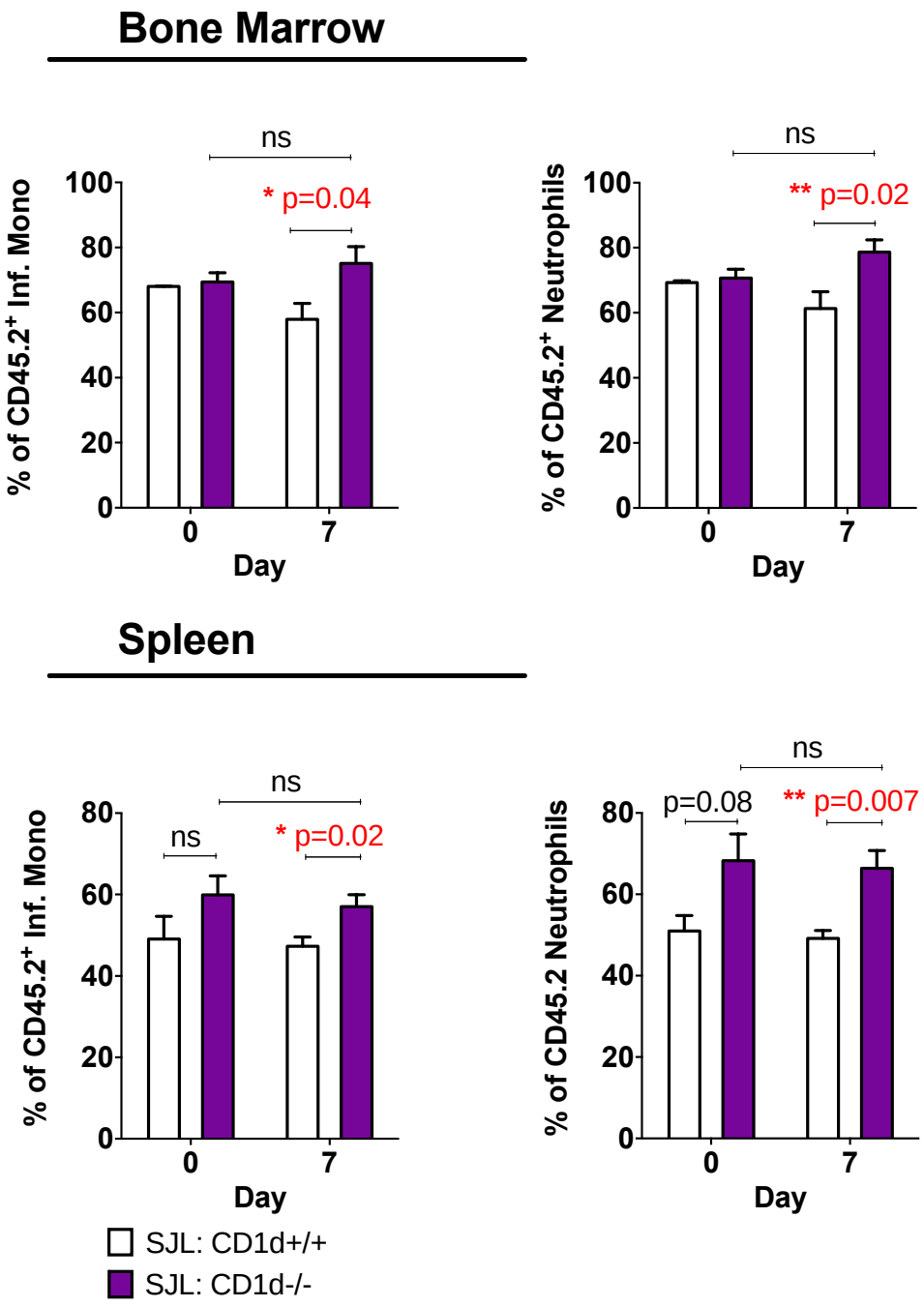


Figure 14. Comparison of the expansion of CD1d$^{+/+}$ compared with CD1d$^{-/-}$ inflammatory monocytes and neutrophils in the bone marrow and spleen of PR8-infected mice.

Graphs summarising the relative expansion of the CD45.1^{+} and CD45.2^{+} (either CD1d^{-} or CD1d^{+} as indicated) within the bone marrow (a) (b) or spleen (c) (d) of PR8-infected mice. Statistics were performed using the student's t test. Error bars represent standard deviation.

3.9. Cell sorting and quality control of microarray samples

Although further investigation is required, FACs analysis of cellular infiltration has indicated that a CD1d-dependent interaction may not be the predominant mechanism regulating the infiltration of cells into the lungs following influenza. In the absence of iNKT cells, influenza infection **(Figure 6)**, and additionally systemic SAA injections [330] mice have shown to induce the expansion of myeloid cells with an overactive, suppressive phenotype, which can be unfavourable during disease resolution [41, 127, 330]. In light of this functional plasticity of myeloid cells observed in the absence of iNKT cells, we aimed to address how CD1d-interaction with iNKT cells could mould the functional capacity of these myeloid cells; we therefore sorted $CD1d^{-/-}$ or $CD1d^{+/+}$ inflammatory monocytes and neutrophils from the lungs of PR8-infected SJL: $CD1d^{-/-}$ or the SJL: $CD1d^{+/+}$ chimeric mice, respectively, to carry gene expression profiling. Genetic profiling of these cells would provide further evidence of whether the responsive capacity of these cells differed in response to influenza in the presence or absence of direct interactions with iNKT cells via CD1d-signalling.

In order to sort pure populations from the lungs, we firstly gated on live cells, and excluded NK, $CD3^+$ T lymphocytes and B lymphocytes prior to gating on inflammatory monocytes (**Figure 15a**: gate P5) ($Ly6C^{high}$ $Ly6G^-$) and neutrophils **(Figure 15a**: gate P6) ($Ly6C^{int}$ $Ly6G^{high}$). Each population was then split and sorted into $CD45.1^+$ and $CD45.2^+$ cells, thus allowing discrimination between the wild-type ($CD1d^{+/+}$) and $CD1d^{-/-}$ cell populations **(Figure 15b)**. The overall gating hierarchy is shown in **Figure 15c**.

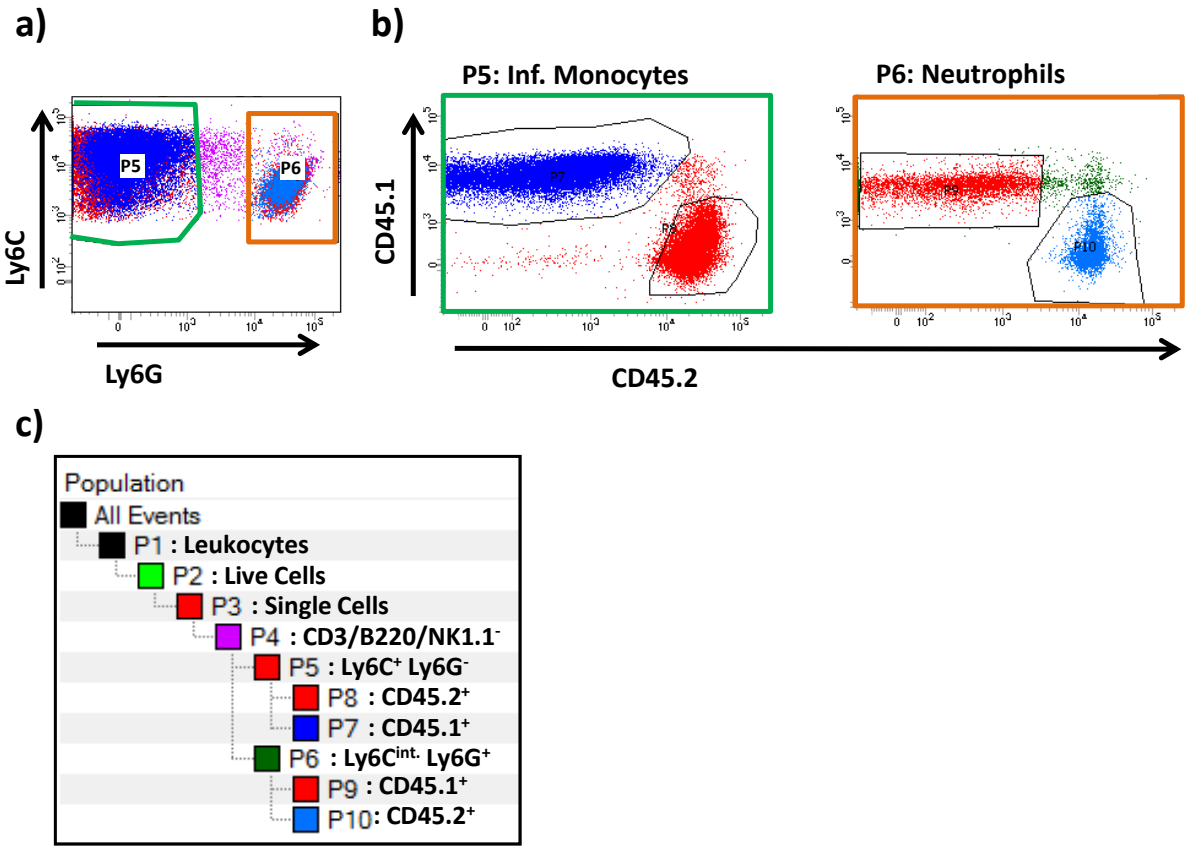


Figure 15. The gating strategy used for cell sorting of lung monocytes and neutrophils.

Cells were isolated from the lung using collagenase digestion, stained, and sorted using fluorescence activated cell sorting (FACS). To achieve the final populations shown (a)(b), cells were gated on live cells (aqua$^-$ cells), singlet's (based on FSC-W vs FSC-H) and finally, Ly6C and Ly6G populations are shown as percent of CD3/B220/NK1.1$^-$ cells (c). Gates were set using FMO controls.

Prior to running the sorted samples on the Affymetrix microarray, it was essential to ensure that the quantity and quality of RNA was suitable for the microarray preparation steps. RNA quality as assessed through the “RNA integrity number” (RIN) of the RNA samples. The RIN values of all samples showed high and comparable integrity (RIN>7), indicating that all

samples were suitable for hybridisation (**Appendix** 2). Data was then pre-processed by Stephen Taylor of the computational Biology Research Group (CRBG), and statistical analysis assistance was given by Emmanouela Repapi, also of the CRBG. The Robust Multi-array Average (RMA) algorithm was used to process and normalise raw array data **(Methods, 2.22d)**. Plotting the normalised log2 intensities across all sample chips indicated high quality data and consistent profiles across our sample set **(Appendix 3.)**

We next ran hierarchical clustering and principle component analysis. Hierarchical clustering (HCL) uses standard statistical algorithms to arrange genes according to similarity in pattern of gene expression, thus genes with similar expression patterns are grouped together and are connected by a series of branches (dendrogram) [333]. HCL of our data shows segregation of samples into four main clusters, infected and naïve inflammatory monocytes, and infected and naïve neutrophils (**Figure 16a**). Within the four cellular clusters, there is evidence of the $CD1d^{+/+}$ and $CD1d^{-/-}$ cells clustering together; this segregation is observed most clearly in PR8- infected monocytes (**Figure 16**- labelled in red).

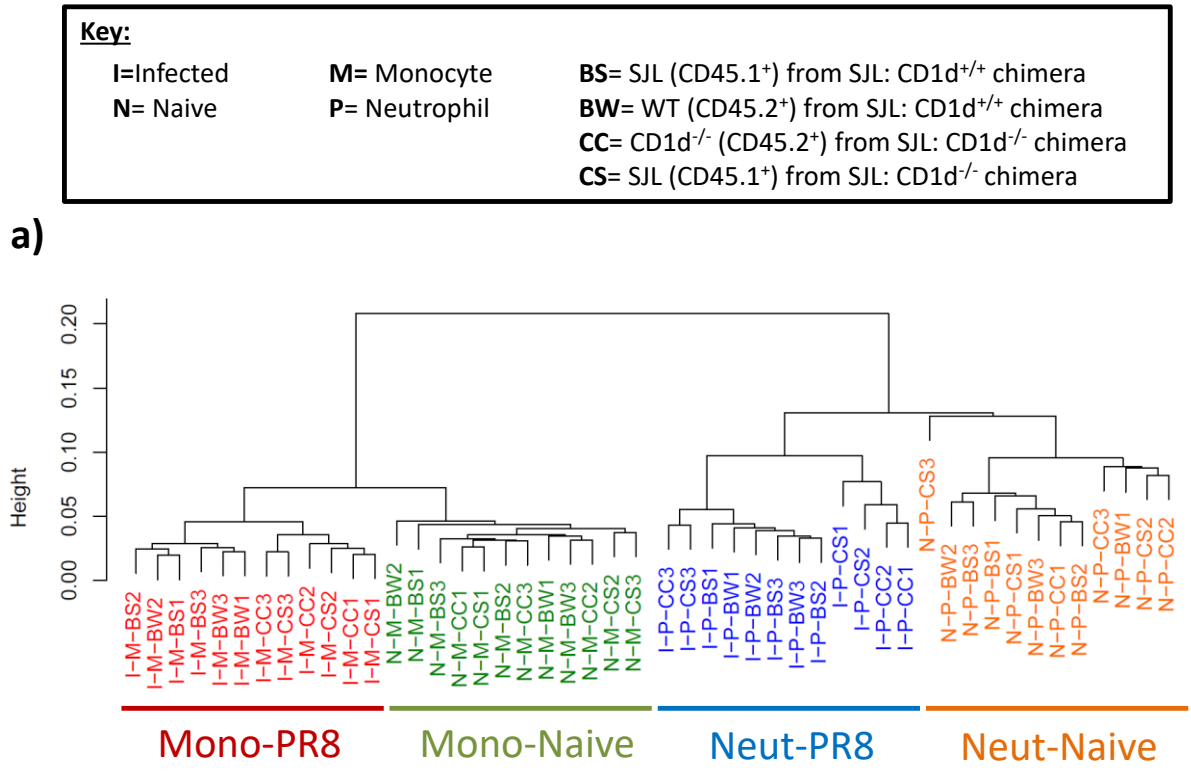


Figure 16. Expected segregation of naïve and infected samples using hierarchical clustering.

Hierarchical clustering (HCL) was used to identify any outliers in the sample groups, and to assess factors varying between samples. (a) HCL demonstrates clear segregation of inflammatory monocyte and neutrophil populations in naïve and infected conditions.

Principal Component Analysis (PCA) provides an alternative method to visualise the correlations of expression between arrays; data are projected on several axes (or components) and are ordered by decreasing significance. As such, strong correlations of expression signals between samples are depicted by tighter clusters of genes on a PCA plot. We firstly ran PCA on total inflammatory monocytes and neutrophils, in the naïve and infected state, irrespective of their genotype **(Figure 17a)**. Samples show a clear dichotomy between monocytes and neutrophils, and into infected and naïve treatment groups, consistent with the previous HCL data **(Figure 16a)**. Interestingly, neutrophils exhibit a

greater diversity in their clustering, as compared with monocytes, in both naïve and infected cells **(Figure 17a-blue lines)**. Since these general PCA plots do not account for cellular genotypes (i.e. the CD1d proficiency of the cells), we generated further PCA plots for inflammatory monocytes **(Figure 17b)**, and neutrophils **(Figure 17c)** separately, to decipher whether clustering between CD45.1^{+} cells, and either wild-type (CD1d$^{+/+}$) or CD1d$^{-/-}$ CD45.2^{+} cells from each chimera could account for the spread of variance. Firstly, it is important to note that CD1d$^{-/-}$ and CD1d$^{+/+}$ CD45.2^{+} cells clustered comparably, but distinctly from their SJL-CD45.1^{+} controls, which are indicated in with green and orange, respectively.

We observed tight clustering for the inflammatory monocytes isolated from naïve chimeras, however there was greater variability in the inflammatory monocytes isolated from infected chimeras, which form a distinct cluster to the naïve cells **(Figure 17b)**. In naïve inflammatory monocytes, CD1d$^{-/-}$ and CD1d$^{+/+}$ cells cluster similarly, however, we found greater variance in the infected inflammatory monocytes, with CD1d$^{-/-}$ cells (blue triangles) clustering separately to the CD1d$^{+/+}$ cells (red triangles). The observed clustering patterns in inflammatory monocytes **(Figure 17c)**, suggests that there is differential gene expression patterns following infection, as expected, and additionally that there could be differences between the wild-type (CD1d$^{+/+}$) and CD1d$^{-/-}$ populations following PR8 infection. PCA of neutrophils demonstrated tight clustering within the naïve groups, and similarly to inflammatory monocytes, a more diverse variance following infection **(Figure 17c)**. Neutrophils isolated from infected chimeras have clustered into two groups, which predominantly, with the exception of two samples, contain cells from either the SJL: CD1d$^{-/-}$ chimera, or the SJL: CD1d$^{+/+}$ chimera **(Figure 17c)**. We observed one sample "N-Neut- (SJL: CD1d$^{-/-}$) SJL CD1d$^{+/+}$" (green square), which did not cluster with the other CD1d$^{-/-}$ neutrophils from the SJL: CD1d$^{-/-}$ chimera; the reason for this is currently unclear, and the impact of this sample was monitored during later analysis. Taken together these data show that overall the groupings fall into the expected clusters, and confer there is no unexpected bias across the samples, providing technical reassurance that the data can be further analysed.

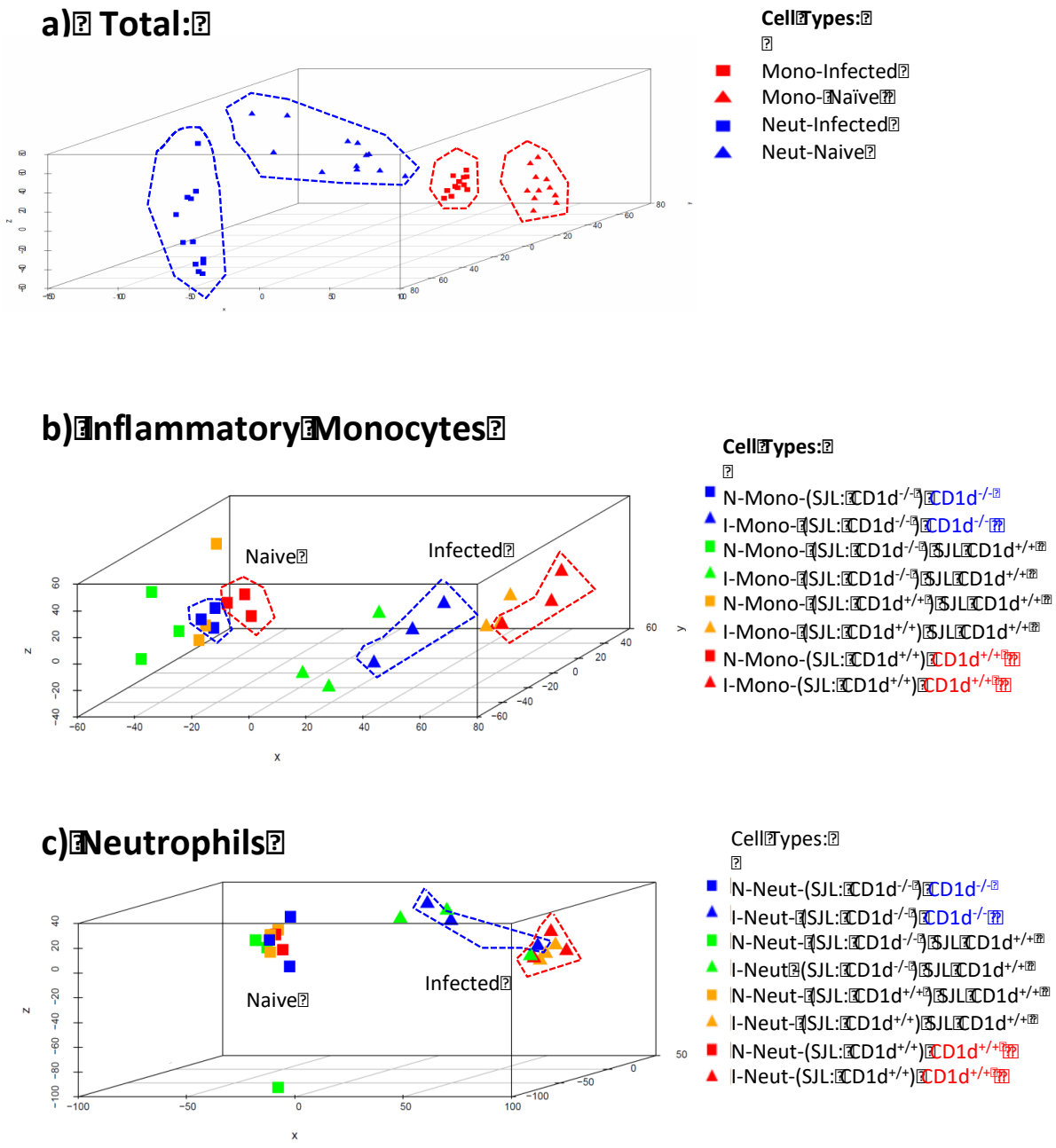


Figure 17. Expected segregation of naïve and infected samples using principle component analysis.

Principle component analysis (PCA) was used to identify any outliers in the sample groups, and to assess factors varying between samples. (a) PCA analysis shows clear clustering of naïve and infected cells in both neutrophils and inflammatory monocytes, with infected cells demonstrating a greater degree of variability, as compared with naïve cells. Subsequent

analysis was carried out for inflammatory monocytes (b) and neutrophils (c) with regards to their genotype. HCL and PCA plots were generated by Steven Taylor (CRBG).Abbreviations: N, naïve; I, infected; Mono, inflammatory monocyte; Neut, neutrophil.

3.10. Investigating the effect of CD1d-interactions on the gene expression profiles of inflammatory monocytes or neutrophils following IAV infection

To further assess the contribution of CD1d-signalling to gene profiling of myeloid cells, the fold changes in gene expression, and significance of the resulting upregulation or down regulation in $CD1d^{-/-}$ inflammatory monocytes and neutrophils, as compared with their $CD1d^{+/+}$ counterparts, was performed in the software 'R', using the package Limma [305] **(Methods, 2.22d)**.

3.10.a. The role of CD1d expression on neutrophil gene expression profiles

Following statistical analysis, which identified genes with a p value < 0.05, and a Log2 fold change (Log2FC) greater than 1.5, a total of 131 genes of known function were significantly upregulated, and a total of 70 genes of known function were downregulated. The top 30 differentially expressed genes, and the top 10 down-regulated genes in $CD1d^{-/-}$ neutrophils, as compared with $CD1d^{+/+}$ cells are shown in

Table 2. Interestingly, despite the bias towards $CD1d^{-/-}$ neutrophils in the periphery and lungs or naïve mice following engraftment, as shown by FACS (**Figure 10d**, **e**), we observed no significantly upregulated genes in $CD1d^{-/-}$ neutrophils isolated from the lungs of naïve mice, and only 5 altered genes in monocytes (discussed later in **Figure 20**). Given the lack of significantly altered genes observed in naïve mice, and our interest in the PR8-mediated

cellular response, we focus our analysis and discussion on the genes modulated in either population following 7 days of influenza infection.

Gene Assignment	Log2FC	Adjusted P Value	Gene description
Edn1	4.05	0.033	Endothelin 1
Saa3	4.01	0.042	Serum Amyloid 3
Klhl6	3.41	0.028	Kelch-Like Family Member 6
Atp6v0d2	3.28	0.047	Atpase, H^+ Transporting, Lysosomal 38kda, V0 Subunit D2
Bcl2l15	2.91	0.031	BCL2-Like 15
Entpd3	2.88	0.033	Ectonucleoside Triphosphate Diphosphohydrolase 3
Lipg	2.85	0.035	Endothelial Lipase
Ifnb1	2.83	0.036	Interferon Beta
Rab15	2.82	0.042	Ras-Related Protein Rab-15
Prnp	2.67	0.047	Prion Protein
Fmnl2	2.61	0.041	Formin-Like 2
Il12a	2.60	0.042	Interleukin 12A
Lyrm4	2.58	0.046	LYR Motif Containing 4
Msh3	2.54	0.033	Muts Homolog 3
Tm4sf19	2.52	0.047	Transmembrane 4 L Six Family Member 19
Sdhaf1	2.51	0.044	Succinate Dehydrogenase Complex Assembly Factor 1
Chi3l3	2.47	0.043	Mouse Chitinase 3-Like 3
Rps6ka2	2.40	0.036	Ribosomal Protein S6 Kinase
Dpep2	2.37	0.034	Dipeptidase 2
Syne1	2.34	0.038	Spectrin Repeat Containing, Nuclear Envelope 1
Mt1	2.32	0.033	Melatonin Receptor 1A
Crispld2	2.32	0.040	Cysteine-Rich Secretory Protein LCCL Domain Containing 2
Kcnma1	2.31	0.049	Potassium Large Conductance Calcium-Activated Channel, Subfamily M, Alpha Member 1

Mrpl52	2.30	0.040	Mitochondrial Ribosomal Protein L52
Dhfr	2.28	0.046	Dihydrofolate Reductase
Inhba	2.27	0.033	Inhibin, Beta A
Hilpda	2.24	0.033	Hypoxia Inducible Lipid Droplet-Associated
Ptpn22	2.24	0.038	Protein Tyrosine Phosphatase, Non-Receptor Type 22
Cldn1	2.19	0.035	Claudin 1
Dock10	2.17	0.040	Dedicator of Cytokinesis 10
Cxcl3	2.17	0.041	Chemokine (C-X-C Motif) Ligand 3
Gzmb	-1.62	0.044	Granzyme B
Hist1h2ak	-1.70	0.115	Histone Cluster 1, H2ak
Map4k2	-1.82	0.021	Mitogen-Activated Protein Kinase Kinase Kinase Kinase 2
Ptplad2	-1.84	0.028	Protein Tyrosine Phosphatase-Like A Domain Containing 2
Cd300lb	-1.86	0.022	CD300 Molecule-Like Family Member B
Tmem123	-1.89	0.022	Transmembrane Protein 123
Atp1a3	-1.90	0.019	Atpase, Na^+/K^+ Transporting, Alpha 3 Polypeptide
Emr4	-1.97	0.052	Epidermal Growth Factor (EGF)-TM7
Kif23	-2.13	0.021	Kinesin Family Member 23
Dck	-2.14	0.019	Deoxycytidine Kinase

Table 2. Top 30 upregulated, and top 10 downregulated genes observed in CD1d$^{-/-}$ neutrophils isolated from the lungs of PR8 challenged mice

Table showing the top 30 upregulated, and top 10 downregulated genes observed in CD1d$^{-/-}$ neutrophils isolated from the lungs of PR8-challenged mice, as compared with CD1d$^{+/+}$ neutrophils. Values are adjusted to p<0.05 and includes both up and down signalling events. For the complete list of significantly differentiated genes see ***(Appendix 4).***

Initial observations of the top upregulated genes highlight that CD1d signalling can regulate the pro-inflammatory response, with a number of pro-inflammatory factors, such as SAA (Log2FC $^{+}$4.01), IFN-β (Log2FC $^{+}$2.83), IL-12α (Log2FC $^{+}$2.60) and chemotaxis associated factors, such as CXCL3 ((GRO-3) Log2FC $^{+}$2.17) and endothelin-1 (Log2FC $^{+}$4.05).

It will be crucial to validate all of the identified genes in CD1d$^{-/-}$ neutrophils by qPCR or protein expression analysis in our CD1d-chimera model. Interestingly, although we have not yet confirmed assessment of serum amyloid A (SAA) message levels by alternative assays in the chimera model, qPCR analysis of whole lungs removed from *Jα18*$^{-/-}$ or C57BL/6 mice, following 5 day PR8 infection, showed elevated local message levels of SAA3 **(Figure 18a)**, but not SAA1 **(Figure 18b)**, in the absence of iNKT cells.

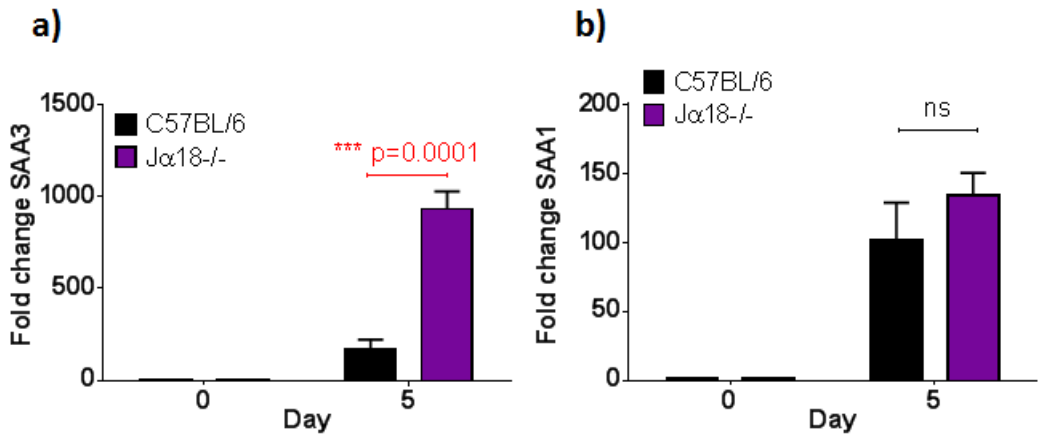


Figure 18. iNKT cells modulate the gene expression of SAA3 in the lungs of PR8 - infected mice.

Whole lungs of PR8 infected mice, following 5 days infection were analysed for ***(a)*** *SAA3 and* ***(b)*** *SAA1 gene expression using qPCR. Fold changes are shown relative to expression at day 0. Graph is representative of two experimental repeats with n=5 mice per group. Statistical analysis was performed using a student's t test.*

We have additionally observed increased SAA release into the BALF of IAV-infected *Jα18*$^{-/-}$ mice **(Figure 19)**; this SAA ELISA did not specify the cross-reactivity of the antibody, and thus, given the homology between the different SAA isoforms [334], further characterisation is required to ascertain the specific form of SAA (i.e. SAA1/2 or SAA3) present in the lung following influenza infection. At present the reported message fold changes, and protein release can unfortunately not be attributed to a particular cell population in these experiments, however taken together these results strengthen the hypothesis for a regulatory role for iNKT cells in modulating SAA3 production locally in the lungs during influenza infection.

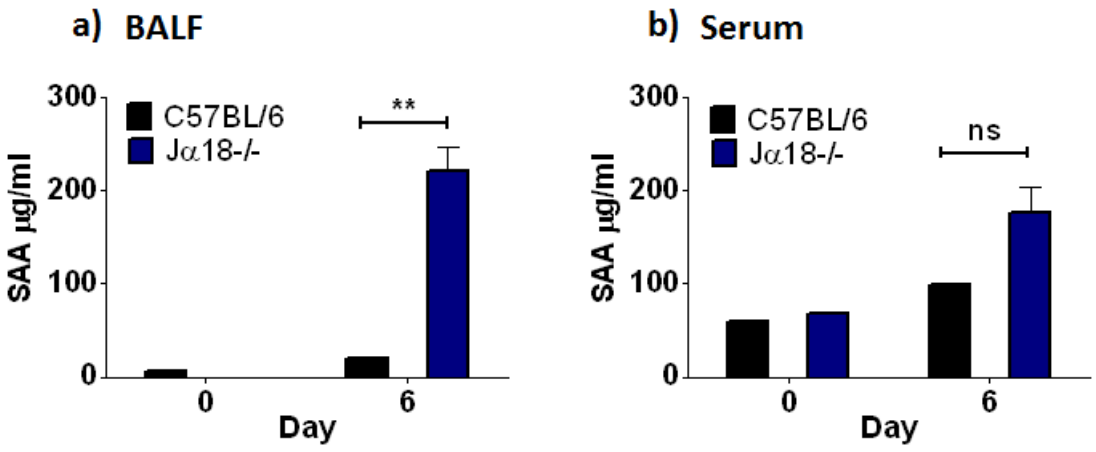


Figure 19. Increased SAA release during PR8 infection in *Jα18*$^{-/-}$ mice.

*Serum and BALF of naïve or PR8-infected C57BL/6 mice were analysed for elevated and SAA (c), by ELISA. Data are shown for two individual experimental replicates, with 3 mice per group. Error bars indicate SEM. Statistical significance was analysed by two-tailed student's t-test for ELISA data, **p<0.01.*

3.10.b. CD1d-expression in neutrophils modulates genes which are involved in multiple signalling pathways

As shown in **(Table 2)**, a broad range of genes are differentially regulated in CD1d$^{-/-}$ neutrophils isolated from PR8 infected mice. One of the key observations upon which we based our study, is the enhanced recruitment and accumulation of myeloid cells in influenza-infected lungs, in the absence of iNKT cells [41, 127, 335, 336]. The precise mechanisms responsible for this accumulation and altered immune pathology are currently unclear, however, as we have discussed previously, this could be attributed to a number of cellular functions including increased ability for migration, extravasation, survival, and in pro-inflammatory or anti-inflammatory signalling which modulates the recruitment of further innate and adaptive immune cells.

To determine how the genes, which we have identified to be modulated by CD1d-signalling (**Table 2**) could be implicated in such immune responses, we processed the data further using MetaCore. MetaCore utilises a number of pre-built networks based on processes, disease biomarkers, and metabolic pathways which have been reported in the literature. This analysis allowed for the identification of biological pathways and networks which could be differently represented in the neutrophil and inflammatory monocyte datasets. The top ten pathway maps, corresponding to genes which were significantly modulated in $CD1d^{-/-}$ neutrophils, as compared with $CD1d^{+/+}$ neutrophils, are shown in (**Table 3**).

	Pathway Maps	p-value	FDR	Ratio	Network Objects
1	Development:TGF-beta-dependent induction of EMT via MAPK	5.00E-06	1.44E-03	5/47	Claudin-1, ITGB3, Endothelin-1, p38 MAPK, p38alpha (MAPK14)
2	Immune response: HSP60 and HSP70/ TLR signalling pathway	2.00E-04	1.78E-02	4/54	iNOS, IL-12 alpha, p38 MAPK, CD69
3	Expression targets of Tissue factor signalling in cancer	2.17E-04	1.78E-02	3/22	GRO-1, Coagulation factor VII, Coagulation factor X
4	Immune response: IL-15 signalling via JAK-STAT cascade	2.48E-04	1.78E-02	3/23	Bcl-XL, sIL-15RA, IL-15RA
5	Immune response: IL-15 signalling	3.87E-04	2.22E-02	4/64	Bcl-XL, sIL-15RA, IL-15RA, p38 MAPK
6	Development: TGF-beta-dependent induction of EMT via SMADs	8.76E-04	4.19E-02	3/35	Claudin-1, Endothelin-1, ID2
7	Development: Flt3 signalling	1.71E-03	5.30E-02	3/44	Bcl-XL, p90Rsk, p38 MAPK
8	Immune response: IL-1 signalling pathway	1.71E-03	5.30E-02	3/44	Endothelin-1, iNOS, p38alpha (MAPK14)
9	Signal transduction: PTMs in IL-17-induced CIKS-independent signalling pathways	1.95E-03	5.30E-02	3/46	p90Rsk, p38 MAPK, p38alpha (MAPK14)
10	Muscle contraction: Relaxin signalling pathway	2.20E-03	5.30E-02	3/48	Endothelin-1, p90Rsk, iNOS

Table 3. Top ten differentially expressed pathways in CD1d$^{-/-}$ neutrophils isolated from the lungs of PR8-challenged mice.

The top pathways altered in the absence of CD1d signalling were generated using MetaCore analysis. Thresholds were set to a Log2FC greater than +/-1.0, with a p value <0.05, and include both up and down signalling events. The 'ratio' demonstrates how many objects out

of the suggested pathway components are significantly increased or decreased. FDR – 'False discovery rate' indicates the proportion of false discoveries among the observed pathways

MetaCore pathway analysis as utilised in **Table 3** is extremely valuable at highlighting single sequential links between genes, and specific signalling pathways which could be modulated by alteration in specific combinations of genes. Importantly, the inflammatory responses during influenza infection comprises a complex network of signalling, with any one gene acting on a number of signalling pathways in parallel; the implication of such pathway-relationships is not addressed in pathway mapping alone. To evaluate the broader implications of the gene expression patterns we have observed, we utilised a more complete analysis method of gene interactions using MetaCore 'process networks' **(Table 4)**.

	Networks	p-value	FDR	Ratio	Network Objects
1	Reproduction: FSH-beta signaling pathway	0.001	0.034	6/160	Activin beta A, IBP, p90RSK3(RPS6KA2), p90Rsk, p38 MAPK, p38alpha (MAPK14)
2	Development:EMT_Regulation of epithelial-to-mesenchymal transition	0.001	0.034	7/225	Claudin-1, ITGB3, Endothelin-1, p90Rsk, p38 MAPK, p38alpha (MAPK14), ID2
3	Inflammation: IL-10 anti-inflammatory response	0.002	0.076	4/87	Bcl-XL, IL-12 alpha, p38 MAPK, p38alpha (MAPK14)
4	Inflammation: Neutrophil activation	0.003	0.076	6/215	GRO-1, GRO-3, RalGDS, iNOS, p38 MAPK, p38alpha (MAPK14)
5	Immune response_Th17-derived cytokines	0.004	0.086	4/98	GRO-1, Bcl-XL, iNOS, p38 MAPK
6	Inflammation: IFN-gamma signalling	0.006	0.100	4/110	IFN-beta, Bcl-XL, iNOS, p38 MAPK
7	Proliferation: Negative regulation of cell proliferation	0.007	0.100	5/184	GPNMB (Osteoactivin), GRO-1, IFN-beta, IBP, IBP6
8	Inflammation: Kallikrein-kinin system	0.007	0.100	5/185	Coagulation factor VII, Coagulation factor X, iNOS, p38 MAPK, p38alpha (MAPK14)
9	Chemotaxis	0.012	0.154	4/137	GRO-1, GRO-3, p38 MAPK, p38alpha (MAPK14)
10	Muscle contraction: Relaxin signalling	0.017	0.188	3/83	Endothelin-1, p90Rsk, iNOS

Table 4. Top ten differentially expressed process networks in CD1d$^{-/-}$ neutrophils isolated from the lungs of PR8-challenged mice.

The top process networks modulated by CD1d-deficiency were generated using MetaCore analysis. Thresholds were set to a Log2FC greater than +/-1.0, with a p value <0.05, and include both up and down signalling events. The 'ratio' demonstrates how many objects out of the suggested pathway components are significantly increased or decreased. FDR – 'False discovery rate' indicates the proportion of false discoveries among the observed pathways.

Interestingly, analysis of pathway components, and the contribution of individually differentially expressed genes suggest that CD1d signalling influences a broad spectrum of

gene expression in neutrophils, which exhibit overlapping functions in a number of pathways relevant to influenza-mediated pathology and disease resolution. Mapping differentiated genes into specific pathways and process networks highlighted a number of common themes; pro-inflammatory signalling, such as IL-15 signalling pathways, neutrophil activation and chemotaxis appear as highly differentiated processes, as does cell proliferation and apoptotic regulation. Identification of such pathways is especially interesting given the prior findings of increased neutrophil infiltration and lung pathology in the absence of iNKT cells (in *Jα18*$^{-/-}$ mice) (**Figure 6**), and the altered cytokine and chemokine release into the lungs of these mice (**Figure 7**). It should be noted that although many of the networks shown in **Table 4** show statistical significance, there is a higher probability for false discovery rate (FDR) in these pathways. This is likely to stem from the small proportion of significantly altered factors as a ratio of the total network objects when analysed at these stringent thresholds. It would thus be important to validate the expression and impact of each factor on these pathways in the future; however, in light of the common genes and immune themes are identified via both mapping techniques, it is probable that many of these signalling pathways will be relevant.

To further illustrate the multifarious activity of CD1d-dependent genes, **Table 5** denotes the top genes which we identified to be significantly upregulated, with a high log2FC, and which were found to map within multiple signalling pathways (**Table 3**) and processes (**Table 4**) in CD1d$^{-/-}$ neutrophils.

	Common Network Objects	Log2FC
1	Endothelin-1	+4.05
2	IFN-beta	+2.82
3	IL-12 alpha	+2.59
4	GRO-1 (CXCL1)	+2.17
5	GRO-3 (CXCL3)	+2.17
6	sIL-15RA	+1.928
7	IL-15RA	+1.928
8	Bcl-XL	+1.64
9	iNOS	+1.88
10	CD69	+1.76

Table 5. CD1d- dependent genes are implicated in multiple signalling pathways

Table *of the pathway and network objects which are significantly upregulated, with a Log2FC > 1.5, a p value <0.05, and which were found to be implicated in many of the top signalling networks.*

3.10.c. The role of CD1d signalling on inflammatory monocyte gene expression profiles

As highlighted by Ron Germain's group, among others, inflammatory monocytes and neutrophils confer distinct functions and signalling profiles during influenza infection [194]. Thus, we next looked to assess the differential gene expression patterns in $CD1d^{-/-}$ inflammatory monocytes following influenza infection. We aimed firstly to identify which genes are upregulated, and secondly to assess whether the gene signatures differ from those observed in neutrophils. Following statistical analysis, which identified genes with a p

value < 0.05, and a Log2FC > 1.5, or < -1.5, a total of 321 genes, of known function, were significantly upregulated, and a total of 225 genes, which had a known function, were downregulated **(Table 6)**.

Gene Assignment	Log2FC	Adjusted P Value	Gene description
Ifnb1	5.27	7.11E-06	Interferon Beta
Cxcl3	4.49	9.40E-07	Chemokine (C-X-C Motif) Ligand 3
Il1r2	4.22	2.57E-06	Interleukin 1 Receptor, Type II
Il6	3.71	1.97E-05	Interleukin 6
Lcn2	3.56	1.86E-07	Lipocalin 2
Fabp7	3.50	1.42E-06	Fatty Acid Binding Protein 7
Mmp8	3.48	9.07E-06	Matrix Metallopeptidase 8
Edn1	3.35	5.21E-06	Endothelin 1
Inhba	3.34	1.41E-06	Inhibin, Beta A
Scrg1	3.26	9.89E-06	Stimulator of Chondrogenesis 1
S100a8	3.25	9.41E-06	S100 Calcium Binding Protein A8
Mrap	3.12	9.41E-06	Melanocortin 2 Receptor Accessory Protein
Lrg1	3.07	1.42E-06	Leucine-Rich Alpha-2-Glycoprotein 1
Slc7a11	3.03	1.97E-05	Solute Carrier Family 7 (Anionic Amino Acid Transporter Light Chain, Xc- System), Member 11
Mertk	2.88	2.04E-04	C-Mer Proto-Oncogene Tyrosine Kinase
Gpnmb	2.87	2.57E-06	Glycoprotein (Transmembrane) Nmb
Slc7a10	2.72	9.89E-06	Solute Carrier Family 7 (Anionic Amino Acid Transporter Light Chain, Xc- System), Member 10
Cd33	2.64	2.82E-05	Sialic Acid Binding Ig-Like Lectin 3 (Siglec3)
Vcan	2.62	9.89E-06	Versican
Fpr1	2.59	1.51E-05	Formyl Peptide Receptor 1
Gm19951	2.58	2.19E-05	No Associated Phenotype
Hamp	2.58	1.66E-03	Hepcidin Antimicrobial Peptide
Dhfr	2.46	5.21E-06	Dihydrofolate Reductase

Tnfrsf23	2.43	1.21E-04	Tumor necrosis factor receptor superfamily, member 23
Tlr3	2.42	9.29E-05	Toll like receptor 3
Dpep2	2.40	4.51E-06	Dipeptidase-2
Lrp12	2.40	5.88E-06	Low Density Lipoprotein Receptor-Related Protein 12
Mmp19	2.38	2.19E-05	Matrix metallopeptidase 19
Clec4d	2.34	1.25E-05	C-Type Lectin Domain Family 4, Member D
Il1a	2.33	5.17E-05	Interleukin 1, alpha
Clec5a	2.33	2.26E-06	C-type lectin domain family 5 member A
Klre1	-2.30	8.40E-06	Killer cell lectin-like receptor subfamily C, member 2
Hsph1	-2.37	1.75E-05	Heat shock 105kDa/110kDa protein 1
Trbv13-2	-2.44	1.23E-05	T cell receptor beta, variable 13-2
Treml4	-2.54	1.79E-09	Triggering receptor expressed on myeloid cells-like 4
Igj	-2.55	4.25E-05	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides
Hspa1b	-2.65	7.75E-09	Heat shock 70kDa protein 1B
Hist1h2ac	-2.80	2.87E-05	Histone cluster 1, H2ac
Ace	-3.18	1.08E-09	Angiotensin I converting enzyme
Emr4	-3.36	4.07E-09	Egf-like module containing, mucin-like, hormone receptor-like 4 pseudo-gene
Retnlg	-3.45	1.74E-06	Resistin like beta

Table 6. Top 30 up-regulated, and top 15 down-regulated genes of CD1d$^{-/-}$ inflammatory monocytes isolated from the lungs of PR8-challenged mice.

Thresholds were set to a Log2FC greater than +/-1.0, with a p value <0.05, and include both up and down signalling events. For the complete list of significantly differentiated genes see ***(Appendix 5)****.*

Initial observations of the top upregulated genes highlight a role for CD1d interactions in the pro-inflammatory response; IFN-β (Log2FC $^{+}$5.27) showed the most significantly upregulated expression in CD1d$^{-/-}$ inflammatory monocytes, as compared with CD1d$^{+/+}$ cells, followed by the chemoattractant CXCL3 ((GRO-3) Log2FC $^{+}$4.49), and the pro-inflammatory factors interleukin 1 receptor (IL-1r2 (Log2FC $^{+}$4.22) and interleukin 6 ((IL-6) Log2FC $^{+}$2.95). Similarly to neutrophils, we once more observed upregulation of the migration factor endothelin-1 (Log2FC $^{+}$3.35).

3.10.d. CD1d$^{-/-}$ inflammatory monocytes exhibit a gene profile indicative of a heightened immune response

As with neutrophils we observed that CD1d$^{-/-}$ inflammatory monocytes exhibit a broad range of genes with a significant Log2FCs, as compared with CD1d$^{+/+}$ inflammatory monocytes (**Table 6**). Subsequent to identifying the most highly differentiated during influenza infection, we again utilised MetaCore analysis to identify the relevant pathway and network mapping which have the potential to be modified by the combination of active genes. The top 10 pathways hits are shown in **Table 7.**

	Pathway Maps	p-value	FDR	Ratio	Network Objects
1	Immune response: HSP60 and HSP70/ TLR signalling pathway	1.69E-06	8.16E-04	8/54	CD14, TLR4, IL-6, IL-10, CD80, CD69, TNF-alpha, iNOS
2	Expression targets of Tissue factor signalling in cancer	1.87E-05	4.52E-03	5/22	Coagulation factor VII, GRO-1, Tissue factor, Coagulation factor X, PLAUR (uPAR)
3	Immune response: IL-1 signalling pathway	5.75E-05	9.27E-03	6/44	IL-1 alpha, IL-6, Tissue factor, TNF-alpha, Endothelin-1, iNOS
4	Immune response: HMGB1/RAGE signalling pathway	1.66E-04	1.61E-02	6/53	IL-1 alpha, TLR4, IL-6, Tissue factor, TNF-alpha, iNOS
5	Immune response: TLR3 and TLR4 induce TICAM1-specific signalling pathway	2.30E-04	1.61E-02	4/20	CD14, IFN-beta, TLR4, TLR3
6	Immune response_TLR2 and TLR4 signalling pathways	2.50E-04	1.61E-02	6/57	CD14, TLR4, IL-6, IL-10, TNF-alpha, iNOS
7	Immune response: Role of PKR in stress-induced antiviral cell response	2.50E-04	1.61E-02	6/57	IFN-beta, TLR4, IL-6, IL-10, TNF-alpha, TLR3
8	Substance P-mediated inflammation and pain in Sickle cell disease	2.91E-04	1.61E-02	5/38	PLC-beta, CD14, IL-6, Tissue factor, TNF-alpha
9	Blood coagulation: Blood coagulation	3.30E-04	1.61E-02	5/39	Coagulation factor XIII A, Coagulation factor VII, Tissue factor, Coagulation factor X, PAI2
10	Immune response: IL-18 signalling	3.32E-04	1.61E-02	6/60	IL-1 alpha, Bcl-XS, IL-6, Fibronectin, TNF-alpha, iNOS

Table 7. Top ten differentially expressed pathways in CD1d$^{-/-}$ inflammatory monocytes isolated from the lungs of influenza-challenged mice.

The top pathways altered in the absence of CD1d signalling were generated using MetaCore analysis. Thresholds were set to a Log2FC greater than +/-1.0, with a p value <0.05. The ratio demonstrates how many objects out of the total suggested pathway components are significantly increased or decreased. FDR (false discovery rate) indicates the proportion of false discoveries among the observed pathways.

The pathways listed in **(Table 7)** indicated that there are numerous genes differentially regulated, which have the potential to contribute towards numerous signalling networks. Remarkably, seven out of the top ten pathways related directly with monocyte activation or innate immune responses during influenza infection. The pathways which exhibited the most significant differentiation in gene expression were HSP60/70 and TLR signalling, with significant upregulation of genes for CD14, TLR4, IL-6, IL-10, CD80, CD69, TNF-alpha, and finally iNOS. IL-1 signalling pathways and HMGB1/RAGE signalling pathways were also highly modified. As with neutrophils, we subsequently mapped our genes of interest with more general signalling networks **(Table 8)**.

	Networks	p-value	FDR	Ratio	Network Objects
1	Chemotaxis	4.11E-08	5.71E-06	16/137	Galpha(q)-specific peptide GPCRs, IL8RB, Galpha(i)-specific peptide GPCRs, C5AR, IL-1 alpha, GRO-2, C3aR, CCR1, IL-10, GRO-1, FPRL1, CCL17, Osteopontin, PLAUR (uPAR), GRO-3, FPR
2	Inflammation: Innate inflammatory response	3.81E-07	2.65E-05	17/181	PLA2, C5AR, PLC-beta1, PLC-beta, IL-1F9, IL-1F6, IL-1 alpha, CD14, sCD14, IFN-beta, C3aR, TLR4, IL-6, Tissue factor, TNF-alpha, iNOS, TLR3
3	Development: Blood vessel morphogenesis	2.22E-06	1.03E-04	18/228	Galpha(q)- GPCRs, Galpha(i)- GPCRs, Neuregulin 1, Galpha(q)-SA GPCRs, IL-1 alpha, Guanylate cyclase A (NPR1), Angiogenin, Galpha(q)-SNL GPCRs, Fibronectin, CCR1, Furin, RAI, PDE, HGF receptor (Met), Endothelin-1, MMP-19, PLGF, Transferrin
4	Inflammation: Neutrophil activation	1.78E-05	6.19E-04	16/215	IL8RB, Galpha(i)-specific peptide GPCRs, PLA2, C5AR, PLC-beta1, PLC-beta, GRO-2, IL-6, IL-10, GRO-1, CD63, FPRL1, TNF-alpha, GRO-3, iNOS, FPR
5	Proliferation: Negative regulation of cell proliferation	4.79E-05	1.33E-03	14/184	Galpha(i)-specific peptide GPCRs, BTG3, IL-1 alpha, GAB1, IFN-beta, IL-6, Oncostatin M, C/EBPalpha, CCR1, GPNMB (Osteoactivin), GRO-1, MAD, IBP, IBP6
6	Proteolysis: ECM remodelling	9.38E-05	2.17E-03	9/85	Antileukoproteinase 1, Fibronectin, MMP-14, SERPINA3 (ACT), Osteopontin, PLAUR (uPAR), PAI2, MMP-19, MMP-8
7	Inflammation: Protein C signalling	1.20E-04	2.38E-03	10/108	PLC-beta1, PLC-beta, IL-1 alpha, Coagulation factor VII, TLR4, IL-6, Tissue factor, TNF-alpha, SPHK1, Coagulation factor X
8	Inflammation: MIF signalling	2.40E-04	3.91E-03	11/140	PLA2, PLC-beta1, PLC-beta, IL-1 alpha, CD14, TLR4, IL-6, ETS, ETS2, TNF-alpha, iNOS
9	Proteolysis: Connective tissue degradation	2.68E-04	3.91E-03	10/119	ADAM8, Antileukoproteinase 1, Fibronectin, MMP-14, SERPINA3 (ACT), Furin, PLAUR (uPAR), PAI2, MMP-19, MMP-8
10	Immune response_Th17-derived cytokines	2.81E-04	3.91E-03	9/98	Bcl-XL, IL-6, Calgranulin A, C/EBP, Calgranulin B, IL-10, GRO-1, NGAL, iNOS

Table 8. Top ten differentially expressed networks in CD1d$^{-/-}$ inflammatory monocytes isolated from the lungs of PR8-challenged mice.

The top process networks modulated by CD1d-deficiency were generated using MetaCore analysis. Thresholds were set to a Log2FC greater than +/-1.0, with a p value <0.05. The 'ratio' demonstrates how many objects out of the total suggested pathway components are

significantly increased or decreased. FDR (false discovery rate) indicates the proportion of false discoveries among the observed pathways.

As shown by the pathway and network maps (**Table 7**) (**Table 8**) a number of genes have the potential to moderate a selection of highly relevant signalling pathways. We found that the genes associated with chemotaxis, innate inflammatory responses and neutrophil activation pathways are the most differentially expressed signalling pathways in $CD1d^{-/-}$ inflammatory monocytes (**Table 8**). As with neutrophils, the pathways modified in **Table 7** and **Table 8** suggest that $CD1d^{-/-}$ inflammatory monocytes exhibit a heightened pro-inflammatory responsiveness, as compared with $CD1d^{+/+}$ inflammatory monocytes during influenza infection.

3.11 CD1d-dependent signalling targets overlapping signalling pathways in neutrophils and inflammatory monocytes following PR8 infection

Pathway mapping of the individual cell populations demonstrated prominent signalling themes occurring in cell signalling in the absence of CD1d interactions. We progressed to determine which signalling pathways iNKT cell interactions may affect jointly and distinctly between monocytes and neutrophils. Using Venn diagrams, we showed that no comparable genes are expressed in naïve $CD1d^{-/-}$ neutrophils or inflammatory monocytes **(Figure 20a)**. Naïve $CD1d^{-/-}$ inflammatory monocytes showed differential expression of 5 known genes, namely, a significant increased fold change in IL-6 expression, and significantly decreased expression of a number of heat shock proteins (HSP). Interestingly, cellular aging and prolonged cytokine exposure, such as exposure to IL-6 and TNFα *in vitro*, have been shown to modulate HSP70 production in monocytes, and thus, the elevated IL-6 message we observe in both naïve and infected mice could account for these observations [337].

In contrast to the minimal gene expression observed in neutrophils and monocytes from naïve mice, in influenza infected mice **(**Figure 20**a)**, we observed a substantial number of genes which are significantly upregulated or downregulated solely in $CD1d^{-/-}$ neutrophils as compared with $CD1d^{-/-}$inflammatory monocytes; indeed, we observed 62 genes upregulated in both neutrophils and inflammatory monocytes **(Figure 20b)**, and 9 genes commonly downregulated **(Figure 20c)**.

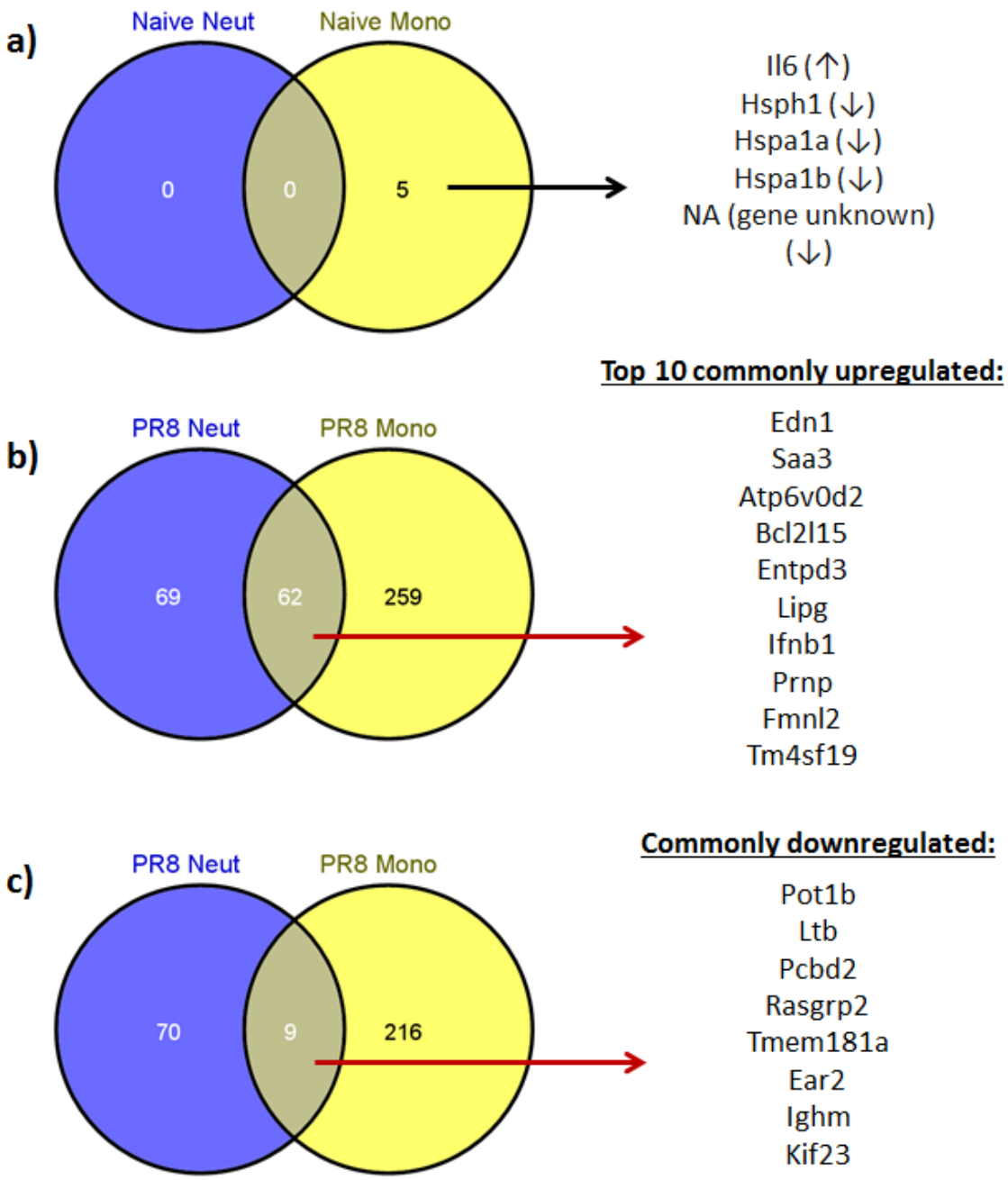


Figure 20. iNKT cell deficiency modulates similar gene pathways in infected monocytes and neutrophils.

a) The total list of significantly altered genes (excluding genes whose function had not yet been characterised) in naïve CD1d$^{-/-}$ monocytes and neutrophils were compared using a Venn diagram. The total list of significantly b) upregulated or c) downregulated genes in

PR8-infected CD1d$^{-/-}$ inflammatory monocytes (mono) and neutrophils (PMN). Thresholds were set to a Log2FC greater than +/-1.0, with a p value <0.05. Venn diagrams were created using VENNY: http://bioinfogp.cnb.csic.es/tools/venny/. (↑) indicates significantly upregulated. (↓). indicates significantly downregulated.

To gain a more comprehensive understanding of how inflammatory monocyte and neutrophils signalling pathways may be related, we performed pathway enrichment analysis of significantly upregulated pathways **(Figure 21)**. We found that the differentially expressed genes of CD1d$^{-/-}$ inflammatory monocytes (orange bars) are more prominent in the contribution to the top signalling pathway; this may stem from the greater number of total genes which were found to be significantly affected by CD1d-deficiency in inflammatory monocytes, as compared with those significantly altered in CD1d$^{-/-}$ neutrophils (blue bars) **(**Figure 21**)**.

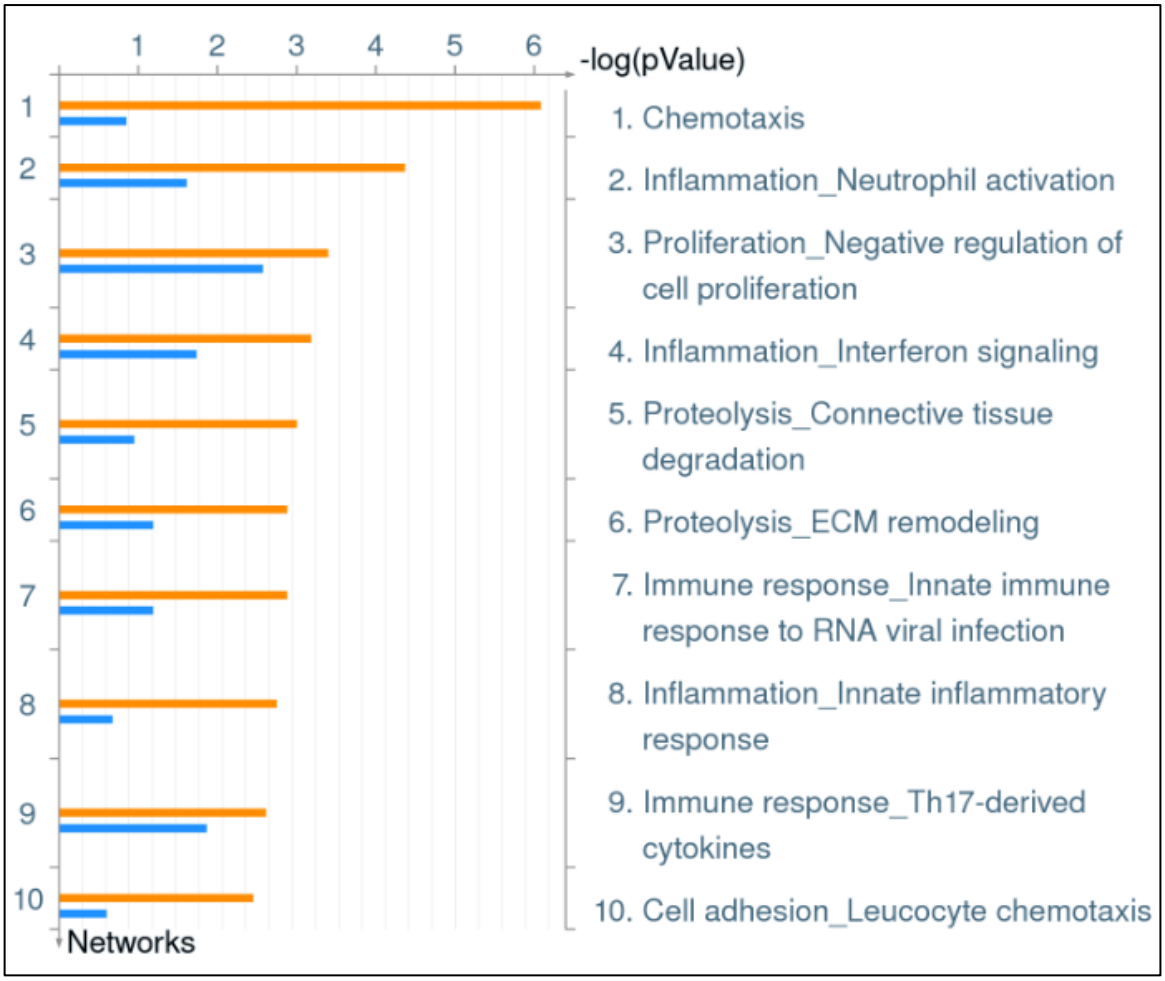


Figure 21. Differentially expressed genes in inflammatory monocytes have a greater potential to impact on infection-associated signalling pathways

The top process networks enriched in CD1d$^{-/-}$ inflammatory monocytes (orange) and neutrophils (blue), were generated using MetaCore. Thresholds were set to a Log2FC greater than +/-1.0, with a p value <0.05.

3.12. Discussion:

A fine regulation of innate cell recruitment and function during influenza infection is fundamental; these innate responders are essential for host response against a variety of pathogens, however, in excess, they can account for tissue destruction and exacerbated lung pathology. The aim of this chapter was to gain a greater understanding of the regulatory capacity of iNKT cells over myeloid cells responding to influenza infection, and to attempt to characterise the mechanisms responsible. In order to further elucidate how iNKT cells afford their control over the innate immune response, we utilised a combination of *in vivo* experiments with iNKT-cell deficient mice (*Jα18*$^{-/-}$), and SJL: CD1d$^{-/-}$ chimeric mice.

3.12.a. iNKT cells afford a protective role during influenza infection

A protective role of iNKT cells during IAV infection is now widely accepted, and has been attributed to a number of functions during IAV-infection, including elevation of IL-12, IL-22 and IFN-γ [292, 329, 336], increased natural killer (NK) cell activation [117] and blocking suppressive activity of myeloid derived suppressor cells [41]. We confirm previous reports of the increased susceptibility of iNKT-cell deficient mice (*Jα18*$^{-/-}$) to influenza infection [41, 127, 292], showing that iNKT cell deficiency leads to increased weight loss and mortality (**Figure 6**). Utilising antibodies specific against neutrophils (Ly6G) and monocytes (Ly6C), in replacement of the non-specific antibody, Gr-1, which was widely used in initial publications analysing these cells, we showed that during influenza infection in the absence of iNKT cells, neutrophils and inflammatory monocytes are the predominant cell populations infiltrating the lungs during the innate response.

3.12.b. CD1d-dependent interactions play a limited role in regulating cell migration during influenza infection.

iNKT cells have demonstrated a role, if still slightly unclear, in myelopoiesis under steady state conditions [321, 326, 327]; the role of iNKT cells in myelopoiesis during infection, however, remains unclear. As such, we utilised a sub-lethal influenza infection in SJL: $CD1d^{-/-}$ and SJL: $CD1d^{+/+}$ chimeras to determine the contribution of CD1d signalling in the immune response to influenza.

Analysis of weight loss over the infection period demonstrated no significant difference in morbidity or mortality when reconstituted with 100% $CD1d^{+/+}$ cells, as compared with 50:50 of $CD1d^{+/+}$: $CD1d^{-/-}$ bone marrow cells (**Figure 11**). In addition, when analysing populations irrespective of CD45.1 or CD45.2 expression, we observed an increased, but comparable total cellular infiltration in the lung between both strains (**Figure 12**). When dissecting the contribution of $CD1d^{-/-}$ and $CD1d^{+/+}$ cells to this cellular infiltrate, we revealed that the IAV-induced expansion of $CD1d^{-/-}$ inflammatory monocytes or neutrophils, although significantly greater than their $CD1d^{+/+}$ counterparts during infection, was not significantly increased when compared with the bias towards $CD1d^{-/-}$ cells already observed in the naïve animals (**Figure 13**). This reduced effect of iNKT cells on myeloid cell recruitment or proliferation is contrary to work by De Santo et al. who demonstrated that SAA injections of CD1d-chimeric mice resulted in the proliferation of IL-10-secreting immune-suppressive neutrophils only in the $CD1d^{-/-}$ cell population. Importantly, it is widely established that influenza infection is associated with the release of a vast array of pro-inflammatory factors, cytokines and chemokines, which function to recruit and activate immune cells in the lung [154]. SAA injections, although strongly pro-inflammatory, are unlikely to induce the same range and extent of signalling as IAV-infection; we could thus speculate that during influenza infection in the chimeric mice, CD1d-signalling can modulate numerous function of immune cells, as shown later in gene profiling, but may not be sufficient to solely regulate the recruitment or

proliferation of $CD1d^{+/+}$ populations. Indeed, a number of studies, including our own, have shown elevated chemokines, such as CCR2 (**Figure 7**) and IL-6, key chemoattractants for monocytes, in the BALF of IAV-infected *Jα18*$^{-/-}$ mice [127], suggesting that chemotactic factors may play a vital role in the recruitment of myeloid cells. Accordingly, as discussed later, we show elevated message levels of a number of chemotactic factors in $CD1d^{-/-}$ inflammatory monocytes and neutrophils, which may exert a reciprocal effect on the $CD1d^{+/+}$ populations during infection. Importantly, iNKT cells release an array of cytokines and chemokines, independent of cell-cell contact; iNKT cells in the chimera which are selected and activated by the $CD1d^{+/+}$ DP thymocytes and APCs, respectively, may provide indirect signalling to regulate the immune response of $CD1d^{-/-}$ and $CD1d^{+/+}$ alike. Indeed, this is fitting with the reports that a range of cytokines and chemokines are produced by activated iNKT, such as IFN-γ, GM-CSF and IL-3, which can affect myelopoiesis, cell survival and recruitment [321].

Interestingly, in contrast to our observations in the periphery, we observed comparable ratios of $CD1d^{-/-}$ to $CD1d^{+/+}$ cells in the bone marrow of naïve mice, which only becomes biased towards $CD1d^{-/-}$ cells following infection **(Figure 14)**. The explanation for the variance in $CD1d^{-/-}$: $CD1d^{+/+}$ cell ratio observed in the periphery compared with the bone marrow of naïve mice is unclear; we speculate that differing stimuli in the bone marrow versus the periphery, which is later modified following influenza infection, could account for the differing proportions of $CD1d^{-/-}$ and $CD1d^{+/+}$ cells observed. In line with this, the bias in engraftment capacity towards $CD1d^{-/-}$ cells following adoptive transfer into irradiated SJL mice additionally requires further investigation **(Figure 10).** This bias was not restricted to neutrophils and inflammatory monocytes; indeed we additionally observed a significant localisation of $CD1d^{-/-}$ cells in NK cells and B cells, but not in CD4 T cells. The observation of greater $CD45.1^{+}$ $CD4^{+}$ T cells is likely to be attributed to the accumulation of host $CD45.1^{+}$ radiation-resistant

T cells [338, 339]. It would be interesting to assess additional T cell subsets to determine whether engraftment of all T cells is affected by CD1d expression. Furthermore, given the reported enhanced absolute numbers of precursor populations in the bone marrow and spleen of $CD1d^{-/-}$ mice, as well as enhanced colony forming capacity of $CD1d^{-/-}$ cells [327], it will be vital to confirm donation bone marrow from $CD1d^{-/-}$ and $CD1d^{+}/^{+}$ contains equivalent proportions of myeloid precursor cells.

3.12.c. CD1d-signalling significantly alters the genetic profile of myeloid cells during influenza infection

Flow cytometry analysis of cellular infiltration implies that CD1d-mediated interactions are non-essential in the migration of inflammatory monocytes or neutrophils to the lungs during influenza infection. Our final approach to assess the role of CD1d-signalling in regulating influenza-induced myeloid cells was to assess the genetic profiles of $CD1d^{-/-}$ or $CD1d^{+/+}$ myeloid cells using microarray array. Data presented here is the first to show differential gene profiles in $CD1d^{-/-}$ myeloid cells compared with $CD1d^{+}/^{+}$ cells following influenza infection. Strikingly, both $CD1d^{-/-}$ inflammatory monocytes and neutrophils exhibited gene profiles characteristic of a heightened inflammatory state. Investigating every gene with significant alterations in expression would have been beyond the temporal constraints of this thesis, so given the background to this project, and the common themes observed in the pathway mapping above **(Table 3)(Table 4),** we instead focused on the implications of CD1d-mediated interactions on a smaller selection of influenza and innate immunity responses, such as chemotaxis, release of pro-inflammatory mediators, and apoptosis, which will be discussed below.

3.12.d. CD1d-signalling defines chemotactic signalling at the message level

A number of chemokines have been implicated in the migration of neutrophils to influenza infection; Brandes et al. published an elegant systems' analysis of chemokine signalling in neutrophils and monocytes during infection with different influenza virus strains. The authors demonstrated that neutrophils and monocytes utilise a feed-forward system to amplify their own recruitment into the lungs, forming cellular focal points, which are often associated with lung pathology in lethal infection [194]. We had hypothesised that iNKT cells could regulate chemotaxis, however FACS data showing equivalent recruitment of both $CD1d^{-/-}$ and $CD1d^{+/+}$ cells did not support this. Intriguingly, however, we observed differential expression of a number of genes involved in chemotaxis in the absence of CD1d-signalling in both inflammatory monocytes and neutrophils. Firstly, we confirmed previous reports of elevated CXCL1 (Log2FC $^{+}$2.17) and CXCL3 (Log2FC $^{+}$2.17) in neutrophils during influenza infection [194], and more importantly, we were the first to show that the expression of both of these chemotactic factors are significantly enhanced in the absence of CD1d signalling. Intriguingly, we did not observe differential expression of other CXC chemokines in our $CD1d^{-/-}$ neutrophils, which were observed in neutrophils by Brandes et al. among others, such as CXCL2, CCL3 and CCL4, implying that CD1d signalling acts specifically on subsets of chemokines. Similarly, chemotaxis was identified as a predominant 'process pathway' corresponding with our differentiated genes in inflammatory monocytes; we observed significant upregulation of CXCL3 (Log2FC of 4.49), as well as upregulation of chemotactic factors CXCL1, CXCL2, CCR2.

In addition to canonical chemokines, we identified a novel role for iNKT cells in regulating endothelin-1 production from both inflammatory monocytes and neutrophils. Endothelin-1 has been shown to act as a potent enhancer of neutrophil migration and adhesion [340]. Few papers have addressed expression of endothelin-1 in cells other than endothelial cells,

with one previous paper reporting LPS, PMA, f-MLP and TNFα-induced upregulation of endothelin-1 expression on human neutrophils [341]; here we show that mouse CD1d$^{-/-}$ neutrophils during influenza infection are able to upregulate endothelin-1 message. In line with our observed elevation of CXCL1 message in CD1d$^{-/-}$ neutrophils (**Table 5**), Zarpelon et al. showed that endothelin-1 induces the production of mediators such as TNFα and CXCL1, which in turn are important in both innate and adaptive inflammation-induced neutrophil migration [342]. Thus, the enhancement of endothlein-1 by neutrophils themselves, if confirmed at the protein level, could confer a positive feedback loop to recruit further neutrophils via endothelin-1 and release of chemokines, such as CXCL1.

The identification of enhanced chemotactic genes in both infected neutrophils and inflammatory monocytes was somewhat intriguing, since we were unable to observe any difference in the percentage, or absolute number (data not shown) of either of these cellular populations by FACS at day 7 post PR8 infection (**Figure 13**) or at day 4 or day 5 infection (data not shown). There are a number of explanations which may account for this difference. It is possible that a difference in cell migration occurred much earlier in infection, days 1-3 for example, and thus at the time point's analysed the difference was no longer present. Furthermore, it is possible that the observed differential gene expression does not translate equivalently at protein level. A number of cytokines and chemokines have been implicated in influenza infection, such as CCL2 and CXCL2 in monocytes and neutrophils respectively, for which we did not observe a significant difference in expression between CD1d$^{-/-}$ and CD1d$^{+/+}$ cells. It is plausible, that despite differential expression of the chemokines such as CXCL1 and CXCL3, the expression of more relevant chemokines, regulated independently of CD1d-signalling, may overcome this signalling. Accordingly, CCL2 has been implicated in monocyte recruitment in influenza infected *Jα18*$^{-/-}$ mice [127], and we have observed elevated CCL2 protein in the BALF of PR8-infected *Jα18*$^{-/-}$ mice (**Figure 7c**).

3.12.e. The pro-inflammatory factor, Serum Amyloid A-3, is significantly amplified in the absence of CD1d-dependent iNKT interactions

The observed upregulation of Serum Amyloid A (SAA) 3 in CD1d$^{-/-}$ inflammatory monocytes and neutrophils, as well as in the influenza infected *Jα18*$^{-/-}$ mice was especially exciting; SAA is a well-known marker of the acute phase response, can be elevated up to 1000-fold during inflammatory states (reviewed by [343]). Furthermore, relevant to our model, SAA levels are elevated in serum of influenza-infected individuals or PR8-infected mice [344]. In mice, three acute-phase isoforms, SAA1, SAA2, and SAA3, have been identified; intriguingly, many studies addressing roles of SAA have focused on systemic SAA 1 or SAA2, two SAA isotypes which are predominantly produced in the liver or adipose tissue. However, SAA3 has more recently been shown to be produced extrahepatically, with production from macrophage cell lines [345]. Bacterial antigens (such as LPS), pro-inflammatory cytokines, including IL-1β, IL-6, and TNFα, and glucocorticoids, alone or in combination, have been shown to induce acute phase SAA synthesis [346, 347]. Locally-produced SAA has demonstrated a number of critical inflammatory responses, in particular, SAA promotes direct antimicrobial effects, ROS generation, phagocytosis of certain bacteria, recruitment and activation of myeloid cells, and finally, in polarisation of T helper type 17 immune responses [348-350]. Finally, SAA drives production of IL-1α, IL-1β, IL-6, IL-23, and prostaglandin E2 (PGE2) and causes dendritic cell (DC) maturation. We observed many of the cytokines listed above as upregulated in CD1d$^{-/-}$ cells, and the functions of these are highly relevant in the immune response to influenza infection [351].

Interestingly, SAA can also act as an endogenous factor that stimulates granulocyte-colony stimulating factor (G-CSF) expression in isolated macrophages and induces neutrophilia in mice in FPR2- or TLR2-dependent manner [351-353]. Of particular interest to this study, De Santo et al. identified a previously unknown role for SAA in regulating the plasticity of neutrophil differentiation, whereby SAA stimulation induced secretion of IL-10 from human

neutrophils [330]. In line with this, SAA3 expression has been reported in monocytic MDSCs (mMDSCs), and it's upregulation increased the survival of mMDSCs, as well as increasing TLR2 signalling, leading to increased autocrine secretion of TNFα, that lead to STAT3 activation and increased suppressive activity [354]. Although we don't observe upregulation of TLR2 in either cell population investigated, in inflammatory monocytes we do observe significant upregulation of TLR4 (Log2Fc of 1.14, $p < 4x10^{-4}$), an alternative receptor for SAA3, [355]. This could represent a highly complex feed-forward model with these cell types, whereby in the absence of iNKT cells, SAA3 production from either cell type can promote the further signalling via TLR recognition.

3.12.f. iNKT cells modulate the pro- and anti-inflammatory immune responses

In keeping with the observed increase of SAA in the absence of iNKT cells, we observe a number of other immune-regulatory factors upregulated in the absence of iNKT cells, which could contribute to an over activated state of the cells in response to influenza. Firstly, CD69 was shown to be significantly elevated in the absence of CD1d expression on both neutrophils and inflammatory monocytes. CD69 is a marker of cellular activation, which can be induced by TLR signalling, and cytokines such as GM-CSF, IFN-γ and IFN-α [356, 357], and has been shown to be upregulated on lung neutrophils following influenza infection [358]. Furthermore, although the specific ligand for CD69 remains to be characterised, analysis of CD69-mediated cellular output induced by crosslinking with anti-CD69 monoclonal antibody strongly suggests that this factor is significant in the biology of hematopoietic cells, and plays a role in nitric oxide production by monocytes, as well as neutrophil degranulation [359, 360]. Consistent with the elevated activated status of these IAV-induced cells in the absence of CD1d-interactions, further gene profiling demonstrated an enhanced upregulation of a number of inflammatory cytokines, namely IL-1β, IFN-β, IL-12α, IL-15, and IL-15RA in neutrophils, and IL-6, IFN-β, IL-1 and IL-1R2 in inflammatory

monocytes. We are yet to validate protein expression of all of these factors, however IL-1β has shown to be elevated in the BALF of influenza infected *Jα18*$^{-/-}$ mice (**Figure 7**), which supports a role for iNKT cells in regulating this factor.

Taken together, the range of genes and pathway maps identified in both neutrophils and inflammatory monocytes supports a role for iNKT cells in regulating polarisation of innate responders, whereby iNKT cell signalling can switch cells between a pro- and anti-inflammatory phenotype. Below, we discuss a further three anti-inflammatory gene signalling pathways which are relevant to immune-dysfunction and lung pathology, and are of particular interest to our model of infection.

Firstly, the anti-inflammatory cytokine IL-10 has been identified in a number of our influenza infection models; we observed upregulation of IL-10 in the BALF during IAV-infection of *Jα18*$^{-/-}$ mice (**Figure 7**), and later we extended this finding by demonstrating that IL-10 message was elevated in inflammatory monocytes and neutrophils isolated from IAV-infected *Jα18*$^{-/-}$ mice (**Figure 8**). Analysis of the CD1d$^{-/-}$ inflammatory monocytes subsequently identified significantly increased IL-10 message in these cells when isolated from the SJL:CD1d$^{-/-}$ chimera (**Table 6**). IL-10 message in CD1d$^{-/-}$ neutrophils was also increased with a Log2FC of $^{+}$1.07, however this gene fell short of significance when compared with expression in CD1d$^{+/+}$ neutrophils (p=0.07). Interestingly, a suppressive role for neutrophils during infection is consistent with previous reports demonstrating the presence of hyper-segmented, IL-10-producing neutrophils, during a number of bacterial infections, such as during infection with *Mycobacterium bovis* [361] and *Yersinia enterocolitica [362]*. IL-10 secreting neutrophils were also recently discovered in the bone marrow of Sendai virus (SeV)-infected mice [363]. More intriguingly, De Santo *et al.* demonstrated that SAA stimulation promoted human neutrophil interaction with invariant natural killer T cells (iNKT), a process which limited the suppressive activity of neutrophils, by reducing IL-10 production, and enhancing the production of the pro-inflammatory cytokine, IL-12 [330]. In light of the observed increase in

SAA in the absence of iNKT cells, it is plausible that IL-10 is released from neutrophils in this model, but is regulated independently of CD1d, via other pro-inflammatory stimulus. Hence, we do not observe significant changes in IL-10 expression in our array analysis

Secondly, we observed substantial upregulation of S100A8 message expression in $CD1d^{-/-}$ monocytes following influenza infection (Log2FC of 3.25, p value < $9.4x10^{-6}$) (**Table 6**). S100A8, which belongs to the family of S100 calcium binding proteins, is commonly expressed together with its dimerization partner S100A9 in circulating neutrophils and monocytes, but not in resting tissue macrophages [364]. Upon elevation of intracellular calcium, S100A8 and S100A9 translocate from the cytosol to the cytoskeleton and the plasma membrane of myeloid cells [365] and are released as a heterodimer to recruit leukocytes to sites of inflammation. Consistent with the combined roles of S100A8/A9, we also observed significantly increased S100A9 in $CD1d^{-/-}$ inflammatory monocytes (Log2FC of 1.12, p=0.004) **(Appendix 5)**. The observed upregulation of S100A8 has impact for a number of reasons; S100A8 and S100A9 have been shown to upregulate SAA3 in pre-metastatic lungs, and thus contribute to the accumulation of myeloid cells, and functioning as a positive feedback regulator for chemoattractant secretion [355]. Furthermore, a paper recently published by Cheng et al. showed that over-expressing S100A9 in haematopoietic precursor cells causes differentiation into $CD11b^{+}Gr1^{+}$ MDSCs, which had strong immuno-suppressive effects on T cells [366]. Likewise, S100A9 down-regulation corresponded with myeloid differentiation into DCs [366].

A final gene identified in our array which was of notable interest was the identification nitric oxide synthase 2 (iNOS/NOS2), an enzyme responsible for the production of nitric oxide (NO), which was significantly elevated in the absence of CD1d-interactions. Furthermore, NOS2 shows to be implicated in five out of the top ten 'process pathways' which correspond most highly with our gene set (**Table 8**). The importance of NOS2 during influenza infection

is shown since mice deficient in NOS2 ($NOS2^{-/-}$), or wild-type mice treated with NOS2-inhibiting compounds, have been shown to exhibit reduced morbidity, reduced mortality, and diminished cytokine production in lung tissue following influenza challenge, as compared with control mice [367-369]. Moreover, highly virulent IAV was cleared from the lungs of $NOS2^{-/-}$ mice much faster than in control mice; the mechanism of virus clearance in the absence of NOS2 activity was shown to be dependent on IFN-**γ** [369]. In support of a role for iNKT interactions in modulating the suppressive capacity of neutrophils, De Santo et al. demonstrated that iNKTs were able to reverse the SAA-induced suppressive activity of human neutrophils, by reducing IL-10 production, and enhancing the production of the pro-inflammatory cytokine, IL-12 [330]. Furtermore, De Santo et al. demonstrated that the immunosuppressive activity, namely Arg1 and NOS2 release, of the $CD11b^{+}Gr\text{-}1^{+}$ cells isolated from the lungs of influenza-infected iNKT-deficient mice can be abrogated by a CD1d- and CD40-dependent crosstalk with iNKT cells [41]. Interestingly we observe significant upregulation of Arg2 (Log2FC of $^{+}1.78$, $p=9.95x10^{-5}$) in $CD1d^{-/-}$ inflammatory monocytes during influenza infection (**Table 6**). More recently MDSCs have been further characterised into granulocytic MDSC (G-MDSC) and monocytic MDSC (m-MDSC); G-MDSC have a phenotype of $CD11b^{+}Ly6G^{+}Ly6C^{low}$, whereas m-MDSC have a phenotype of $CD11b^{+}Ly6G^{-}Ly6C^{high}$ [370, 371]. This overlapping of surface markers demonstrates the lack of clarity in characterising MDSCs compared with canonical myeloid cells. Work presented here expounds upon that done by De Santo et al. [41], since we have shown that both inflammatory monocytes and neutrophils in the lung have the potential to switch to a more suppressive phenotype in the absence of iNKT cells. At present, the developmental or transitional relationship between MDSC and monocytes and neutrophils during viral infection is currently unclear, and thus, further functional characterisation of the $CD1d^{-/-}$ inflammatory monocytes and neutrophils during influenza will be required to address this question.

3.13. Conclusions and Future Objectives

This chapter reveals striking data on the regulatory capacity of iNKT cells over myeloid cells during influenza infection. We have clearly shown differential gene expression of $CD1d^{-/-}$ inflammatory monocytes and neutrophils, as compared with their $CD1d^{+/+}$ controls, which have the potential to correlate to many relevant immune responses during influenza infection. Taken together, this strengthens the hypothesis that iNKT cells are able to modify myeloid cells, via direct CD1d interactions, and this interaction defines their capacity to respond to infection. We have highlighted and discussed only a selection of the differentiated genes which were identified, and which we hypothesise could have implications in iNKT mediated regulation of myeloid cells. The complete set of genes regulated by direct iNKT interactions, however, deserves further exploration; this will provide a more comprehensive understanding of the signalling components involved in iNKT and myeloid cell interactions, and will likely highlight targets of interest on which to base future studies.

Completion of this study has raised some interesting questions which need to be addressed before we have a comprehensive understanding of the regulatory roles of iNKT cells in influenza infection. The highest priority in the future of this analysis is to validate the genes of interest using PCR and protein assessment. This further validation will be completed using qPCR and protein analysis (western blotting, FACS or ELISA), and will ensure that the pathways we hypothesis to be relevant in this model are applicable in the disease model. We have shown that in an identical influenza microenvironment, $CD1d^{-/-}$ inflammatory monocytes and neutrophils exhibit a heightened inflammatory potential.

Unfortunately in the present study we were unable to analyse the percentages of iNKT cells in the two chimera models, SJL: $CD1d^{-/-}$ versus SJL: $CD1d^{+/+}$, however given the cells are present in the same animals, we can assume they would be subjected to the same levels of cytokine and chemokines released from other cells, including those from iNKT cells engrafted the $CD1d^{+/+}$ compartment. We can thus hypothesise that the differential gene

profiles is predominantly attributed to the inability of these cells to interact with iNKT cells via CD1d- interactions. In naïve mice we observed no differentially expressed genes in neutrophils, and only upregulation of IL-6 in monocytes. This suggests iNKT cells can regulate myeloid populations independently of infection, but that iNKT cells afford a large proportion of their regulatory role over CD1d- expressing myeloid cells during infection, where we observed a substantial differentiation of over 100 genes in both neutrophils and inflammatory monocytes.

It remains unclear, however, where the interaction between myeloid cells and iNKT cells occurs to account for this regulation. It is also currently unclear whether the CD1d competent cells are over-compensating for the $CD1d^{-/-}$ populations during infection, or vice versa, which could account for why we fail to observe any difference in weight loss or lung pathology (data not shown), which we observe in $CD1d^{-/-}$ mice infected with influenza [41]. It would be valuable to characterise myeloid cells, either mature, or precursor populations in the periphery and the bone marrow of these chimeric mice, to determine the contribution of local and systemic cytokine and chemokine production, which is associated with influenza infection, and which could overcome the lack of CD1d expression on myeloid cells.

Chapter 4.

Investigating the a role for Fractalkine in influenza-induced lung pathology

Chapter 4: Investigating the a role for Fractalkine in influenza-induced lung pathology

4.0. Introduction

It is widely reported that exacerbated circulatory leukocyte migration and accumulation in the lung vascular endothelium is associated with the structural destruction and remodelling seen during acute and chronic lung diseases. Despite a number of chemokines and cytokines being implicated in cellular infiltration and lung pathology during IAV infection **(Table 1**), the regulation of myeloid cell recruitment and activation during infection with viruses of differing pathogenicity, requires further clarification. Interestingly, increasing reports have proposed that fractalkine (CX3CL1), the sole member of the fourth class of (CX3C) chemokine family, and its receptor (CX3CR1), are implicated in the both the recruitment and the survival of leukocytes during chronic lung inflammatory conditions, such as asthma [372]. The generation of $CX3CR1^{gfp/+}$ reporter mice has been fundamental in elucidating the role of CX3CR1 expression in the steady state, and during inflammation [373]. Although the original characterisation of constitutive CX3CR1-knockout mice reported no apparent defects in immune function [301], the specific role of CX3CL1 and its receptor, CX3CR1 in regulating IAV infection or during IAV-induced lung pathology has not been addressed.

Our main interest in this signalling pathway stems from a number of key properties of CX3CL1 and CX3CR1. Firstly, CX3CR1 is reported to be highly expressed on a diverse range of cells, many of which have been implicated in influenza infection [302]; CX3CR1 is widely accepted to be expressed on monocytes, where high and low expression defines classical, or inflammatory ($CX3CR1^{low}CCR2^{high}Gr\text{-}1^{high}$) and non-classical, or patrolling ($CX3CR1^{high}CCR2^{low}Gr\text{-}1^{low}$) subsets in mice, and additionally on macrophages, DCs, NK cells, and subsets of T and B cells [301, 302]. A recent mouse study by Auffray et al.

demonstrated that patrolling monocytes, which are CX3CR1^{high}Gr1^{low} in mice (equivalent to human CD14^{low}CD16^{high} monocytes) are a subset that predominantly crawls on normal endothelium of blood vessels, entering tissues rapidly upon tissue damage and subsequently generating tissue macrophages [374, 375]. The current dogma suggests that patrolling monocytes enter tissues constitutively via the activity of the chemokine receptor CX3CR1 [376], whereas inflammatory monocytes enter inflamed peripheral and lymphoid tissues via the activity of CCR2 [376, 377]. Interestingly, in humans, CX3CR1^{high} monocytes have exhibited an ability to patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors [378]. Surprisingly, in addition to their patrolling and repair activities, when stimulated with viral stimuli, CX3CR1^{high} monocytes were shown to release high amounts of pro-inflammatory cytokines in a TLR7- and TLR8-dependent, as well as MyD88-dependent manner, which may play an important role in antiviral immunity [378]. Deletion of CX3CR1 using CX3CR1$^{gfp/gfp}$ mice results in moderately reduced patrolling by CX3CR1^{hi}Ly6C^{low} monocytes [374], and also decreases the recruitment of Ly6C^{hi} monocytes to splenic sites of bacterial infection [379]. Few papers have addressed the expression or the role of CX3CR1 and CX3CL1 in neutrophils; however, neutrophils demonstrate expression at the message, and the protein levels, albeit at substantially lower levels when compared with mononuclear cells [302, 380-382]. Whether CX3CR1 expression can modulate the Ly6C monocyte subsets, differentiated macrophages, DCs, or neutrophils during influenza infection has not yet been fully explored.

In contrast to its receptor, CX3CL1 is reported to be predominantly expressed on non-haematopoietic cells [302], namely epithelial cells, endothelial cells and smooth muscle cells. Expression on a selection of haematopoitic cells has also been reported, for example, apoptotic B lymphocytes release CX3CL1, which then promotes the recruitment of macrophages [383]. Another key finding which supports our hypothesised role of this

signalling axis in influenza infection is the expression of CX3CL1 on lung epithelial cells [302]. Notably, CX3CL1 expression can be upregulated in bronchial epithelial cells by IFN-γ stimulation, which subsequently enhances the adhesion capacity of mononuclear cells *in vitro* [384]. In line with this, exposure of human pulmonary endothelial cells to IFN-γ, resistin, cigarette smoke, or shear stress leads to upregulation of CX3CL1 [385-388]. Expression of CX3CL1 on lung tissue during IAV infection has not been reported, but given IFN-γ, among other CX3CL1- known stimuli (as discussed later), represents a prominent cytokine found in influenza infection, it is plausible that these factors could be associated with influenza infection.

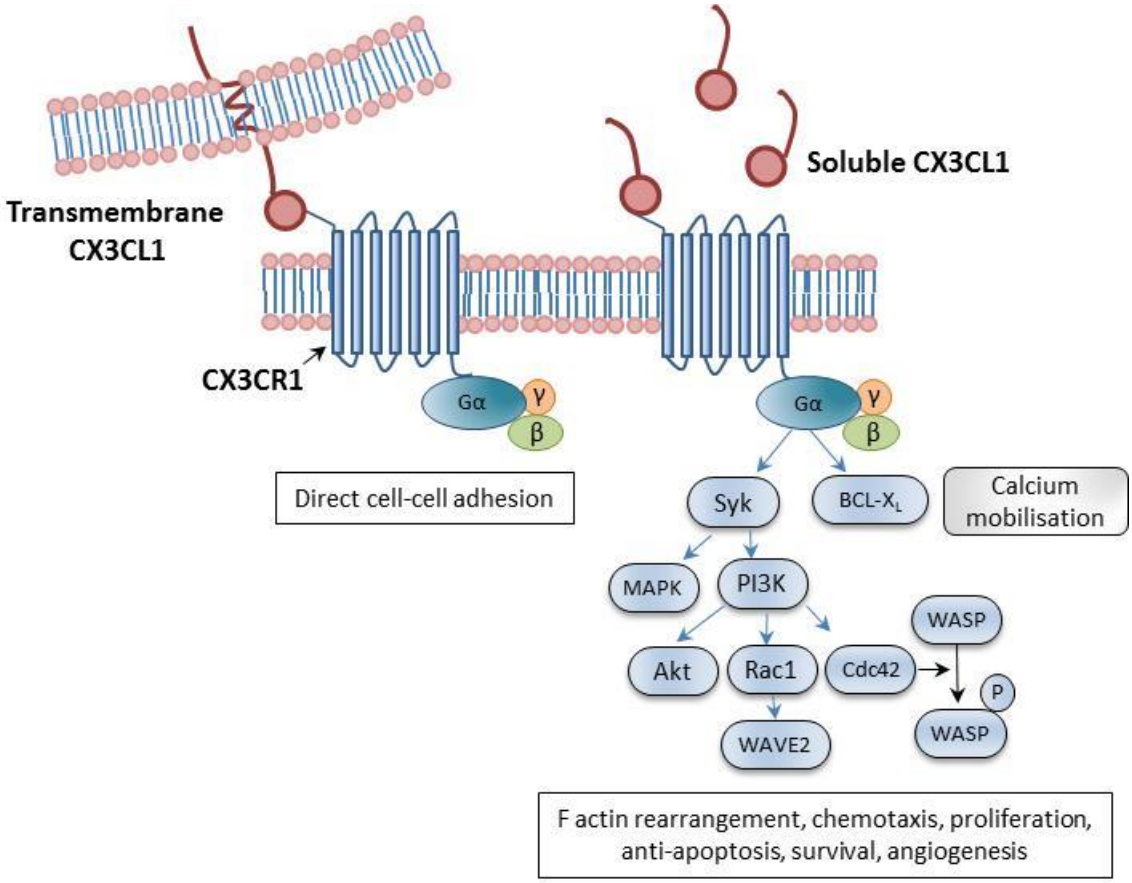


Figure 22. CX3CL1 mediates multiple functions, including cell adhesion, migration and survival of murine and human cells.

Membrane-bound CX3CL1 enables the integrin-independent capture and firm adhesion of leukocytes via the Gαi-coupled receptor, CX3CR1. Cleavage of cell-surface CX3CL1 generates soluble CX3CL1, which binds CX3CR1 and, via signalling intermediates, can induce cell chemotaxis and survival. PI3K activation of Rac-1 and Cdc42 has been implicated in murine macrophage cell line chemotaxis [389], whereas PI3K/Akt, MAPK and BCL-X_L signalling mediates CX3CL1-induced proliferation, migration and survival of epithelial cell lines and microglial cells [390, 391].

A second function of CX3CL1 which is of interest in our model, is the unique ability of CX3CL1 to function as both a membrane-bound chemokine, and following cleavage by a

disintegrin and a metalloproteinase (ADAM)-10 or ADAM 17, as a soluble factor [392]. Soluble CX3CL1 (sCX3CL1) functions as a canonical chemoattractant [393, 394], where sCX3CL1-induced CX3CR1 activation leads to a G-protein-dependent signal transduction pathway, promoting calcium mobilisation [381], F-actin rearrangement [395] and upregulation of integrins [396], via the modulation of a number of downstream effector molecules (**Figure 22**). Furthermore, transmembrane-bound CX3CL1 directly interacts with CX3CR1, thus promoting cell adhesion and endothelial transmigration [397-400], which is essential for immune cell trafficking and accumulation in the lungs. The direct interaction between CX3CL1 and CX3CR1 is not inhibited by pertussis toxin, and is independent of integrins [401] (**Figure 22**). Additionally, it has been shown that fractalkine can indirectly enhance endothelial cell- leukocyte interactions; indeed, stimulation of endothelial cells has been shown to enhance the adhesion and migration of neutrophils, by the upregulation of ICAM-1 [380]. Although there is somewhat limited knowledge of the regulation of CX3CR1 in many cell types and disease models, IFN-γ, MCP-1 and IL-10 can upregulate CX3CR1 on human peripheral monocytes, and on the human leukaemia cell line, THP-1 [402]. Conversely, key modulators of CX3CL1 expression, shown using *in vitro* stimulation of endothelial cells, have been identified as tumour necrosis factor-α (TNFα), interleukin (IL)-1β and interferon-γ (IFN-γ) [403, 404].

Finally, growing evidence suggests that the promotion of cell survival may be a key function of CX3CL1 operating under both homeostatic and inflammatory conditions (reviewed by [405]). In line with this, CX3CL1 has shown to play a key role as a survival factor for monocytes and T cells, in models of atherosclerosis and allergic asthma, respectively [372, 406]. CX3CL1-CX3CR1 interactions have since been shown to be important in a number of relevant diseases to influenza infection; CX3CL1 expression is increased in chronic obstructive pulmonary disease COPD [407], and the expression of CX3CL1 was found to be

strongly associated with the recruitment of CX3CR1^{+} cells into the lung parenchyma of mice during cigarette smoke-induced emphysema [399]. Johnson et al. have additionally reported elevated CX3CR1 and CX3CL1 expression in the lungs of respiratory syncytial virus (RSV)-infected mice [408], and subsequently, using transgenic CX3CR1$^{gfp/gfp}$ mice, which lack CX3CR1 expression [301], the authors demonstrated that the infiltration of Gr-1^{+} cells, as well as NK cells, was dependent on CX3CR1 expression [408]. One last observation which may be noteworthy for our hypothesised role of CX3CR1 during influenza infection, is the finding that alveolar hypoxia, which can result from blocked airways and damage to pulmonary structures for the oxygen-CO_2 exchange, constitutes a stimulus for EC activation and circulating monocyte migration in the lung [409]. A local hypoxic microenvironment may thus serve as a further stimulus to promote the trans-endothelial migration of leukocytes [410].

In light of the literature discussed, and the established migration and accumulation of cells known to express CX3CR1 or CX3CL1 during influenza infection, we examine the expression and relevance of the CX3CR1-CX3CL1 axis during IAV infection. This study will thus determine whether these cells could play a role in the persistence and prolonged activation during highly pathogenic infection.

4.1. Results

4.1. Influenza infection increases infiltration of CX3CR1^{+} cells

As previously discussed, CX3CR1 demonstrates broad expression across a number of haematopoietic cells in the naïve state, which can play a role during infection, inflammation and cancer. To assess whether CX3CL1-CX3CR1 signalling was relevant during influenza

infection we firstly utilised CX3CR1$^{gfp/+}$ mice [301] to assess the localisation and expression levels of CX3CR1 on infiltrating cells in IAV-infected lungs (**Figure 23a**).

FACs analysis of the innate and adaptive immune cells of the lungs in the naïve mouse demonstrated that CX3CR1 is expressed on a broad range of cells. In particular, we found CX3CR1-expressing inflammatory monocytes, interstitial macrophages, alveolar macrophage and B cells in the naïve mice, and following influenza infection, these CX3CR1-expressing populations were expanded in the lung (**Figure 23b**). Although a minor subset of neutrophils, as well as patrolling monocytes, DCs, CD3$^+$ T cells and NK cells expressed CX3CR1, we did not observe a significant change in expression following influenza infection (**Figure 23b**).

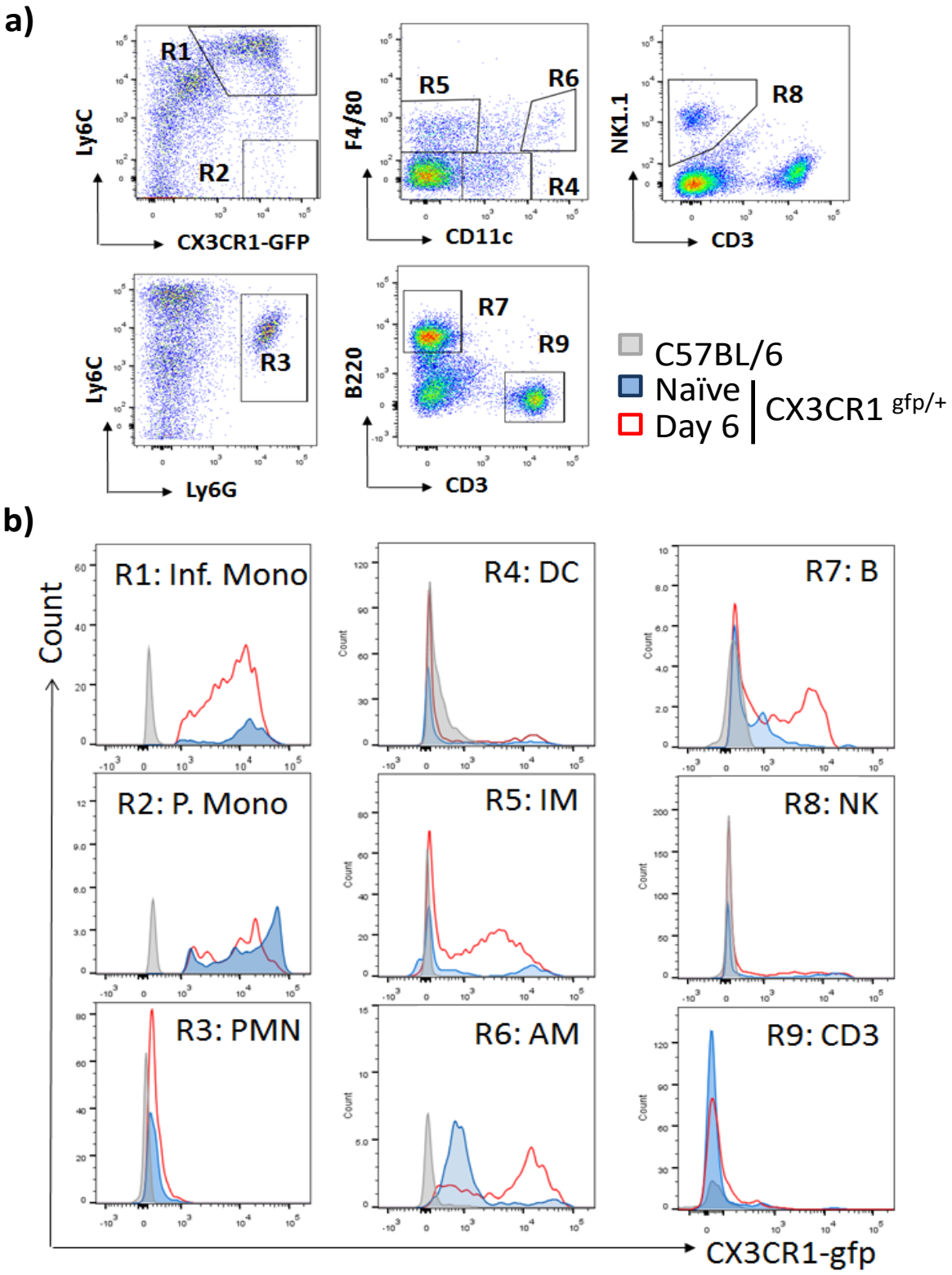

a)
R1
R2
Ly6C
CX3CR1-GFP
R5
R6
R4
F4/80
CD11c
R8
NK1.1
CD3
R3
Ly6C
Ly6G
R7
R9
B220
CD3
C57BL/6
Naïve
Day 6
CX3CR1 gfp/+
b)
Count
R1: Inf. Mono
R4: DC
R7: B
R2: P. Mono
R5: IM
R8: NK
R3: PMN
R6: AM
R9: CD3
CX3CR1-gfp

Figure 23. Analysis of CX3CR1 expression on cell populations infiltrating the lungs of PR8-infected CX3CR1$^{gfp/+}$ reporter mice.

(a) Gating strategy used to identify the cells infiltrating the lungs in naïve or influenza infected mice. (b) Representative histograms showing the expression of CX3CR1-gfp *on cells infiltrating the lungs in naïve or influenza infected mice. Plots are representative of 3 experimental repeats. P. Mono, patrolling (classical) monocytes; Inf. Mono, non-classical (inflammatory) monocytes; PMN, neutrophils; DC, dendritic cell; IM, interstitial macrophage; AM, alveolar macrophage; NK, natural killer cell; B, B cell; CD3, CD3$^+$ T cell.*

4.2. CX3CR1-deficient mice exhibit enhanced resistant to influenza infection

Figure 23 confirms the expression of CX3CR1 in the lung during influenza infection. To determine whether this expression has a functional impact on disease progression, as shown with other infection or inflammation models [399, 408], we infected both CX3CR1$^{gfp/gfp}$ mice and CX3CR1$^{gfp/+}$ with PR8, and assessed weight loss and the infiltration of cells in the lung, which are strong indicators of the intensity of infection (**Figure 24**.). Strikingly, we observed a significantly greater resistance of CX3CR1$^{gfp/gfp}$ mice to influenza infection, with CX3CR1$^{gfp/gfp}$ mice demonstrating a reduced, or delayed, loss of weight (**Figure 24a**), and an increased overall survival rate (**Figure 24b).** In addition, we assessed the total infiltration of leukocytes in the lung, and also as a control, in the bone marrow, and found that there is a significantly greater infiltration of immune cells in the lungs in CX3CR1-sufficient animals (CX3CR1$^{gfp/+}$) compared with their CX3CR1$^{gfp/gfp}$ controls (**Figure 24c, d**). We did not observe any difference in the bone marrow; these data firstly indicate the cellular effect of CX3CR1 is local to the lungs, but additionally, the comparable numbers of cells in the naïve mice in both the lungs and bone marrow is consistent with these mice exhibiting normal phenotype in the naïve state [301].

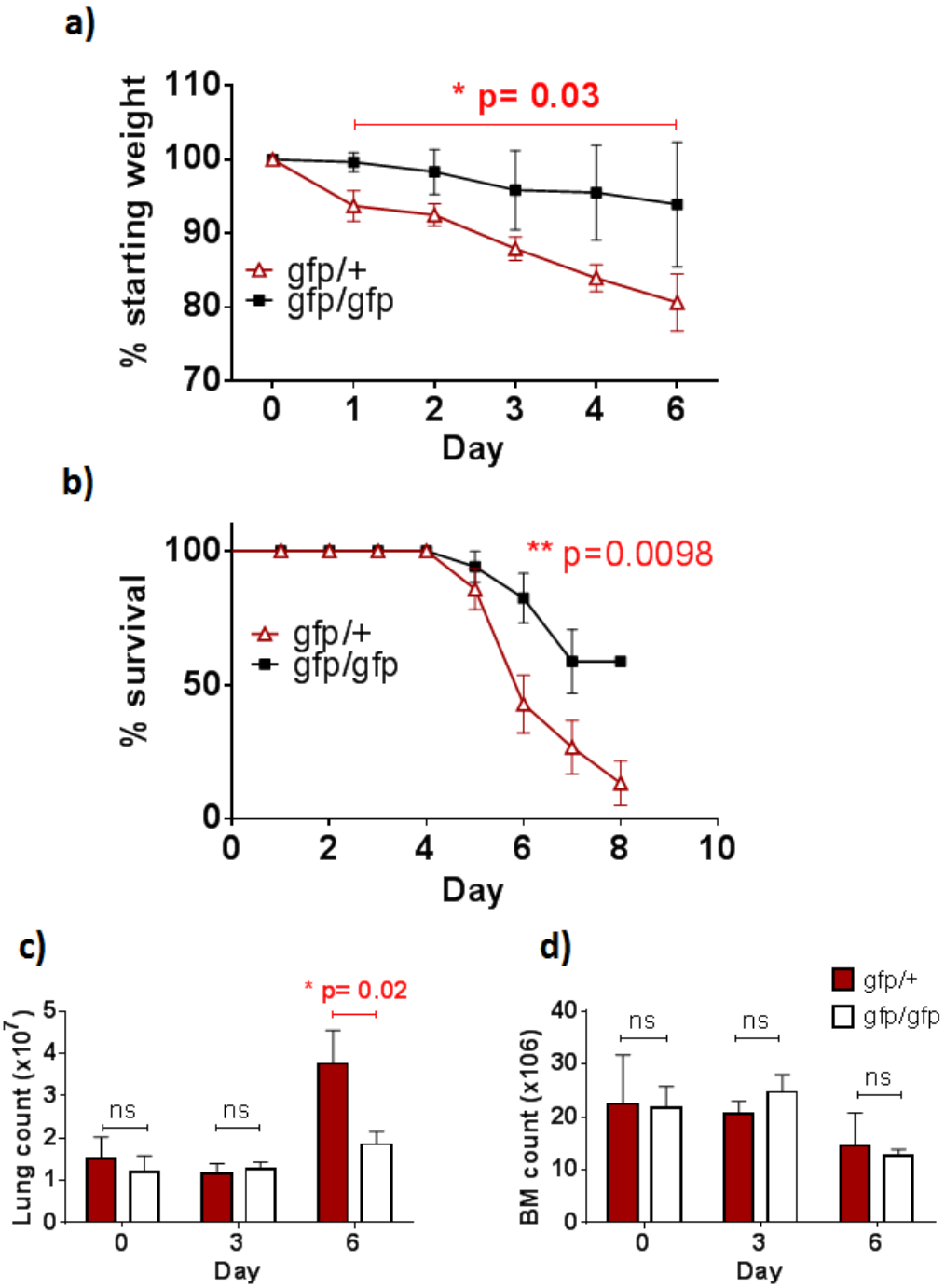


Figure 24. CX3CR1-depletion correlates with reduced weight loss, and increased survival during PR8 infection.

(a) Weight changes in mice over the PR8-infection period. Mice were weighed every day, and values given are a percentage of the starting weight (b) Survival curve of CX3CR1$^{gfp/gfp}$ or CX3CR1$^{gfp/+}$ mice following infection with 1x10^{5} PFU of PR8. Data combine three

independent experiments. Graphs showing the absolute cell counts in the (c) lung and (d) bone marrow following influenza infection. Cells were isolated as previously described, and manually counted using a haemocytometer. Data representative of 5 experimental repeats, 3-5 animals per group. For (a) and (b), statistical analysis was performed using a one-way ANOVA. For (c) and (d), statistical analysis was performed using a two tailed student's t test. (: p<0.05, **p<0.01). Error bars represent standard deviation.*

4.3. CX3CR1 expression enhances accumulation of myeloid cells in the lung during influenza infection

We have shown in chapter 3. that excessive accumulation of immune cells, particularly innate responders such as monocytes and neutrophils, correlates with increased lung pathology and worsened disease outcome **[41, 127] (Figure 6)**. In light of both our observation of CX3CR1 expression on the surface of many influenza-associated immune cells (**Figure 23b**), and the reported function of the CX3CR1-CX3CL1 signalling in cell migration and survival, our next step was to assess how the expression, or lack of expression, of CX3CR1 affected the infiltration of influential innate and adaptive immune populations during influenza infection (**Figure 25**).

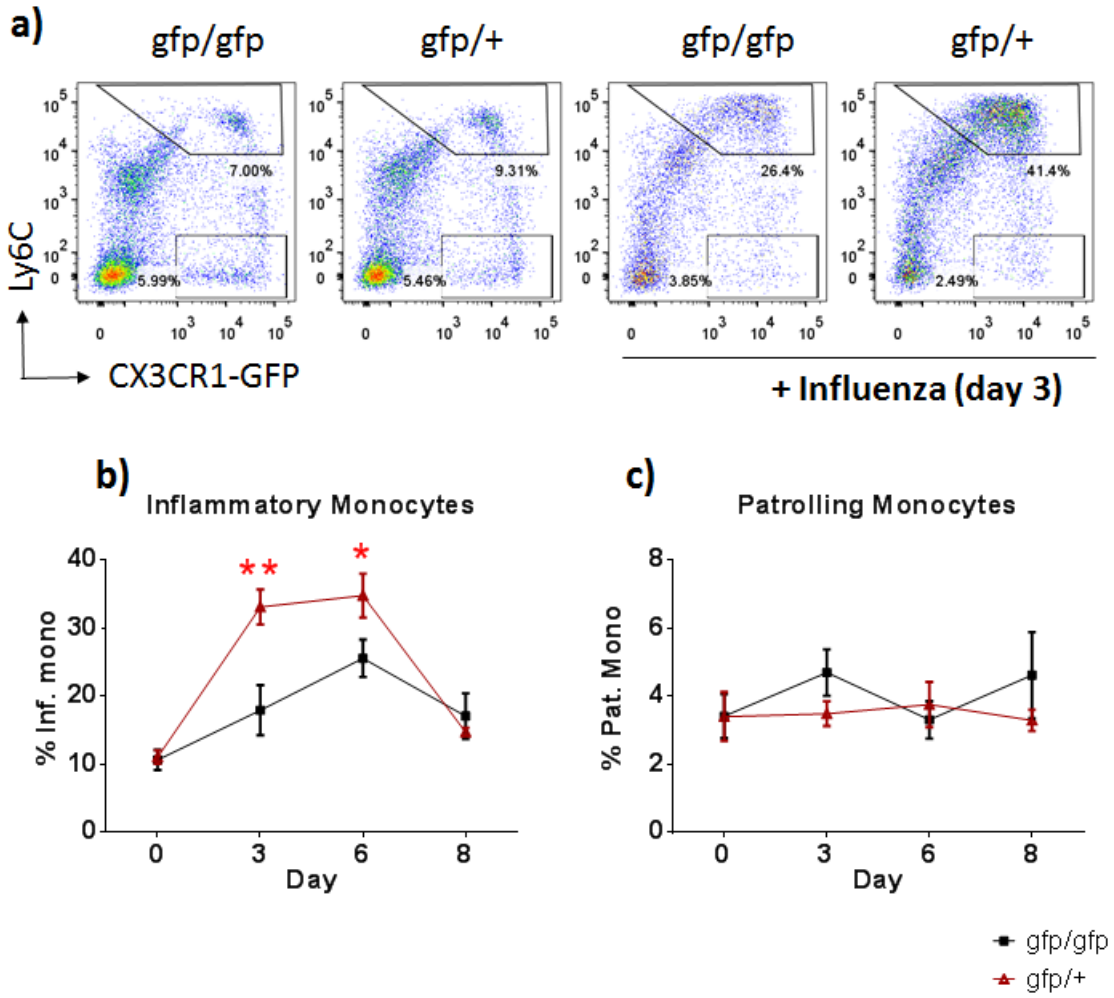


Figure 25. Analysis of the effect of CX3CR1-deficiency on monocyte infiltration into the lungs of PR8-infected mice.

*(*a) Representative FACS plots of monocyte infiltration into the lungs of naïve or influenza infected CX3CR1$^{gfp/+}$ or CX3CR1$^{gfp/gfp}$ mice. Graphs summarising infiltration of inflammatory monocyte (b), or patrolling monocyte (c) in the lungs of naïve or PR8-infected CX3CR1$^{gfp/+}$ (red line) or CX3CR1$^{gfp/gfp}$ (black line) mice (1x10^5 PFU). Plots are representative of 5 experimental repeats, with 3-5 animals per experiment. Inf. Mono, inflammatory monocyte; Pat. Mono, patrolling monocytes. Statistical analysis was performed using a one-way ANOVA (*: $p<0.05$, **$p<0.01$). Error bars represent SEM.

Remarkably, our data demonstrated that CX3CR1-deficiency during influenza infection significantly reduced the infiltration of inflammatory monocytes both at day 3 and day 6 following influenza infection, with cell numbers returning to comparable levels by disease resolution at ~ day 8 (**Figure 25b**). Intriguingly, we notice no difference in the percentages of patrolling monocytes; this is thought-provoking since these cells are CX3CR1^{high}, compared with CX3CR1^{low} expression in inflammatory monocytes [376]. Nonetheless, despite their reported patrolling behaviour, which allows rapid tissue invasion by monocytes in case of damage and infection, and differentiation into macrophages [374], patrolling monocytes are not the predominant monocyte population involved in influenza infection, which may explain why this population is not significantly altered in either strain (**Figure 25c).**

In line with the known reciprocal relationship between neutrophils and monocytes during the innate immune response ([411]), we progressed to assess the infiltration of neutrophils in the absence of CX3CR1 expression (**Figure 26).** As with inflammatory monocytes, we showed that recruitment of neutrophils is diminished in the absence of CX3CR1 expression (**Figure 26b**), with minimal recruitment of neutrophils in CX3CR1$^{gfp/gfp}$ mice, compared with 10-15% in CX3CR1$^{gfp/+}$ mice.

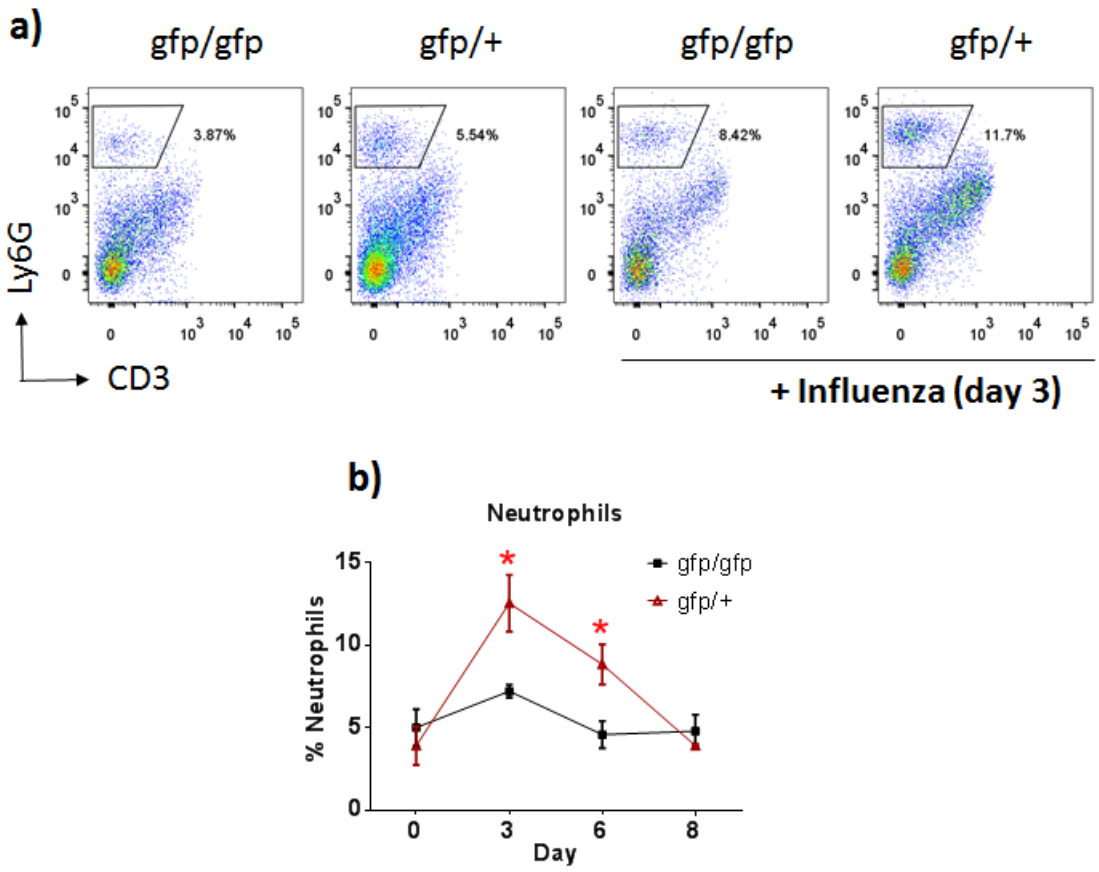


Figure 26. Analysis of CX3CR1-deficiency on neutrophil infiltration into the lungs of PR8-infected mice.

(a) Representative FACS plots of neutrophil infiltration into the lungs of naïve or PR8-infected CX3CR1$^{gfp/+}$ or CX3CR1$^{gfp/gfp}$ mice. (b) Graph summarising infiltration of neutrophils into the lungs of naïve or influenza infected (1x10^5 PFU) CX3CR1$^{gfp/+}$ (red line) or CX3CR1$^{gfp/gfp}$ (black line) mice. Plots are representative of 5 experimental repeats, with 3-5 animals per experiment. Statistical analysis was performed using a one-way ANOVA (: p<0.05). Error bars represent SEM.*

In addition to the infiltration of monocytes and neutrophils during influenza infection, infiltration and activation of APCs, such as DCs and macrophages is vital for the activation of

the adaptive immune response. We have previously demonstrated that CX3CR1-deficiency alters the initial immune response to influenza **(Figure 24)**, and also that CX3CR1 is expressed on DCs and macrophages (**Figure 23b**). Taken together, these data suggest that CX3CR1-deficiency is likely to directly or indirectly impact the subsequent immune response to influenza. FACs assessment of cells infiltrating the lung demonstrated that, like inflammatory monocytes and neutrophils, in the absence of CX3CR1 expression interstitial macrophages (IMs) and DCs are also significantly reduced in the lung following PR8 infection (**Figure 27a&c**). In line with work by other groups [412, 413], we observed decreased alveolar macrophages (AMs) following influenza infection (**Figure 27b**), however no significant difference was observed between the two strains ($p=0.06$). Interestingly, although we observed a proportion of $CD8^+$ T cells NK cells to express CX3CR1 (**Figure 23**), we observed no difference in the percentages of $CD3^+$ T cells, $CD8^+$ T cells or NK cells following PR8 infection (**Figure 27d-f**).

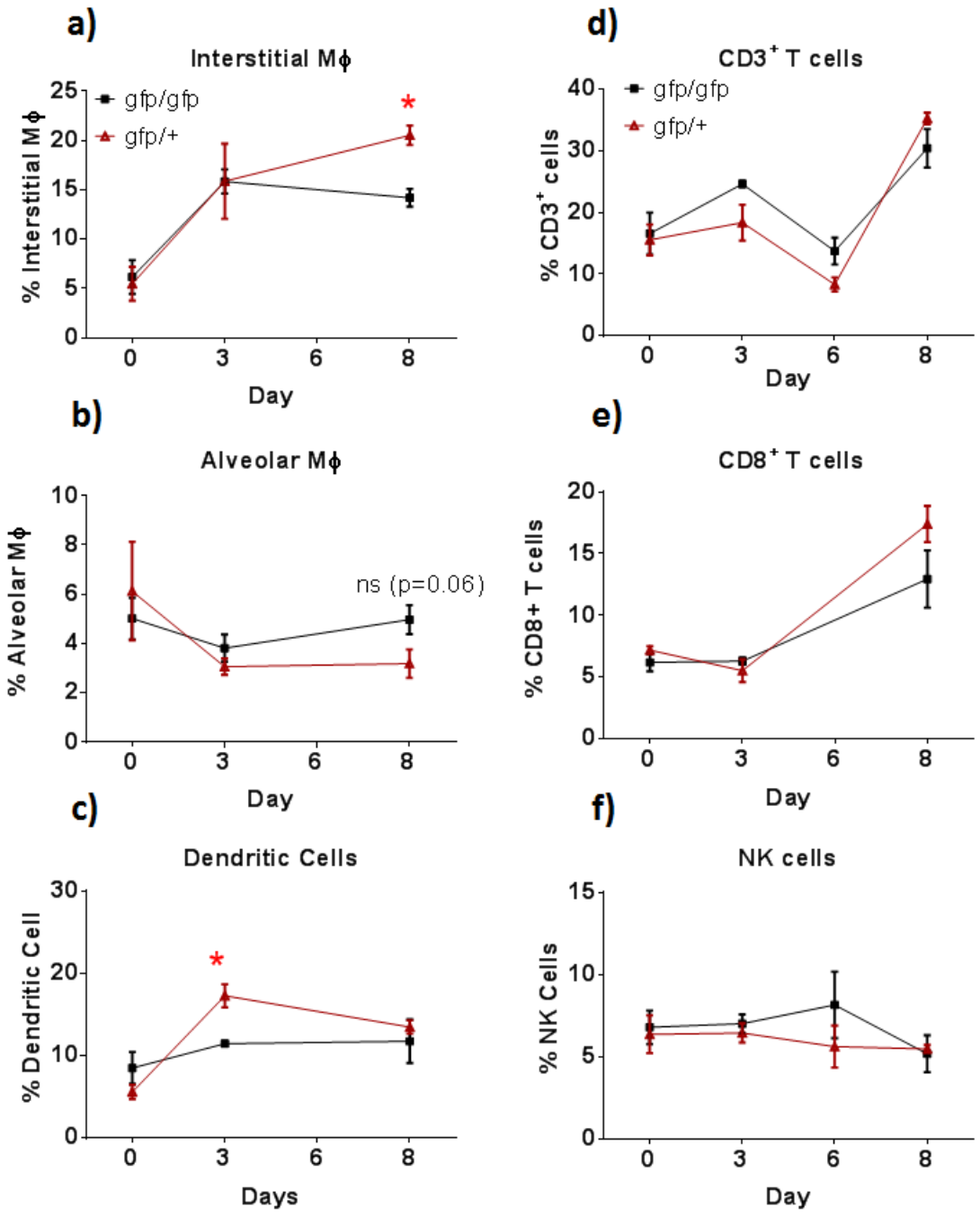


Figure 27. Analysis of the effect of CX3CR1-deficiency on antigen presenting cells and adaptive immune cells during influenza infection.

Graphs showing the infiltration of (a) interstitial macrophages (b) alveolar macrophages (c) DCs (d) CD3$^+$ T cells (e) CD8$^+$ T cells and (f) NK cells into the lungs of naïve or PR8-infected CX3CR1$^{gfp/+}$ (red line) or CX3CR1$^{gfp/gfp}$ (black line) mice. Plots are representative of 3

experimental repeats, with 3-5 animals per experiment. Statistical analysis was performed using a one-way ANOVA (: p<0.05, **p<0.01). Error bars represent SEM.*

4.4. CX3CR1-depletion does not affect cell localisation in the periphery during PR8 infection

Although CX3CR1 is not reported as the predominant chemokine associated with egress of cells from the bone marrow, it has been implicated in monocyte release from the bone marrow following chemotherapy, and is also commonly associated with leukocyte extravasation from the blood [414]. To determine whether the difference in infiltration of myeloid cells was localised to the lungs, or resulted from either a systemic difference between the two mouse strains, or retention of cells elsewhere in the body, we assessed the percentages of inflammatory monocytes, patrolling monocytes and neutrophils in the spleen and bone marrow (**Figure 28**).

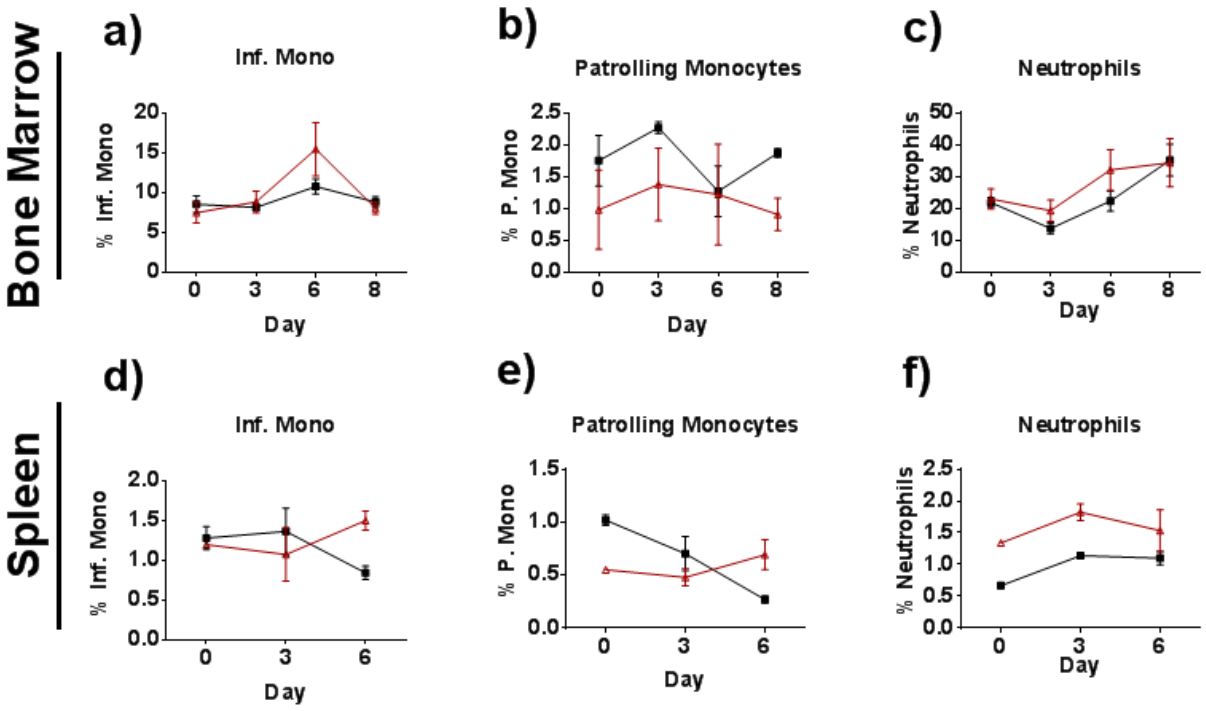


Figure 28. CX3CR1-deficiency does not affect myeloid cell numbers in the spleen or bone marrow during influenza infection.

(a-f) Graphs showing the percentages of inflammatory monocytes, patrolling monocytes and neutrophils in the bone marrow or spleen of naïve or PR8-infected CX3CR1$^{gfp/+}$ (red line) or CX3CR1$^{gfp/gfp}$ (black line) mice. Plots are representative of 3 experimental repeats, with 3 animals per group. Inf. Mono, Inflammatory monocyte; P Mono, Patrolling monocytes; Error bars represent SEM. Statistical analysis was performed using a one-way ANOVA (: p<0.05, **p<0.01).*

Figure 28 shows firstly that PR8 infection did not affect the expansion of monocytes in the bone marrow or spleen, and there was no effect of the absence of CX3CR1 on the percentages of either inflammatory monocytes, or patrolling monocytes (**Figure 28a, b, d, f**). Secondly, although we saw an expansion of neutrophils in the bone marrow following PR8 challenge, we observed no significant difference in CX3CR1$^{gfp/gfp}$ or CX3CR1$^{gfp/+}$ neutrophils

in either the bone marrow or in the spleen (**Figure 28c, f**). We additionally observed no difference in macrophages, DCs, T cells or NK cells (data not shown). This suggests that our observation of decreased cells in the lungs in the absence of CX3CR1 expression is a local effect on recruitment or survival of cells.

4.5. Comparable cytokine and chemokine responses in the absence of CX3CR1-signalling

The above data highlighted two important points. Firstly, in the absence of CX3CR1 expression, we still observed a degree of immune cell accumulation, suggesting that additional factors other than CX3CR1 play a role in the migration or accumulation of cells in the lungs **(**Figure 24**)(Figure 25)(Figure 26)(Figure 27**). Secondly, the accumulation of cells we observed in $CX3CR1^{gfp/+}$ mice correlated with a worsened disease outcome **(**Figure 24**)** as compared with $CX3CR1^{gfp/gfp}$ mice. Therefore, in an attempt to characterise factors which may be implicated in CX3CR1-independent infiltration of innate cells in $CX3CR1^{gfp/gfp}$ mice, and factors which may contribute towards IAV-induced mortality in $CX3CR1^{gfp/+}$ mice we utilised luminex analysis of the BALF fluid in each strain (**Figure 29**).

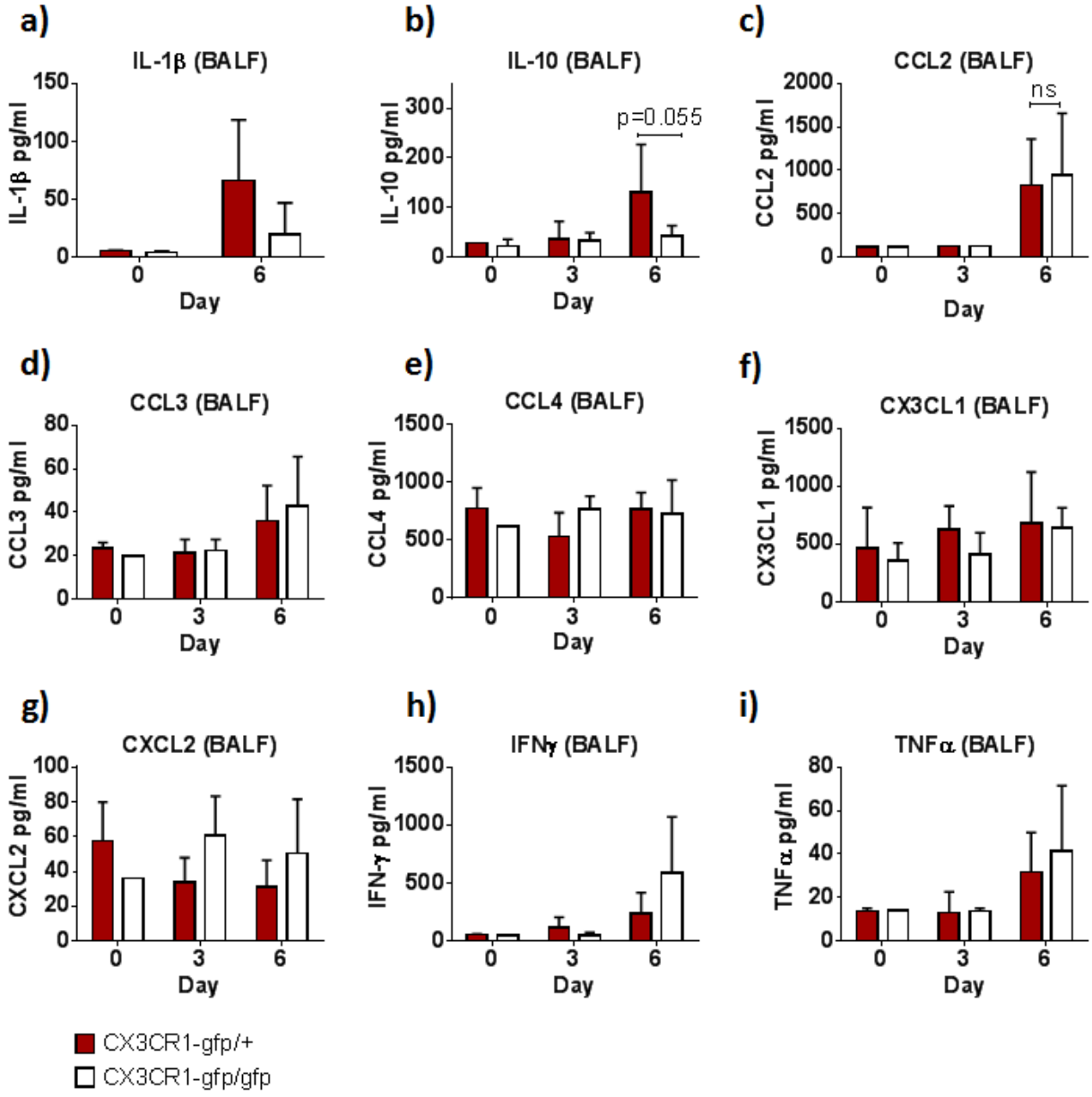


Figure 29. Comparable chemokine and cytokine release into the BALF of influenza infected CX3CR1$^{gfp/gfp}$ and CX3CR1$^{gfp/+}$.

Mice were infected with 1.0 x 10^5 PFU PR8 for 3 or 6 days, and BALF was removed and analysed using a luminex to assess for cytokine and chemokine release. IL-10, IL-1β and CX3CL1 were assessed using ELISA. Data represent two experimental repeats, 3 mice per group. Statistical analysis was performed using a student's t test. Error bars represent standard deviation.

Analysis of the BALF suggested there is no significant difference in chemokine or cytokine release between CX3CR1$^{gfp/gfp}$ and CX3CR1$^{gfp/+}$ mice (**Figure 29**). Notably, CCL2, an important chemokine in monocyte migration during influenza infection [127], is elevated following infection, but is comparable between the two strains. IL-1β and IL-10 appeared to be slightly elevated in CX3CR1$^{gfp/+}$ mice, as compared with CX3CR1$^{gfp/gfp}$ mice, however the difference does not reach significance (**Figure 29a, b**).

4.6. Lack of CX3CR1 expression does not affect CTL responses or viral clearance

CD8$^+$ cytotoxic T lymphocytes (CTLs) are a vital component of the adaptive immune response to influenza infection. CD8$^+$ T cells showed minimal expression of CX3CR1 in naïve or infected CX3CR1$^{gfp/+}$ mice (**Figure 23**), however we are currently unaware of how CX3CR1-deficiency could affect the function of adaptive immune cells, which can be determined by the presence and maturation state of APCs. Therefore, we progressed to determine whether differential infiltration of influenza-specific CD8$^+$ T cells could contribute to the worsened outcome in CX3CR1$^{gfp/+}$ mice. Despite the differences in accumulation of APCs infiltrating the lungs of CX3CR1 transgenic mice, we observed comparable infiltration of NP-specific CD8$^+$ T cells (**Figure 30**).

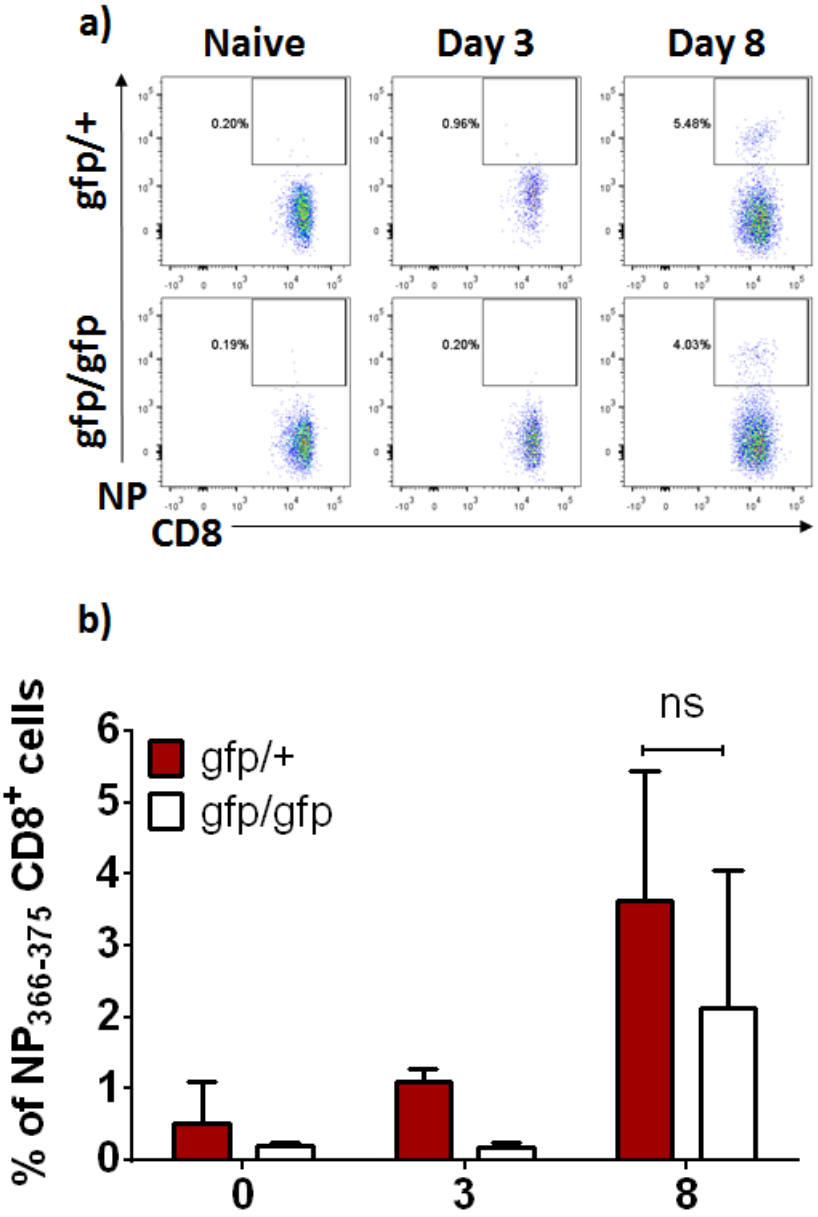


Figure 30. CX3CR1- deficiency does not affect the infiltration of virus-specific CD8⁺ T cells.

The percentage of NP-specific CD8⁺ T cells was analysed at day 3 and day 8 following infection with 1.0x10⁵ PFU of PR8 using flow cytometry. a) Representative FACS plots of the percentage of NP-specific T cells gated on CD3⁺ CD8⁺ cells. b) Graphical summary of the percentage of NP-specific T cells. 3 animals were used per experimental group. Error bars represent standard deviation. Statistical analysis was carried out using student's t test.

To further assess the impact of CX3CR1 on the IAV-induced immune response, and whether the absence of CX3CR1 expression, or the CX3CR1-mediated reduction in innate cell recruitment could affect viral clearance, we next assessed the viral load in the lungs following infection in either influenza-infected CX3CR1$^{gfp/gfp}$ or CX3CR1$^{gfp/+}$ mice (**Figure 31**). We observed no clear difference in viral load at day 3-post infection. Conversely, we found that CX3CR1$^{gfp/gfp}$ mice had cleared the virus, whereas CX3CR1$^{gfp/+}$ had a small but significant viral load still present (**Figure 31a**).

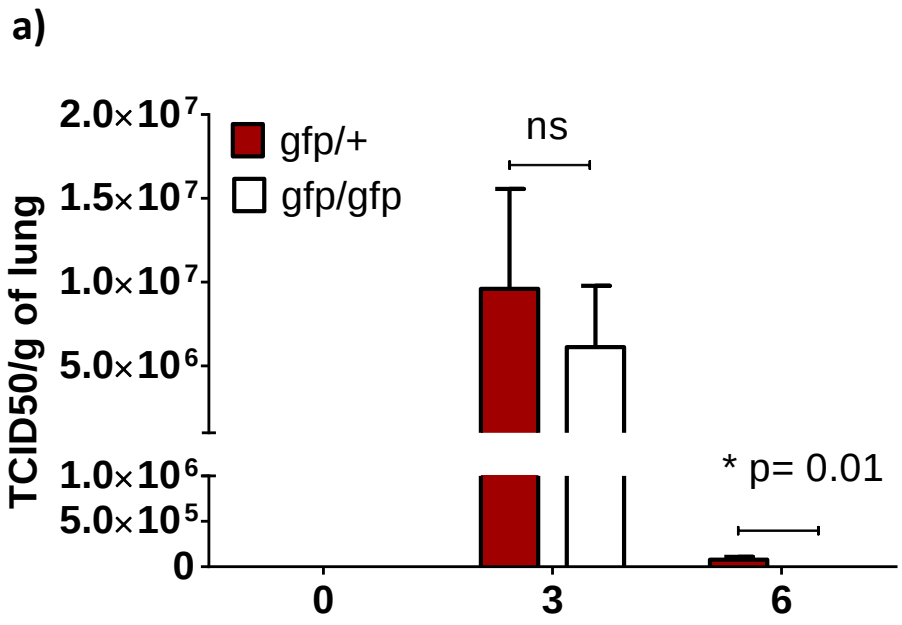


Figure 31. Viral titers in the lungs of influenza infected CX3CR1$^{gfp/gfp}$ or CX3CR1$^{gfp/+}$ mice.

(a) Lung homogenates were assayed for viral load 3 and 6 days after infection. Results were calculated using TCID50 analysis followed by haemagluttination assay, and viral loads were normalised to weights of lung. Representative of one experimental repeat, with 3 mice per experimental group. Error bars represent standard deviation. Statistical analysis was performed using the student's t test.

4.7. CX3CL1 expression is elevated in the absence of iNKT cells

As described throughout this work, the causes of the exacerbated immune response in the absence of iNKT cells remain unclear. Since we have now demonstrated that CX3CR1 expression promotes innate cell accumulation during influenza infection, we questioned whether this signalling pathway could be implicated in the recruitment of cells observed in the absence of iNKT cells. The dysregulation of chemokines in this infection model is a plausible suggestion, since we, and others, have shown that chemokines such as CCL2 (MCP-1) are

elevated in the absence of iNKT cells **(Figure 7)** [127]. As yet, CX3CR1-CX3CL1 signalling has not been investigated during influenza infection in the absence of iNKT cells.

To firstly address this hypothesis, we analysed expression of the fractalkine ligand, CX3CL1 in the lung of influenza infected C57BL6 or *Jα18*$^{-/-}$ mice. Excitingly, and in support of the expression of CX3CL1 on lung epithelial cells [302], we observed significantly elevated message levels following infection with both PR8 (H1N1) or X31 (H3N2) influenza in *Jα18*$^{-/-}$ mice, where expression was significantly greater than in influenza-infected C57BL/7 mice (**Figure 32a, b**). In line with this result, we also observed elevated release of soluble CX3CL1 into the BALF following PR8 infection in the absence of iNKT cells (**Figure 32c**). Adam10 and Adam17 have been reported to facilitate the cleavage of CX3CL1 into the soluble form [382, 392, 394]. To assess whether these factors were also differentially modulated in the absence of iNKT cells, we carried out preliminary analysis of expression of these using qPCR (**Figure 32d, e**). Unlike CX3CL1, we observed no change in message expression of either factor in response to influenza infection, and no difference between the C57BL/6 or *Jα18*$^{-/-}$ mice.

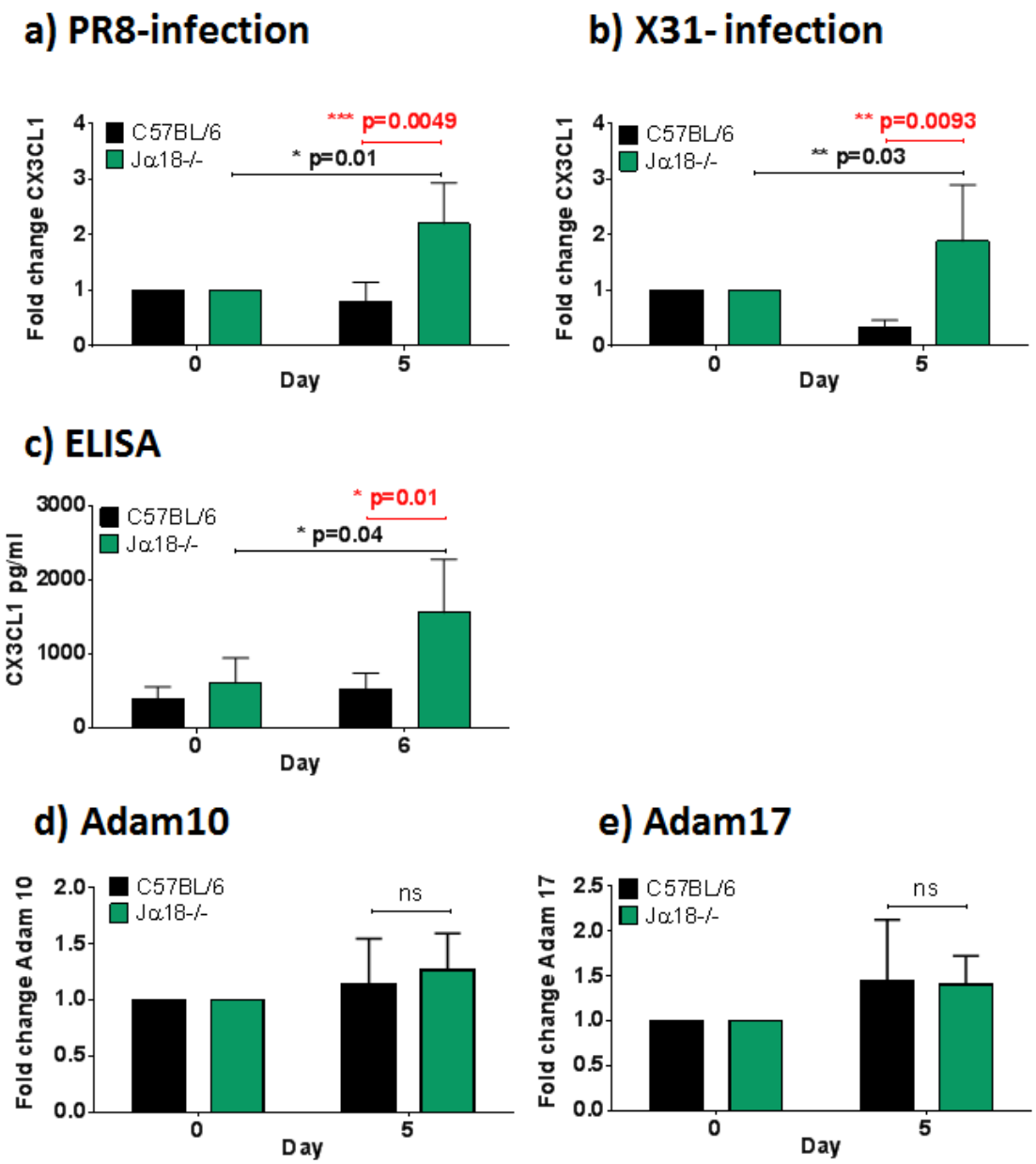


Figure 32. CX3CL1 expression in whole lung is elevated in the absence of iNKT cells

Whole lungs were removed from naïve or PR8-infected (a) or X31-infected (b) mice and analysed for CX3CL1 message expression by qPCR. (c) sCX3CL1 release into the BALF following PR8 infection in C57BL/6 or Jα18$^{-/-}$ mice. qPCR analysis of Adam10 (d) and Adam17 (e) expression. qPCR samples were normalised to the relevant naïve control. Plots are representative of 2 experimental repeats, with a total of 6 animals analysed. Error bars

represent SEM. Statistical analysis was performed using a student's t test (p<0.05, **p<0.01 ***p<0.005).*

In an attempt to characterise which cells were most likely signalling via the CX3CR1-CX3CL1 axis during influenza infection, we sorted innate immune cells from the lungs of *Jα18*$^{-/-}$ mice, namely inflammatory monocytes and neutrophils, and assessed them by qPCR. Although haematopoietic cells are predominantly though to express CX3CR1, in light of the recent discoveries of CX3CL1 expression in a number of lymphocyte populations, we analysed both CX3CR1 and CX3CL1 expression (**Figure 33**).

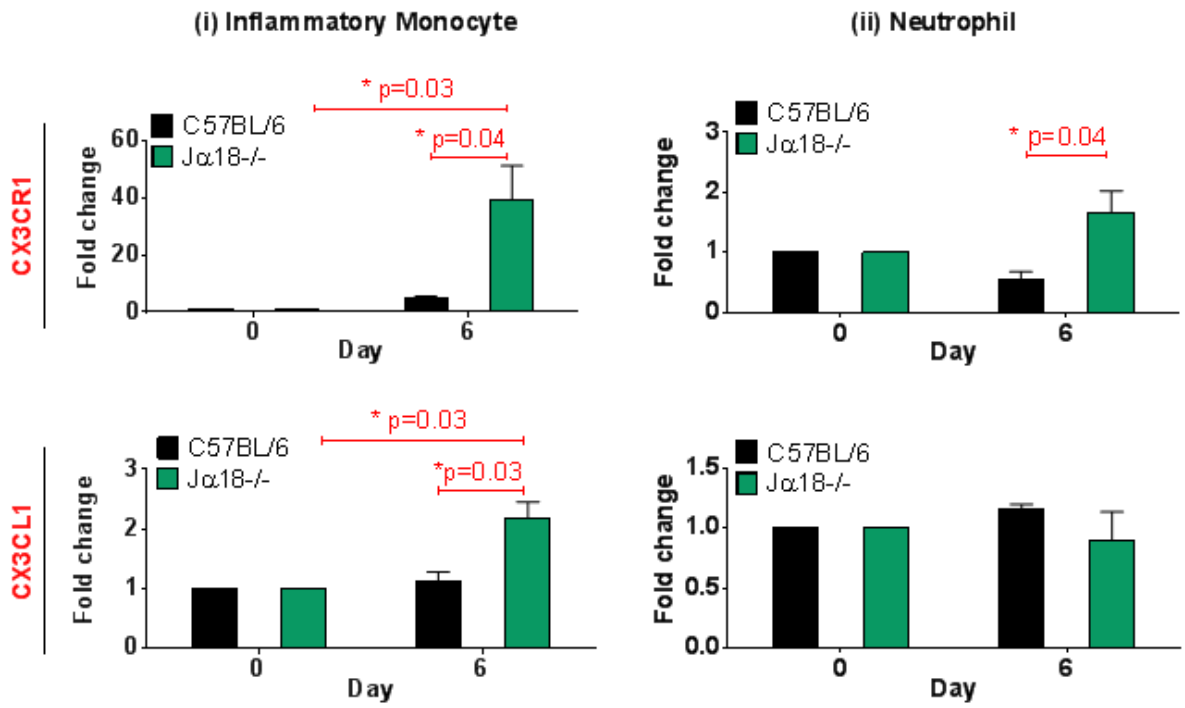


Figure 33. Greater expression of CX3CL1 message in inflammatory monocytes and neutrophils isolated from the lungs of *Jα18*$^{-/-}$ mice compared with C57BL/6 mice.

Mice were infected with 1.0x10^5 PFU PR8 for 6 days. Inflammatory monocytes (i) and neutrophils (ii) were sorted by FACS, and analysed for CX3CR1 and CX3CL1 expression by qPCR. Samples were normalised to their respective naïve control. N= 3-5 mice per group. Statistical analysis was performed using a student's t test (p<0.05, **p<0.01). Error bars represent SEM.*

Excitingly, we observed significantly elevated message levels of CX3CR1 and CX3CL1 in inflammatory monocytes isolated from *Jα18*$^{-/-}$ mice, as compared with C57BL/6 mice. We observed a significant elevation of CX3CR1 in neutrophils isolated from *Jα18*$^{-/-}$ mice, however this was not significantly greater than the expression observed in naïve *Jα18*$^{-/-}$ mice. We additionally observed no difference in CX3CL1 message in neutrophils. Granulocytes, such as mast cells, eosinophils, and neutrophils, continuously synthesize cytokines from

early stages of cell development, and then package these together with other inflammatory mediators into secretory granules or small vesicles, which can be later released following appropriate stimulation (reviewed by [415]). Additionally, it has been shown that the cytoplasmic tail of CX3CL1 facilitates its constitutive clathrin-mediated endocytosis, regulation which enables intracellular storage of a sizable pool of presynthesized CX3CL1 that protects the chemokine from degradation by metalloproteases at the plasma membrane [416]. As such, the fact that we failed to see transcriptional differences in neutrophils does not mean that CX3CL1 expression cannot be altered at the protein level. We therefore assessed the surface expression of inflammatory monocytes and neutrophils from the lungs of influenza infected C57BL/6 and *Jα18*$^{-/-}$ mice. Unfortunately, antibody staining using anti CX3CR1 on murine myeloid cells turned out to be highly non-specific, and thus, we focused on staining for the ligand, CX3CL1.

In support of the findings showing elevated message expression of CX3CL1, we found that both inflammatory monocytes and neutrophils significantly elevated CX3CL1 surface expression following influenza infection (**Figure 34a**). Furthermore, we found that cells isolated from *Jα18*$^{-/-}$ mice exhibited a greater infiltration of CX3CL1^{+} cells, as compared with C57BL/6 mice, with the greatest percentage of cells observed at day 6 post PR8-infection (**Figure 34b**).

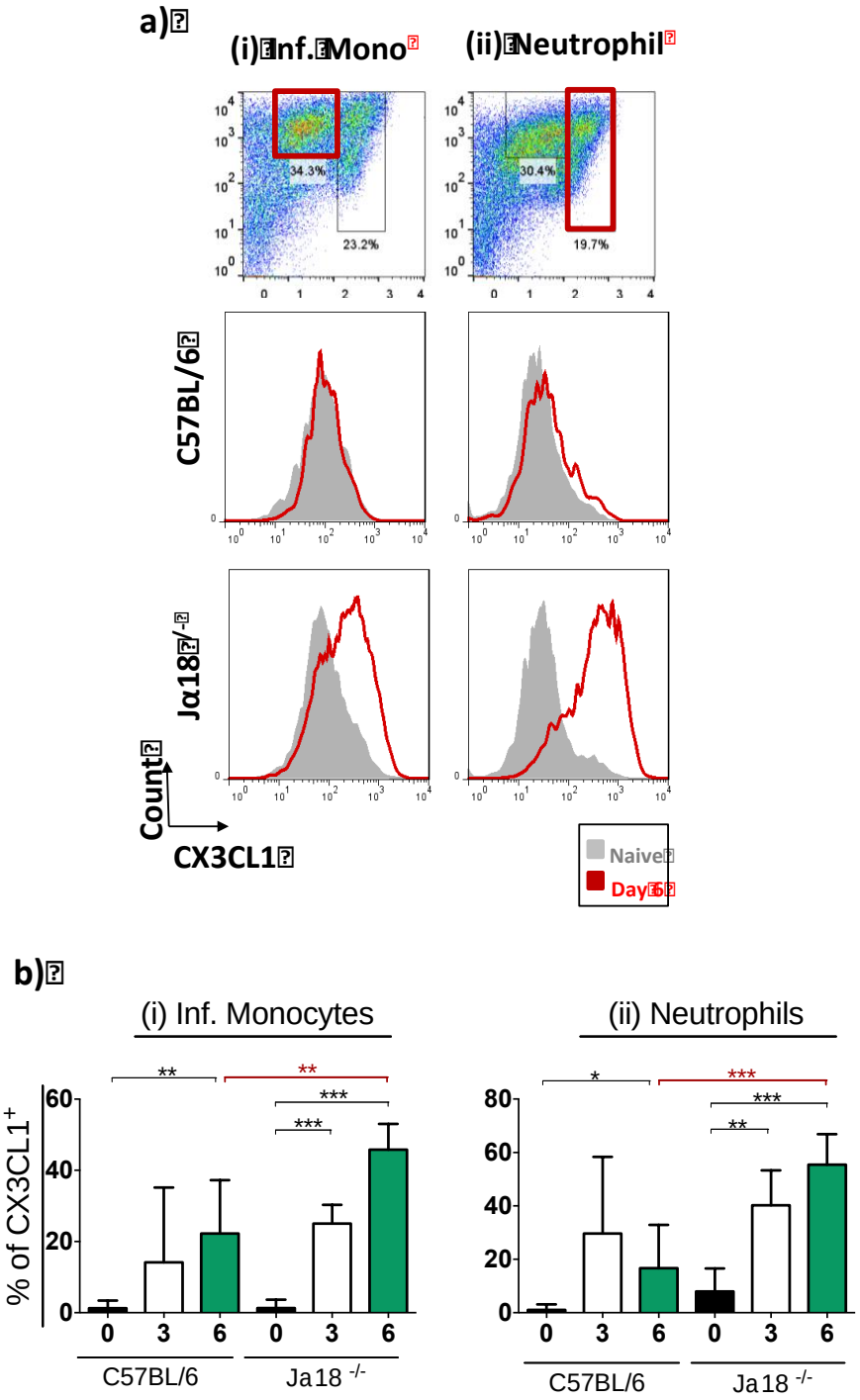


Figure 34. Greater infiltration of CX3CL1^{+} cells in the lungs *Jα18*$^{-/-}$ mice compared with C57BL/6 mice

(a) Whole lungs were removed from naïve or PR8-infected mice and analysed for CX3CL1 expression by FACs. Plots are representative of 2 experimental repeats, with a total of 6

animals depicted. Error bars represent SD. Statistical analysis was performed using a student's t test.: p<0.05, **p<0.01 ***p<0.005.*

4.8. Serum amyloid A (SAA) can modulate CX3CL1 expression in neutrophils, *in vitro*

A number of cytokines have been reported to elevate CX3CL1 expression, including IL-10, IL-1β, IFN-γ and TNF-α [381]. We have shown previously that following influenza infection in *Jα18*$^{-/-}$ mice, there is elevated IL-10 and IL-1β expression, as well as elevated SAA levels **(Figure 19)**. To our knowledge, SAA has not been identified as a regulator of the CX3CR1-CX3CL1 pathway, however neutrophils have previously shown to respond significantly to SAA stimulation, demonstrating enhanced cytokine release and migration [352, 353]; in view of this, and the potent upregulation of CX3CL1 on neutrophils during influenza infection in *Jα18*$^{-/-}$ mice, we investigated SAA signalling in neutrophils, *in vitro*. To assess the contribution of SAA on CX3CL1 upregulation, we firstly harvested and purified bone marrow-resident neutrophils from naïve C57BL/6 mice, and stimulated with recombinant SAA. We consistently achieved high CD11b^{+}Ly6C^{+}Ly6G^{+} cell purity (>97%) by this method, and granulocytic morphology was confirmed using H&E staining **(Figure 35a)**. As a negative control, and to assess whether upregulation of CX3CL1 was SAA-specific, or resulted from non-specific neutrophil activation, we stimulated neutrophils with Pam3Cys, an alternative agonist for the SAA receptor, TLR2 [417]. We found that SAA stimulation induced CX3CL1 expression on Ly6G^{+} neutrophils, with SAA stimulation resulting in a small, but significant increase in CX3CL1 message expression, as compared with naïve cells and Pam3CSK stimulated cells (**Figure 35b**). As a positive control for SAA stimulation, we measured neutrophil IL-10 message expression (**Figure 35c)** and release into the supernatant by ELISA (**Figure 35d**), confirming both stimulants were functional.

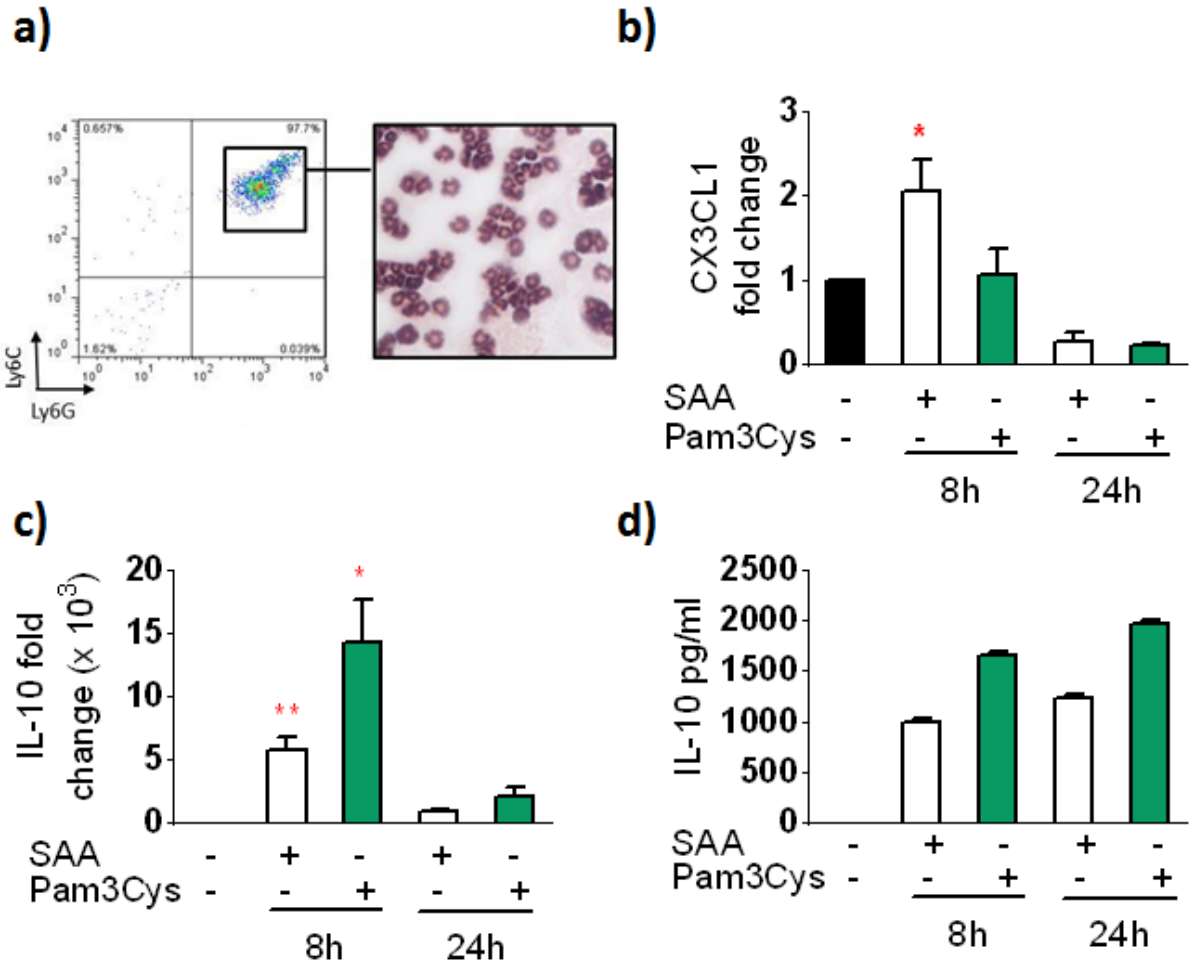


Figure 35. Murine bone marrow-resident neutrophils upregulate CX3CL1 message in response to SAA stimulation, *in vitro*.

Ly6G$^+$ neutrophils were isolated and purified from the long bones of C56BL/6 mice. a) Representative flow cytometry analysis demonstrating >97% of neutrophils purified from C57BL/6 bone marrow by Ly6G$^+$ MACs bead separation are CD11b$^+$Ly6C$^+$Ly6G$^+$. Granulocytic morphology of CD11b$^+$Ly6C$^+$Ly6G$^+$ cells was confirmed using H&E staining. Relative expression of CX3CL1 (b) and IL-10 (c) message levels following stimulation with 2μM SAA or 10μg/ml Pam3Cys, for 8h or 24h, in vitro, are analysed by qPCR. Samples were run in triplicate, using the ΔΔCt method of comparison. Data were normalised to HPRT reference gene, and subsequently to the 'untreated' group at each respective time-point. (d) Bar graph showing SAA and Pam3Cys-induced release of IL-10 from Ly6G$^+$ neutrophils, in

*vitro. IL-10 release into the supernatant was analysed by ELISA. Data representative of three experimental repeats. Error bars represent SEM. Statistical significance was analysed by two-tailed student's T-test with Welch's correction for unequal variance *p<0.05, **p<0.01.*

Utilising anti-CX3CL1 antibodies (**Figure 36ai**) or CX3CL1-mcherry reporter mice (**Figure 36aii**), we demonstrated, using FACS analysis, that 24 hour SAA-stimulation of bone marrow-derived neutrophils *in vitro* causes a significant elevation in CX3CL1 protein expession (**Figure 36b**). This was later observed using confocal microscopy, again using neutrophils isolated from CX3CL1-mcherry reporter mice (**Figure 36c**).

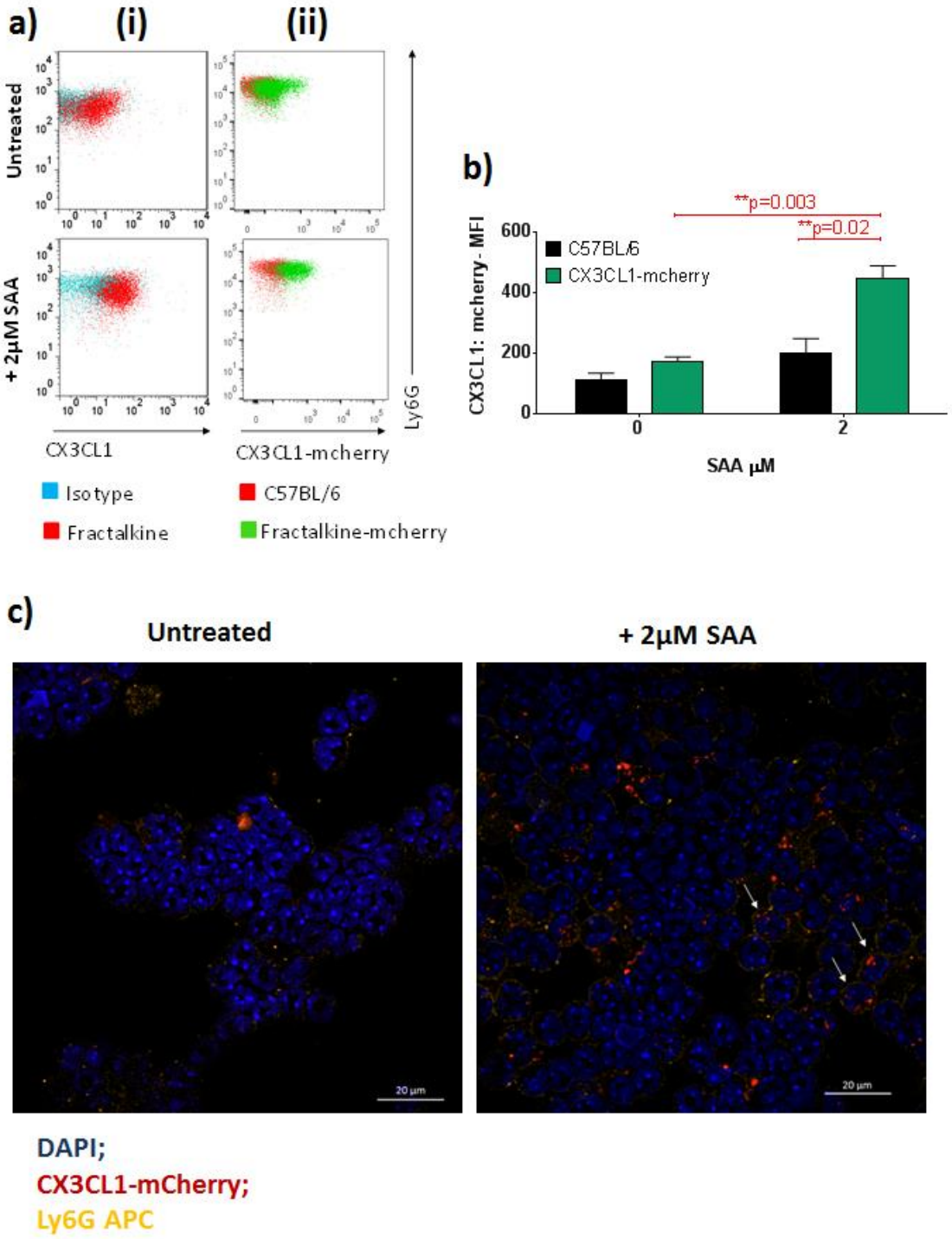


Figure 36. Bone marrow resident neutrophils upregulate CX3CL1 in response to SAA stimulation, *in vitro*.

(a) Representative FACS analysis demonstrating increase in CX3CL1 following SAA stimulation in vitro, using anti CX3CL1 antibodies (i) or CX3CL1-mcherry reporter mice (ii).

(b) Graph summarising FACs expression of CX3CL1-mcherry in neutrophils following 24h, 2μM SAA stimulation of bone marrow-derived neutrophils from CX3CL1-mcherry mice. Error bars indicate SEM. Statistical analysis was performed using student's t test. (c) Confocal image of naïve or SAA stimulated bone marrow purified neutrophils from CX3CL1-mcherry reporter mice. Examples of neutrophils with punctate CX3CL1-mcherry staining are highlighted with white arrows.

4.9. Accumulation of myeloid cells during influenza infection in the absence of iNKT cells is independent of CX3CR1-signalling

We have thus far demonstrated that CX3CR1-deficiency hampers cellular recruitment during influenza infection, and subsequently, that the upregulation of both CX3CL1 and CX3CR1 correlates with the exacerbated accumulation of cells in the lungs of influenza-infected, iNKT cell deficient mice. In light of the above findings, we questioned whether the CX3CR1-CX3CL1 signalling axis was a contributory factor in the accumulation of innate cells in the absence of iNKT cells during influenza infection **(Figure 6)**. To overcome the complications with non-specific antibody staining of CX3CR1 expression on murine myeloid cells, we generated double knockout mice (DKO) which were deficient in both iNKT cells and in CX3CR1 expression (*Jα18*$^{-/-}$CX3CR1$^{gfp/gfp}$). As a control we also generated iNKT deficient CX3CR1 heterozygous mice (*Jα18*$^{-/-}$CX3CR1$^{gfp/+}$). Utilising these DKO mice during influenza infection would determine whether the absence of CX3CR1 expression on infiltrating myeloid cells could reverse or reduce the excessive recruitment of myeloid cells observed during influenza infection in the absence of iNKT cells (**Figure 6**) [41, 127].

Prior to use, we confirmed successful iNKT cell depletion in the DKO and control animals via FACs analysis (**Figure 37a**), as well as correct CX3CR1-gfp expression by genotyping (data

not shown). Furthermore, since CX3CR1 is expressed on a broad range of cells, we evaluated naïve DKO or control mice for developmental defects which may have resulted from the breeding programme; mice appeared healthy, and litters were of expected sizes. Subsequent FACs analysis of the bone marrow and lung suggests there were no developmental defects in inflammatory monocytes, patrolling monocytes, neutrophils **(Figure 37b-g)**, or T cells, B cells and NK cells (data not shown), when compared with naïve CX3CR1$^{gfp/gfp}$ or CX3CR1$^{gfp/+}$ mice, or with C57BL/6, *Jα18*$^{-/-}$ mice (data not shown).

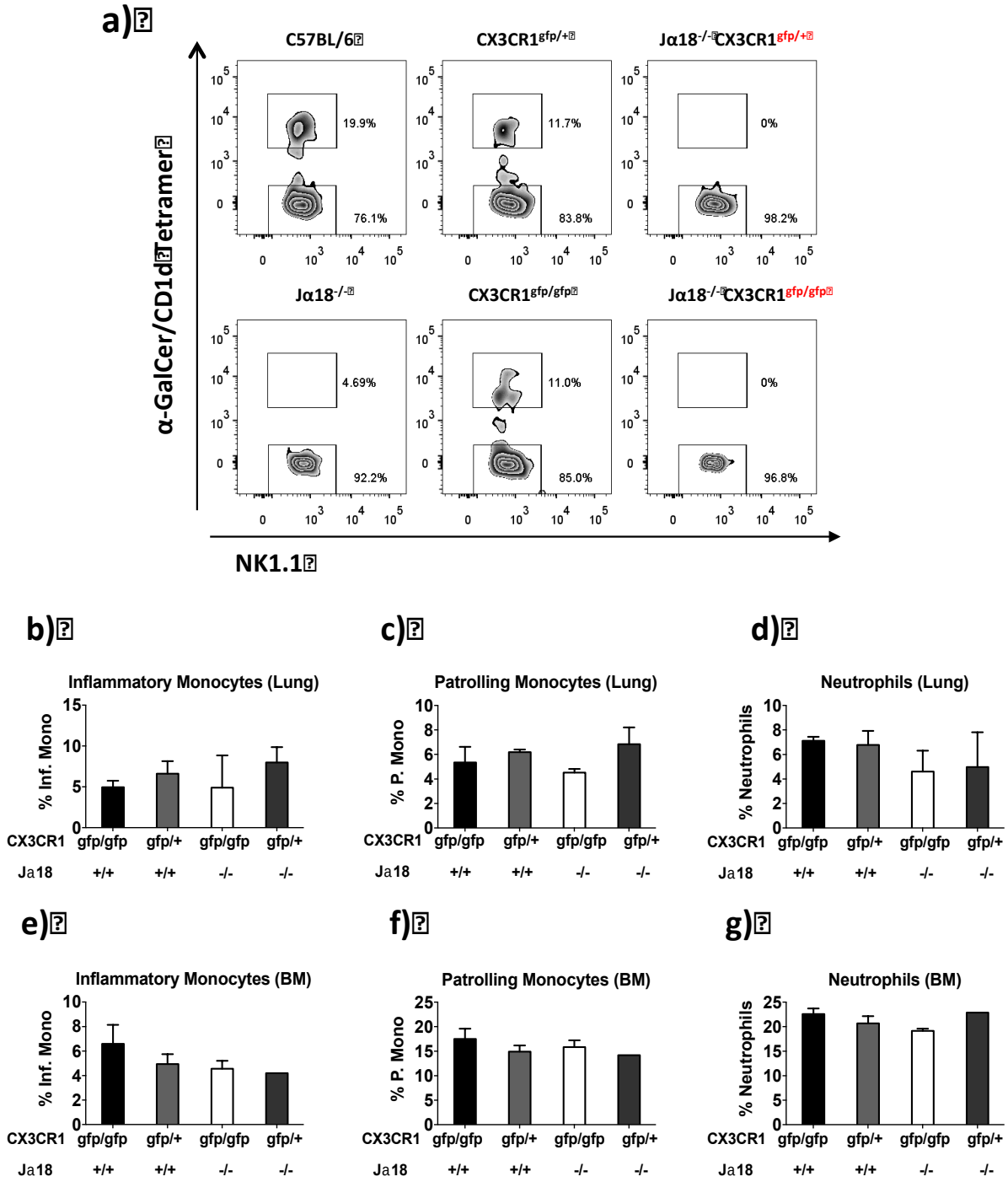


Figure 37. *Jα18*$^{-/-}$CX3CR1$^{gfp/gfp}$ double knockout mice do not exhibit defects in myeloid cell development.

(a) Bone marrow was removed from naïve DKO and control mice and analysed for iNKT cells by FACs. (b) Lungs and bone marrow (BM) were removed from naïve DKO and control mice and analysed for myeloid cell infiltration expression by FACs. Plots are representative of 2

experimental repeats, with 3 animals per group. Statistical analysis was performed using a student's t test. Error bars represent SD. BM, Bone Marrow.

We next infected our DKO and control mice with PR8 to determine whether the absence of CX3CR1 expression, particularly on myeloid cells, rescued the pathogenic phenotype previously observed in IAV-infected *Jα18*$^{-/-}$ mice.

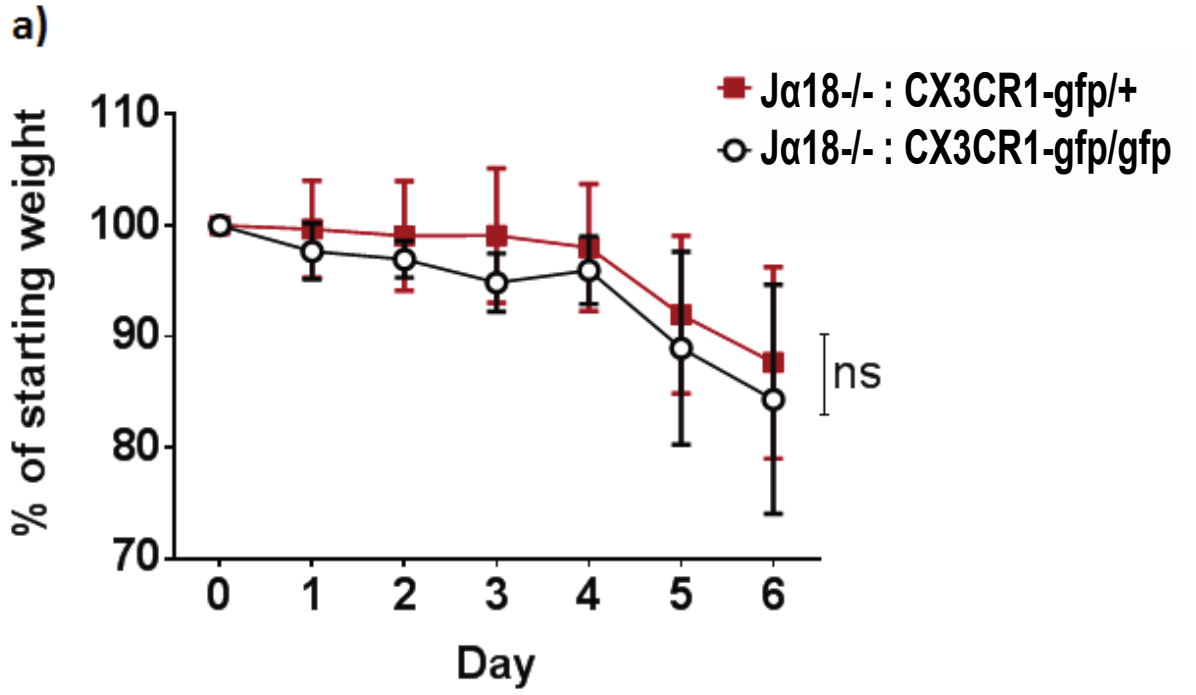


Figure 38. CX3CR1-depletion does not rescue PR8-induced mortality in *Jα18*$^{-/-}$ mice.

Jα18$^{-/-}$*CX3CR1*$^{gfp/gfp}$ *and Jα18*$^{-/-}$*CX3CR1*$^{gfp/+}$ *mice were infected with 1x10*^{5} *PFU of PR8; mice were weighed every day during the infection period, and values given are a percentage of the starting weight. Statistical analysis was performed using a two-way ANOVA.*

Analysis of weight loss during the infection period suggested that both *Jα18*$^{-/-}$CX3CR1$^{gfp/gfp}$ and *Jα18*$^{-/-}$CX3CR1$^{gfp/+}$ mice remain highly susceptible to influenza infection **(Figure 38a**). To confirm whether the absence of iNKT cells, although not affecting mortality, could modulate cellular infiltration, we progressed to assess the populations of innate cells infiltrating the lungs (**Figure 39**).

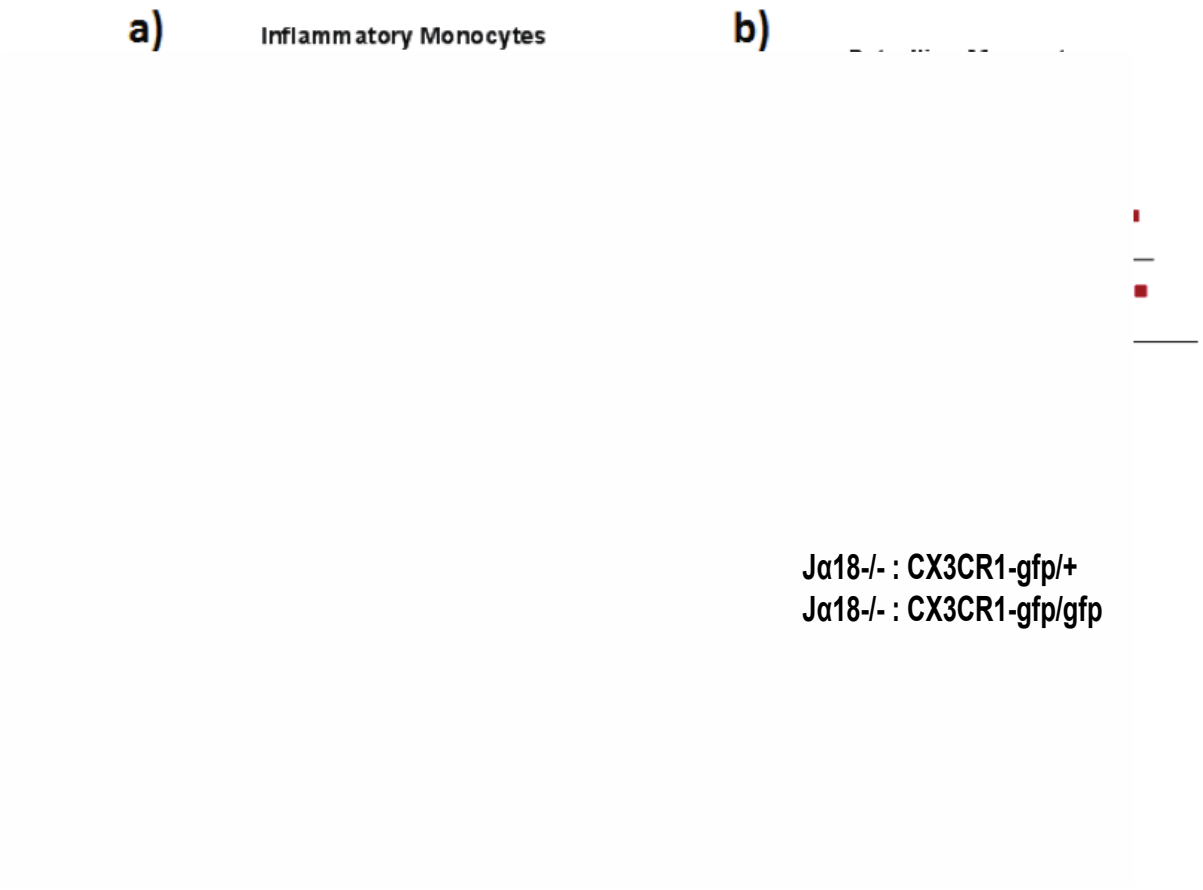


Figure 39. CX3CR1-depletion does prevent infiltration of inflammatory monocytes during IAV infection in *Jα18*$^{-/-}$ mice

Graphs summarising the infiltration of (a) inflammatory monocyte, (b) patrolling monocyte, and (c) neutrophils in the lungs of naïve or PR8-infected (1x10^{5} *PFU*) *Jα18*$^{-/-}$*CX3CR1*$^{gfp/gfp}$ *and Jα18*$^{-/-}$*CX3CR1*$^{gfp/+}$ mice. *Plots are representative of 2 experimental repeats, with 3-5*

animals per experiment. Statistical analysis was performed using a student's t test (p<0.05, **p<0.01).*

FACs analysis of inflammatory monocytes, patrolling monocytes and neutrophils in the lung demonstrated that the absence of CX3CR1 expression in *Jα18*$^{-/-}$ does not counteract the infiltration of myeloid cells, or the lethal outcome of influenza infection. Inflammatory monocytes were the most prominent cellular population infiltrating the lungs, however the percentage of cells in both *Jα18*$^{-/-}$CX3CR1$^{gfp/gfp}$ and *Jα18*$^{-/-}$CX3CR1$^{gfp/+}$ were comparable. We observed no change in the percentage of patrolling monocytes following infection, and a small increase in neutrophils, however, again with no difference between the DKO and control strain.

4.10. Discussion

In this chapter we have demonstrated three important findings. Firstly, we identified a novel role of the CX3C chemokine CX3CL1 and its receptor CX3CR1 in the immune response to influenza. We show that CX3CR1 signalling plays an essential role in migration and accumulation of cells in the lungs following influenza infection, and that the depletion of CX3CR1 reduces myeloid cell infiltration, correlating with an improved disease outcome. Secondly, we addressed whether this pathway was implicated in the observed excessive recruitment of myeloid cells during influenza infection of iNKT-cell deficient mice. We were unable to confidently assess CX3CR1 expression in the *Jα18*$^{-/-}$ influenza infection model, however excitingly, we showed that the sole ligand of CX3CR1, CX3CL1, was significantly increased on inflammatory monocytes and neutrophils following influenza infection in the absence of iNKT cells, as compared with wild-type animals. Notably, later analysis using CX3CR1-iNKT cell double knockout mice demonstrated that CX3CR1-signalling was

redundant in the exacerbated infiltration of inflammatory monocytes and neutrophils, as mortality and monocyte and neutrophil infiltration in *Jα18*$^{-/-}$CX3CR1$^{gfp/gfp}$ and *Jα18*$^{-/-}$ CX3CR1$^{gfp/+}$ was comparable. Finally, utilising *in vitro* stimulation assays, we identified a novel role for SAA in modulate innate immune cells, whereby SAA stimulation was able to upregulate CX3CL1 expression on neutrophils, *in vitro*; these preliminary findings further demonstrate the diverse functions of SAA, and support a model where elevation of inflammatory mediators in highly virulent flu promote activation and recruitment of innate immune cells.

4.10.a. CX3CR1 enhances trafficking of innate immune cells during influenza infection

As discussed previously, CX3CL1-CX3CR1 signalling can modulate an array of downstream functions **(Figure 22)**; it is unclear from our current data how CX3CR1-expression exerts its effect in promoting accumulation of cells during influenza infection. CX3CL1 is especially unusual insofar as it occurs both as an integral membrane adhesion protein and a conventional soluble chemokine [382, 392, 394]. In light of the observed increase in soluble CX3CL1 in the lung upon infection **(Figure 32)**, we could hypothesise that influenza infection promotes the formation of a CX3CL1 gradient in the lung, which promotes the extravasation of CX3CR1-expressing cells. Importantly, we are yet to address whether soluble CX3CL1 is elevated systemically in the blood during infection, which may also contribute to migration of CX3CR1^{+} immune cells. Such a chemotactic mechanism is plausible, and has been shown with T cell migrating into the gut during inflammatory bowel disease (IBD) [418], and for MHC class II^{+} DCs allowing migration in the corneal epithelium [419]. Further investigation should assess transmembrane expression of CX3CL1 since transmembrane CX3CL1 assists adhesion and extravasation of CX3CR1^{+} cells, such as monocytes [384, 420], which could also account for the increased localisation of inflammatory monocytes and neutrophils in the lungs of CX3CR1$^{gfp/+}$ mice.

An alternative hypothesis which could account for the accumulation of cells following infection in CX3CR1$^{gfp/+}$ mice, as compared with CX3CR1$^{gfp/gfp}$ mice, is that CX3CR1-signalling promotes survival of CX3CR1 expressing cells during influenza; accordingly, the chemokine receptor CX3CR1 has shown to be vital in LP macrophage homeostasis in a murine IBD model, where CX3CR1-deficiency caused enhanced IL-17A-dependent colitis [421]. Furthermore, a pro-survival role of CX3CR1-CX3CL1 signalling has been observed in monocytes during atherosclerosis, whereby CX3CR1-deficiency reduced monocyte recruitment, and thus, reduced plaque formation. This phenotype could be reversed following forced survival signalling in CX3CR1-deficient monocytes [406]. Further analysis of pro and anti-apoptotic signalling components, such as the Bcl protein family, in the presence or absence of CX3CR1 signalling would help elucidate this mechanism.

This chapter has predominantly focused on the role of CX3CR1 expression on inflammatory monocytes and neutrophils recruitment and function during influenza infection. Notably, we also observed a significant expansion of interstitial macrophages and DCs in influenza infected CX3CR1$^{gfp/+}$ mice, as compared with CX3CR1$^{gfp/gfp}$ mice (**Figure 27**). The literature on the origins of tissue monocytes, macrophages and DCs during homeostasis and inflammation is highly complex [422], and not the predominant focus of this thesis. It has been reported, however, that infiltrating monocytes can differentiate into a number of cell populations, including include monocyte-derived DC (moDC) and interstitial macrophages (IMs), which serve as an obligate intermediate between blood monocytes and alveolar macrophages (AMs) [423, 424]. Furthermore, it has been shown that lung DC can develop from both Gr1^{high}CX3CR1^{int} and Gr1^{low}CX3CR1^{high} monocyte subsets; conversely, parenchymal lung macrophages originate from CX3CR1^{high}Gr1^{low} monocytes, notably however, Gr1^{high}CX3CR1^{int} monocytes could acquire this potential upon conversion into Gr1^{low}CX3CR1^{high} cells [423, 425]. This chapter presents only preliminary analysis of these

populations; $CCR2^+$ monocyte-derived populations, such as inducible nitric oxide synthase (iNOS)-producing DCs (TipDCs), are known to accumulate significantly during lethal influenza infection, and can play a fundamental role in influenza-induced lung pathology, but also in the magnitude of the $CD8^+$ T cell priming [426, 427]. In light of such observations, it will be important to further investigate macrophage and DC subsets in the lung, utilising precise markers for identification, to determine the precise contribution of $CX3CR1^+$ monocytes and CX3CR1 expression on macrophages and DCs' function in influenza infection.

4.10.b. CX3CR1 signalling is redundant in highly pathogenic viral infections

Notably, we found that although CX3CR1 deficiency reduces weight loss and increased survival, it does not ensure complete survival of mice since 50% of mice still succumbed to influenza infection (**Figure 24**). Additionally, CX3CR1 deficiency did not entirely ablate the infiltration of innate immune cells during infection; indeed, a substantial infiltration of inflammatory monocytes and neutrophils still occurred in PR8-infected $CX3CR1^{gfp/gfp}$ animals (**Figure 25**)(**Figure 26**). It may be important to note that $CX3CR1^{gfp/gfp}$ mice were still losing weight at 8 days post-infection when the experiments ended; we could thus speculate that CX3CR1-depletion merely delays the recruitment of cells and resulting pathology in the lung following infection. In line with this hypothesis, Johnson et al., demonstrated markedly delayed lymphatic trafficking of CX3CR1-deficient DCs *in vivo* using oxazolone-induced skin-hypersensitivity model [428].

The latter experiments of this chapter show that iNKT cell-deficient mice exhibit elevated infiltration of inflammatory monocytes and neutrophils, which excitingly, expressed CX3CL1 (**Figure 32**). Intriguingly, when DKO mice ($Ja18^{-/-}CX3CR1^{gfp/gfp}$) were infected with PR8, the absence of CX3CR1 expression did not rescue the pathogenic phenotype commonly

associated with influenza infection in *Jα18*$^{-/-}$ **(Figure 38)(Figure 39**). Infection of CX3CR1$^{gfp/gfp}$ mice with higher doses of influenza, such as 1x10^{8} PFU PR8, showed that CX3CR1$^{gfp/gfp}$ mice exhibited comparable mortality and cell infiltration to CX3CR1$^{gfp/+}$ mice (data not shown). It is known that during infection with highly lethal influenza [154], and during infection in the absence of iNKT cells **(Figure 6)** [127], there is an exaggerated cytokine and chemokine response in the lungs. Collectively, these data suggest that CX3CR1 expression plays a role in myeloid cell recruitment at low infectious doses, but in the presence of more pathogenic viral infections, CX3CR1 signalling is redundant, and other, currently unknown, signalling factors likely play a more prominent role. This is not entirely unexpected since a variety of immune regulators have been implicated in monocyte and neutrophil migration, during influenza infection (**Table 1**). Whilst we did not observe any significant differences in chemokine signalling during influenza infection in CX3CR1$^{gfp/gfp}$ mice, as compared with CX3CR1$^{gfp/+}$ mice **(Figure 29)**, we are yet to assess whether this is the case in the DKO animals.

4.10.c. CX3CR1 signalling as regulator of innate cell functions?

In addition to the varying cell numbers in the absence of CX3CR1 expression, we are yet to assess the functional capacity of the infiltrating cells in either CX3CR1-sufficient (CX3CR1$^{gfp/+}$), or CX3CR1-deficient (CX3CR1$^{gfp/gfp}$) mice. We observed comparable viral clearance and infiltration of influenza-specific T cells between the two strains (**Figure 30**) (**Figure 31**), thus we hypothesise that the differential disease outcome in CX3CR1$^{gfp/gfp}$ mice versus CX3CR1$^{gfp/+}$ mice is likely to be attributed to increased lung pathology. Importantly, the current study requires further functional analysis on the infiltrates in the two strains to confirm this hypothesis. Assessment of cytokines and chemokines released in the BALF did not identify any significant alterations in the type I or type II cytokine panels; there are some indications of greater IL-10 and IL-1β release in CX3CR1$^{gfp/+}$ mice, however these did not

reach significance, so it is unclear whether differential expression of these factors contributor to the disease outcome. An increasing number of publications have illustrated the importance of characterising CX3CR1-dependent functionality of cells; CX3CL1 stimulation of murine macrophages has been shown to enhance ROS production, in a CX3CR1-dependent manner *in vitro* [429], and additionally, CX3CL1 signalling was shown to promote extension of trans-epithelial dendrites by monocytes and/or DCs within intestinal lamina propria to enhance antigen sensing in the intestinal lumen [430]. Further support of a functional impact of CX3CR1 has been shown in murine and human NK cells; NK cells with high CX3CR1 expression are more cytotoxic than are NK cells with negative or low receptor expression [431], and preferentially migrate toward CX3CL1 [432]. We did not observe differential recruitment of NK cells between the mouse strains; nonetheless, such observations highlight the importance of additional functional analysis of both NK cells, and other $CX3CR1^{+}$ populations to determine their impact on disease progression.

4.10.e. SAA, a novel regulator of CX3CL1-CX3CR1 signalling and cell migration?

Data presented in this chapter also introduced an additional role for SAA in the response to influenza infection. Earlier work in our laboratory demonstrated that SAA induces a crosstalk between iNKT cells and neutrophils [330], and in the previous chapter we presented the novel finding of SAA1/2 release, and SAA3 message expression in inflammatory monocytes and neutrophils over the course of influenza infection. We now extend these findings by demonstrating that direct SAA stimulation can modulate CX3CL1 expression of neutrophils, *in vitro*. Preliminary data suggests SAA could induce this expression via TLR signalling (data not shown), however the precise downstream signalling mechanism has not been elucidated. One of the key observations we reported in the previous chapter is that SAA3 is upregulated during PR8 infection in the absence of iNKT cells **(Figure 19)**. We have now shown that CX3CL1 is also upregulated in the absence of iNKT cells *in vivo*, however data suggests that

the CX3CR1-CX3CL1 pathway is not solely required for recruitment of myeloid cells to the lung in PR8-infected *Jα18*$^{-/-}$ mice.

SAA exhibits a number of relevant functions implying that SAA could be a central factor determining the immune response, and in linking the observations of the current chapter. SAA has been shown to enhance neutrophil migration [433], neutrophil activation [434] and GCSF release [353], via FPR2 [352] or TLR2 [417] signalling, respectively. SAA can act both systemically, signalling directly on leukocytes, or alternatively, SAA has been shown to function locally during COPD, where it induces MCP-1, IL-8 and GM-CSF levels from lung epithelial cells which then promotes the recruitment of leukocytes [434]. Notably, synthetic SAA3 peptide, which we identified in the previous chapter to be elevated during influenza infection in the absence of iNKT cells, like SAA1/2 has been shown to stimulate cell migration and activation of p38 in a manner dependent on TLR4, MD-2, and MyD88 [435]. Intriguingly, SAA not only initiates lung inflammation via ALX/FPR2 but can allosterically modify this receptor so that it no longer transduces pro-resolving signals from endogenous lipoxins, that would normally promote tissue healing [436]. Finally, SAA3 expression has been shown to increase the survival of mMDSCs, and additionally, SAA3 expression can increase TLR2 signalling, leading to increased autocrine secretion of TNFα, ultimately leading to STAT3 activation and increased suppressive activity of mMDSCs [354]. We commonly observe SAA stimulation to enhance the survival of both human and murine neutrophils in culture (R.M. McEwen-Smith and C. De Santo, unpublished observations).

In light of the increasingly reported role of CX3CL1 on cell survival [372, 399, 406], SAA-mediated upregulation of CX3CL1, and signalling via CX3CR1 expression, may contribute towards the pro-survival, and increased migratory phenotype observed following sub-lethal infection. These observations, which demonstrate a novel role of both SAA and the CX3CR1-CX3CL1 signalling axis in innate cell inflammatory responses and disease resolution data,

further highlight the importance of a tight balance between the pro and anti-inflammatory immune response. Investigation into other effects of CX3CR1-CX3CL1 and SAA signalling on cell migration, functionality and survival warrant further experimentation.

4.11. Future perspectives

A large limitation of using CX3CR1-deficient mice in our investigations is the inability to assess the role of CX3CR1-deficiency on specific cell populations. We have shown CX3CR1 to be expressed on a wide range of myeloid and lymphoid populations, all of which can be implicated in the IAV-immune response, and thus, it is impossible to confirm, at present, which cells are directly or indirectly affected by CX3CR1 expression. Previous studies have demonstrated a complex and reciprocal relationship between monocytes and neutrophils during influenza infection [437]; Wareing et al. demonstrated that $CCR2^{-/-}$ mice displayed reduced monocyte, eosinophil and lymphocyte infiltration, but increased neutrophil infiltration in response to influenza infection [195]. Furthermore, in a model of kidney inflammation, both monocyte and neutrophil recruitment were severely decreased CX3CR1-deficient mice, although it was suggested that in this model that disruption of monocyte recruitment was responsible for the abrogated recruitment of neutrophils [438].

In vitro assays utilising cell specific depletion of CX3CR1 during influenza infection will be beneficial in elucidating the interactions between cells of the innate and adaptive immune system, and for identifying mechanisms responsible for CX3CR1-dependent cell accumulation. Although we found that the CX3CL1-CX3CR1 signalling pathway loses its impact during lethal or exacerbated infection, modulation of this axis in concert with other factors may be effective in controlling innate immune responses during infection. Indeed, modulating CX3CR1 expression has shown to be protective in atherosclerosis by reducing infiltration of monocytes and thus reducing plaque formation [439]. Relevantly, anti-CX3CR1 therapeutics have already shown to be beneficial in vaccination and treatment of RSV, where

antibodies blocking CX3CR1-RSV G-protein interactions are successful in preventing G protein-mediated chemotaxis and lung pathology [440]. We hope that the results shown here, and future studies in this field will assist in clarifying the mechanisms regulating cell infiltration, activation and survival of both the innate and adaptive immune cells during influenza; a greater understanding of these interactions will be fundamental in developing effective therapeutics to combat lung pathology and secondary bacterial infections resulting from highly virulent influenza infection.

Chapter 5.

A role for iNKT cells in enhancing viral-specific T cell memory during influenza vaccination

Chapter 5. A role for iNKT cells in enhancing virus-specific T cell memory during influenza vaccination

5.0. Introduction

5.1. Pseudotyped viruses as an alternative influenza vaccine

One of the main challenges of vaccination is to design and produce vaccines, with or without an adjuvant, which are effective against a range of influenza subtypes, and can reduce, or slow the spread of novel viruses in the population. An ideal solution would be a vaccination strategy which could promote both influenza-specific antibodies, and CTL-based immunity; currently approved vaccines for seasonal influenza predominantly target the generation of anti-HA neutralising antibodies; these vaccines, however, are inefficient in the event of heterotypic challenge, i.e. following HA mutation or the introduction of new HA isotypes into circulation (reviewed by [441]). Live attenuated viruses have been shown to induce cross-protective T cells, and to promote greater protection against drifted seasonal strains than subunit vaccines, however there are concerns that re-assortment of the HA vRNA into seasonal strains could occur [442-444].

Recently, pseudotyped influenza virus vaccines have become a promising alternative vaccine [445]; pseudotyped viruses infect cells as efficiently as wild-type influenza, providing a rigorous, but contained infection, with a complete set of conserved viral proteins, which are necessary for recognition by CTLs [220, 446]. This characteristic is highly beneficial for the generation of heterotypic immunity. Furthermore, given that pseudotyped viruses are unable to replicate due to inactivation of the vRNA encoding HA [447, 448], they pose less of a risk for re-assortment [445].

5.2. The role of iNKT cells in heterotypic immunity

The use of T cell adjuvants during vaccination is not unheard of; iNKT cells hold a unique role at the interface of the innate and adaptive immune systems, and their ability to direct the maturation of DCs and B cells suggests that iNKTs could be harnessed to boost the antigen-specific immune response against both microbial pathogen and tumour antigens alike (reviewed by [286]). Interestingly, α-GalCer adjuvants given in conjunction with an influenza vaccine, suggest that iNKT cells promote the generation of an influenza-specific immune response (reviewed by [296, 297, 449]). Notably, although a number of studies have shown increased protection with iNKT cell adjuvants, many studies use homologous challenge; pre-existing neutralising antibodies are likely to be the main mechanism for immune protection in such models, and it is difficult to decipher the role and interactions of iNKT cells and CTLs [295, 296]. Ultimately, the role, and mechanism/s of iNKT cells in mediating homotypic and heterotypic immunity is still largely unclear.

Utilising a vaccination strategy of pseudotyped virus (S-FLU) [445], this chapter addresses the importance of iNKT cells in regulating the adaptive immune response to influenza infection, and subsequently, aims to elucidate impact of iNKT cells in vaccination-induced heterotypic immunity. We are comparing homotypic and heterotypic challenge model by priming mice with S-FLU, and challenging with either A/Puerto Rico/8/34 (PR8) or A/HKx31 (X31). The X31 virus contains the six internal genes of PR8, however X31 expresses the H3N2 surface proteins, compared with H1N1 proteins expressed by PR8; this means that neutralising antibodies against either surface HA or NA of one strain, will be ineffective against the HA or NA of the alternative strain, and immunity will derive from influenza-specific CTLs.

5.1. Schematic overview of S-FLU inoculation and challenge strategy

In this chapter we will address the roles of iNKT cells in boosting heterotypic immunity to influenza virus, by using S-FLU vaccination **(Figure 40)** in wild-type or iNKT-deficient mice (*Jα18*$^{-/-}$ mice). If such a role is identified, we will attempt to dissect the mechanisms and cellular-interactions responsible for protection. These data will be important in directing future adjuvant and vaccination design.

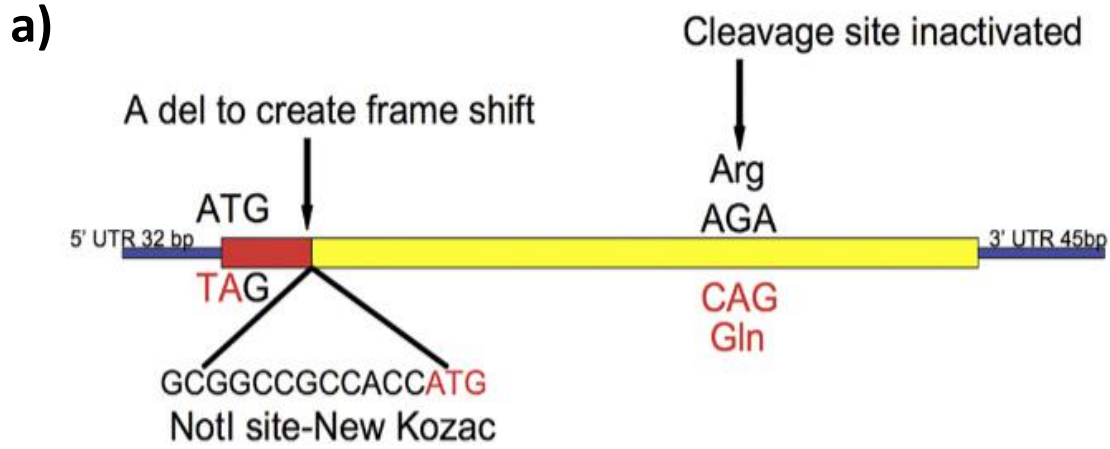


Figure 40 Schematic diagram of the design of S-FLU.

(a) "The HA sequence was derived from the vRNA of A/PR/8/34 Cambridge (Winter et al, 1981) (GenBank CAA24272.1) and was modified by replacing the original ATG start codon with TAG (bases A33T and T34A) to suppress translation of the signal sequence. A single base at the end of the signal sequence at position 83 was removed to ensure that if the original ATG was reconstituted by back mutation, it would read out of frame. Nucleotide 83 was replaced with the sequence GCGGCCGCCACCATG which contains an idealized Kozac sequence containing a NotI site to initiate protein synthesis beyond the signal sequence, and to provide a cloning site for insertion of any desired sequence. Finally, as a further safety mutation, the Arg codon (positions 1063 to 1065, AGA) was changed to CAG to encode Gln and inactivate the HA cleavage site (Chen et al, 1998)" (S-FLU methodical description and image modified from [445]).

The S-FLU vaccination was administered at two week intervals, and influenza challenge was given at 42 days after the initial inoculation **(Figure 41**). We opted for a vaccination strategy with two inoculations prior to challenge, since clinical studies have established that two doses of influenza vaccine, such as TIV, are more efficient at eliciting protective antibody titers in immunologically naïve children [450], and is more suitable for sensitive patients, such as young children, the elderly, or those with respiratory conditions. The route of administration can impact virulence [451], and thus we used intranasal administration for both the vaccine and challenge.

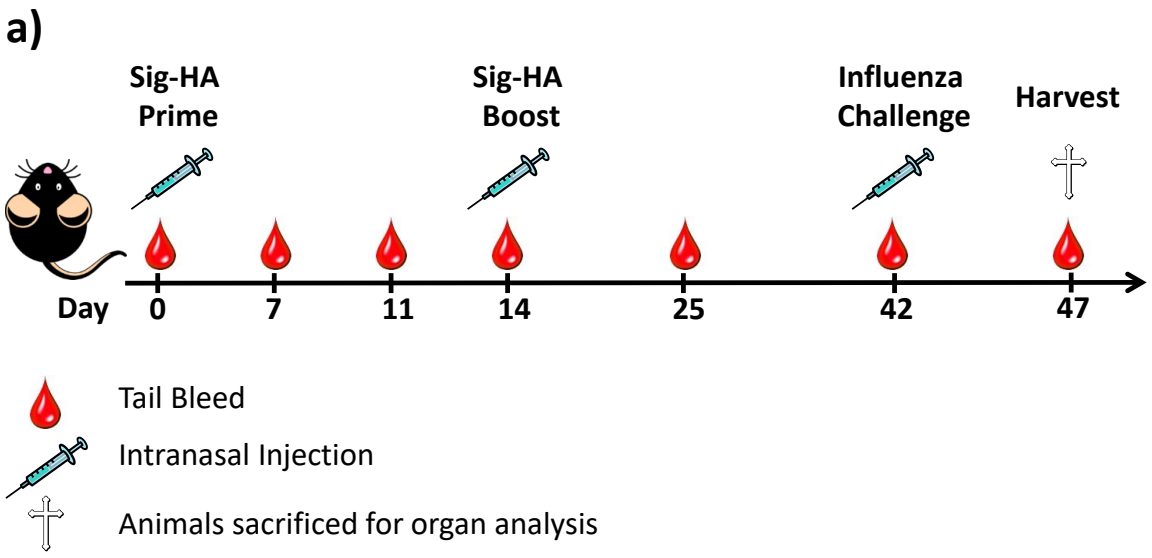


Figure 41 Schematic overview of S-FLU inoculation and challenge strategy.

Female C57BL/6 or Jα18$^{-/-}$ mice, aged between 6 and 10 weeks, were inoculated intranasally with 32 HAU of S-FLU on Day 0, followed by an S-FLU booster inoculation (also 32 HAU) on Day 14. Viral challenge was administered intra-nasally on Day 42 (6 weeks post-prime). Mice

were bled (alternating time-points between parallel experiments) prior to injections, at the time points indicated.

5.2. S-FLU inoculation is not pathogenic in wild-type or *Jα18*$^{-/-}$ mice

In light of the greater susceptibility of *Jα18*$^{-/-}$ mice to viral infections, we firstly addressed the pathogenicity of S-FLU in both wild-type (C57BL/6) or *Jα18*$^{-/-}$ mice. This is important since the Cambridge strain of PR8 (from which S-FLU is designed) affords increased virulence for mice [452], and even when administered at low doses, results in viral replication in the lung, severe clinical symptoms and immediate weight loss. Although S-FLU inoculation has been shown to be non-pathogenic [445], to check for pathogenicity of S-FLU inoculation in either the wild-type or *Jα18*$^{-/-}$ mice, both strains were inoculated with 32 HAU of S-FLU, or viral growth media (VGM) as a control, and weights, as well as health scoring (data not shown), were measured daily to assess for clinical symptoms **(Figure 42)**.

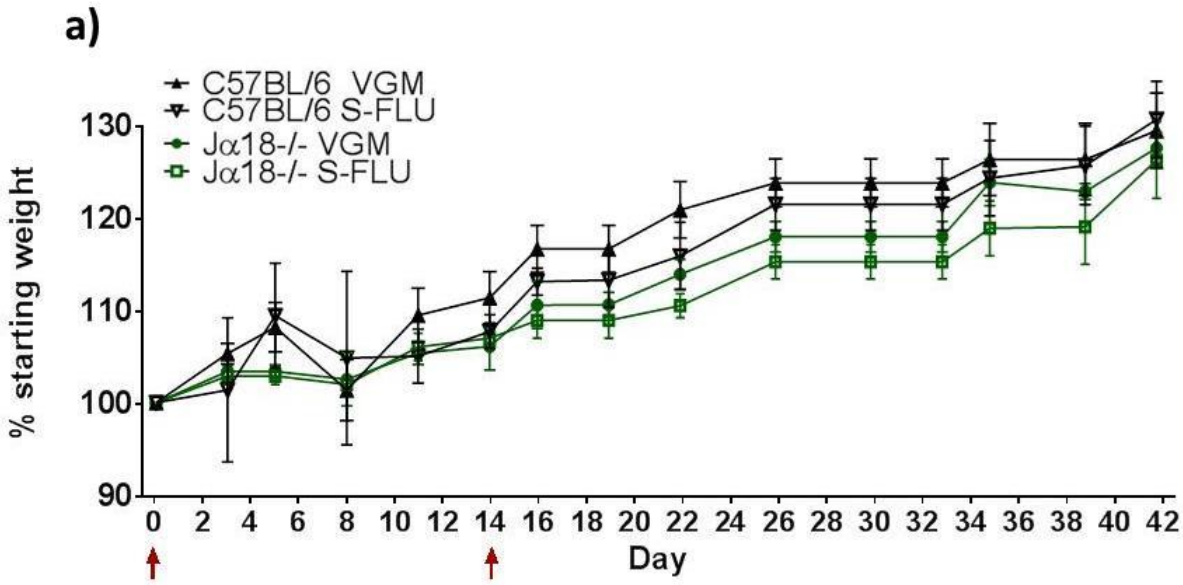


Figure 42. S-FLU inoculation is not pathogenic in either wild-type or *Jα18*$^{-/-}$ mice.

Female C57BL/6 or Jα18$^{-/-}$ *mice, aged between 6 and 10 weeks, were inoculated intra-nasally with 32 HAU of S-FLU on Day 0, followed by an S-FLU booster inoculation (also 32 HAU) on Day 14. Mice were weighed through the vaccination period, and weights are given as a percentage of starting weight. Error bars represent standard deviation. Days of S-FLU administration are indicated by red arrows. Data representative of 2 experimental repeats, with 6-10 animals per group. VGM, viral growth media; S-FLU, pseudotyped influenza.*

Consistent with Powell et al. weight analysis and scoring indicated that neither S-FLU nor VGM inoculation caused weight loss **(Figure 42)**, and mice from either strain exhibited no signs of sickness. This suggested that the vaccination is safe to use in both strains of mice, and we thus proceeded to challenge mice with influenza.

5.3. Innate immune responses to S-FLU inoculation, in the periphery

It is widely established that influenza infection in mice causes expansion and infiltration of innate immune cells, namely neutrophils, inflammatory monocytes and macrophages into infected lungs [195, 198], which can be dependent of iNKT cell signalling [41, 292]. Our group have published data showing that in addition to cell expansion or migration during influenza infection, iNKT cells can modulate the polarisation of myeloid cells between a pro-inflammatory or suppressive phenotype [41, 330]. As such, it is vital to consider all influenza-induced cell populations during infection, since it has been shown that the CTL effector responses, such as cytotoxicity or release of inflammatory mediators, can be dictated by the target cell type encountered [453]. Indeed, neutrophils have been shown to serve as APCs to virus-specific T cells during influenza infection, and may thus influence the T cell response [454]. In light of these observations, we firstly looked to assess how S-FLU inoculation modulates components of the innate immune response. Due to limited numbers of mice within the current study, we were unable to assess cellular infiltration locally in the lungs during the vaccination period, and this will be further addressed in future studies. We instead used periodical tail bleeds, and focused on cellular changes in the periphery following inoculation **(Figure 43)**, where it is also possible to see the cellular alterations following infection or vaccination [455, 456].

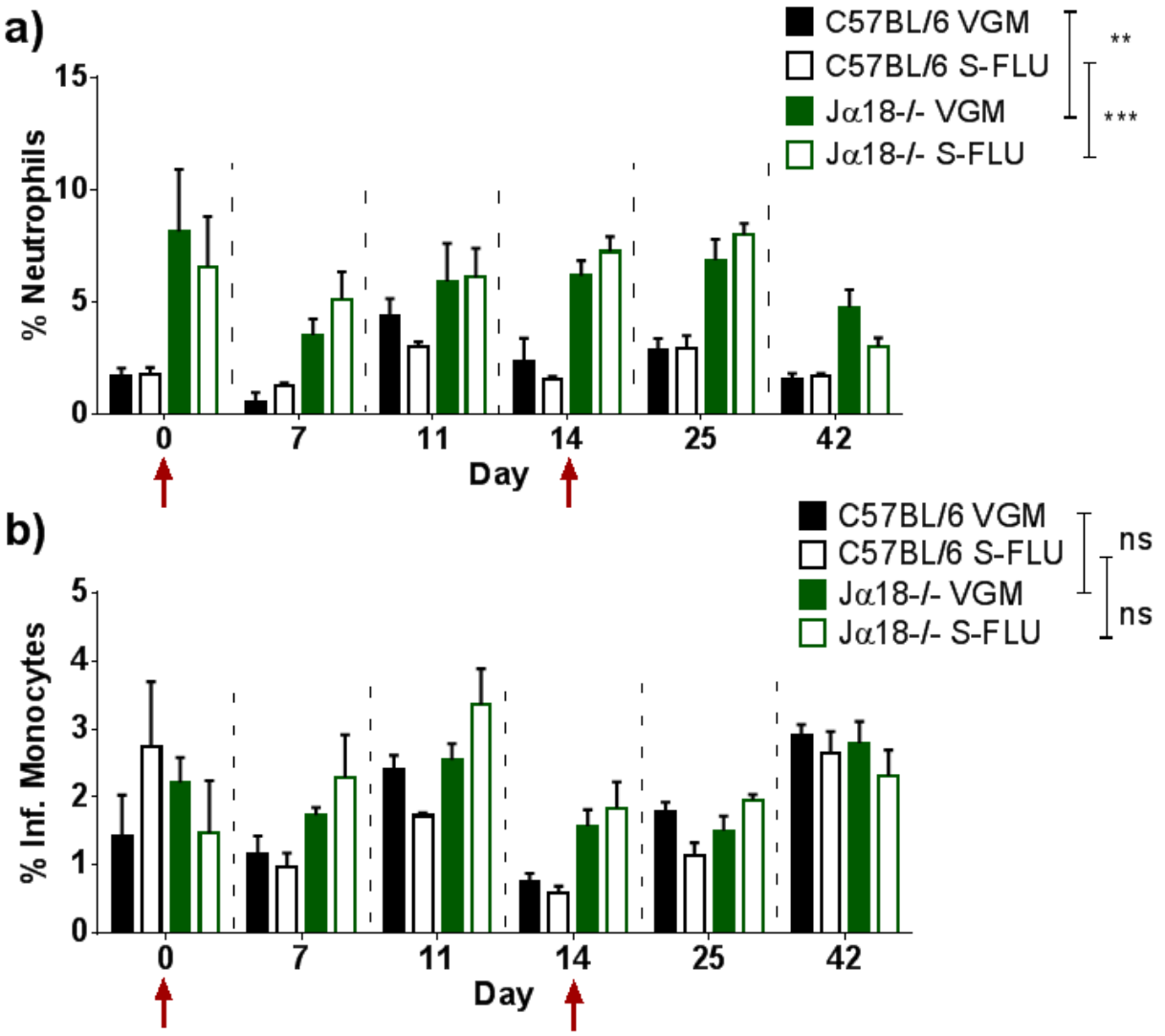


Figure 43. S-FLU inoculation does not affect myeloid cell percentages in the periphery of wild-type or *Jα18*$^{-/-}$ mice.

The percentage of inflammatory monocytes and neutrophils in the periphery was assessed using FACS. Values given are a percentage of total live cells. Data are representative of two experimental repeats, with 6-10 animals per group. Error bars represent standard deviation. Statistical significance was evaluated using a two-way ANOVA with multiple comparisons (p<0.05, ** p<0.01, *** p<0.001). Days of inoculation are denoted by red arrows. Inf. Monocytes, inflammatory monocytes.*

FACS analysis of the peripheral blood myeloid populations demonstrated that *Jα18*$^{-/-}$ mice exhibited a significantly greater percentage of neutrophils, as compared with wild-type mice ($p<0.01$ for VGM-treated and $p<0.001$ for S-FLU-treated) (**Figure 43a**). Despite this elevation in neutrophil numbers in the absence of iNKT cells, we observed no significant effect of S-FLU administration on expansion of peripheral neutrophils or monocytes in strain, over the vaccination period. Next, although we observed a greater degree of variability among the percentages of inflammatory monocytes, we observed no significant difference between the strains, and no effect of S-FLU inoculation (**Figure 43b**). Time-points chosen for analysis of the peripheral blood were optimised to observe the expansion of lymphocytes, and thus, it is possible that alterations in myeloid cell numbers, which are the initial cells recruited during influenza infection, could occur at earlier time-points; this will be addressed in future studies. Taken together, this data suggests that S-FLU does not substantially affect expansion of inflammatory monocyte or neutrophil populations, in the periphery, either in the presence, or absence of iNKT cells.

5.4. The response of peripheral lymphoid cells to S-FLU inoculation in the absence of iNKT cells

The majority of vaccines are targeted at modulating the adaptive immune response, boosting the memory response for protection against re-infection. To address the impact of S-FLU inoculation on lymphoid cells following S-FLU inoculation, we firstly assessed the total percentages of lymphoid cells ($CD3^+$, $CD8^+$ and $B220^+$) in the periphery using FACS (**Figure 44**). Although there was a degree of variability in the percentage of $CD3^+$ cells in the periphery, we did not observe any effect of S-FLU inoculation, nor any significant differences between wild-type or *Jα18*$^{-/-}$ mice **(Figure 44a**). Both $CD8^+$ T cells, and B cells were significantly reduced in *Jα18*$^{-/-}$ mice inoculated with either VGM (for $CD8^+$ cells only), and with S-FLU. A reduction of B cells in viral infection has previously been reported, and could be

attributed to cell cycle arrest and apoptosis of bone marrow B cells, which could thus reduce emigration of B cells into the periphery, or alternatively, a migration of these cells into the mediastinal lymph nodes [457]. Furthermore, it is known that secretion of IFN-γ and IL-12, resulting from activation of iNKT cells and DCs, selectively induces bystander activation of NK cells, B cells, and $CD4^+$ T cells and $CD8^+$ cytotoxic T lymphocytes [458-460], which could account for some of the reductions in lymphocytes observed between naïve wild-type and the naïve *Jα18*$^{-/-}$ mice.

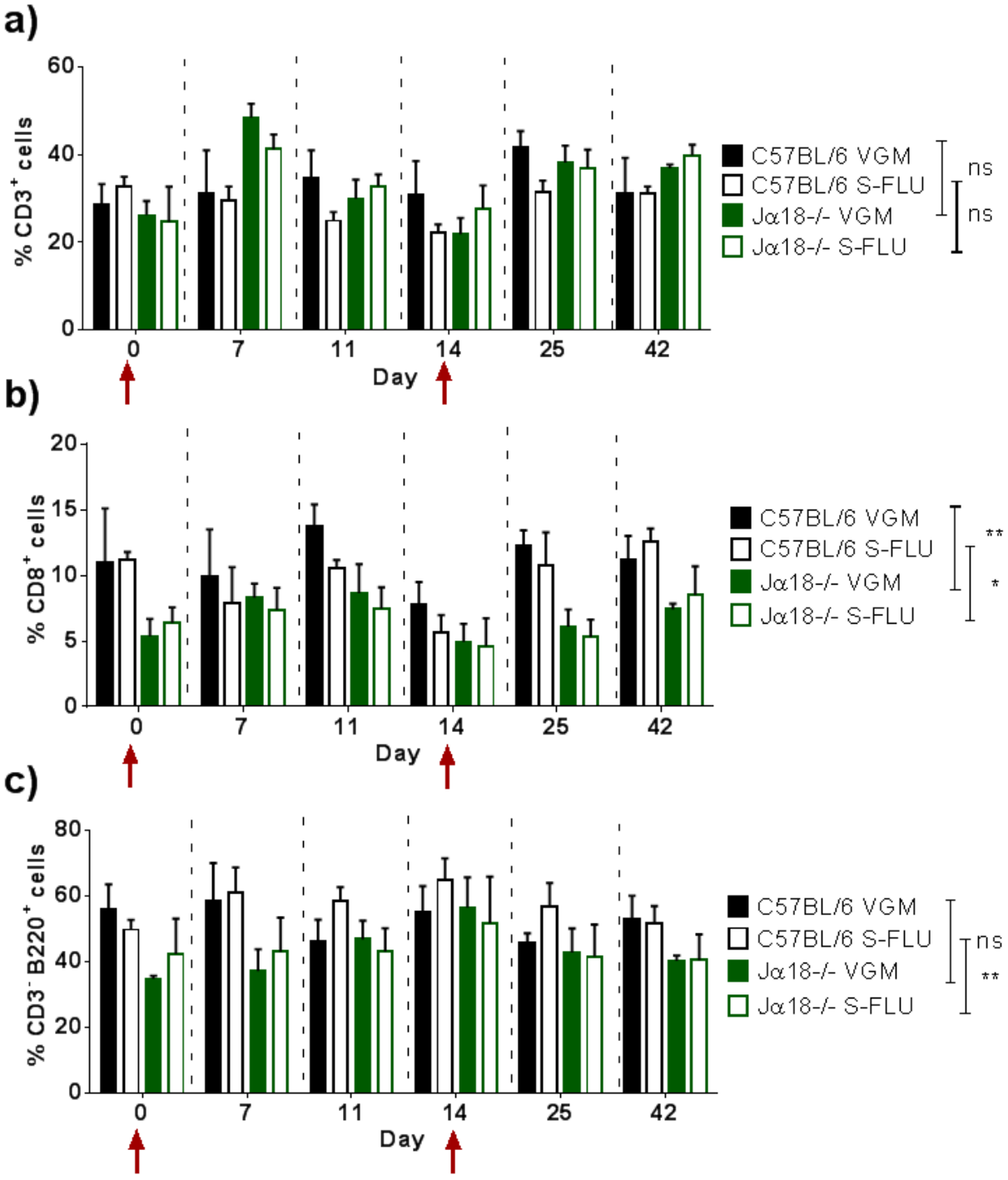


Figure 44. Analysis of the effect of S-FLU on peripheral lymphoid cells in *Jα18*$^{-/-}$ and wild-type mice.

The percentage of T cells and B cells in the periphery was assessed using FACS. Values given are a percentage of total live cells. Error bars represent standard deviation. Days of S-

FLU inoculation are denoted by red arrows. Statistical significance was evaluated using a two-way ANOVA with multiple comparisons (p<0.05, ** p<0.01, *** p<0.001).*

5.5. *S-FLU-inoculated Jα18$^{-/-}$* mice *exhibit significantly reduced longevity of NP-specific CD8^{+} T cells in the periphery, compared with wild-type animals*

It is widely accepted that the induction of heterotypic immunity by influenza correlates significantly with the induction of cytotoxic T cell responses in the lower respiratory tract [461-463]. As well as significant localisation to the lung, influenza-specific CD8^{+} cells have been shown to expand in the periphery, showing increased numbers in the blood, spleen and liver following infection and/or vaccination [464]. Since in the present study we were unable to assess the local recruitment of influenza-specific T cells in the lung throughout the vaccination period, as an alternative, we measured CD8^{+} T cells specific for the conserved H-2D^{b}-restricted nucleoprotein (NP)-peptide 366-374 in the peripheral blood during the inoculation period. Interestingly, we found that despite observing comparable percentages of NP^{+}CD8^{+} T cells immediately after the first inoculation in the *Jα18$^{-/-}$* mice as compared with the wild-type mice, at later time points, namely after the S-FLU booster inoculation on day 14, the percentage of NP^{+}CD8^{+} T cells was significantly reduced in *Jα18$^{-/-}$* mice as compared with their wild-type controls **(*Figure 45*a, b)**. Since we already observed slightly reduced CD8^{+} cells in the periphery of naïve *Jα18$^{-/-}$* transgenic mice **(Figure 44b)**, to ensure the phenomena we see above is not purely due to the CD8^{+} phenotype in this transgenic model, we also investigated the number of NP^{+} cells as a percentage of total CD8^{+} cells **(**Figure **45c)**. Both analysis methods demonstrate a significant reduction in NP^{+}CD8^{+} T cells during the memory phase of vaccination ($p < 0.05$), but not immediately following the primary inoculation. We hypothesise from this data, therefore, that although both strains of mice can initially promote a strong NP-specific CTL response, in the absence of iNKT cells (in *Jα18$^{-/-}$* mice), there is a reduced ability to maintain the NP^{+}CD8^{+} memory population.

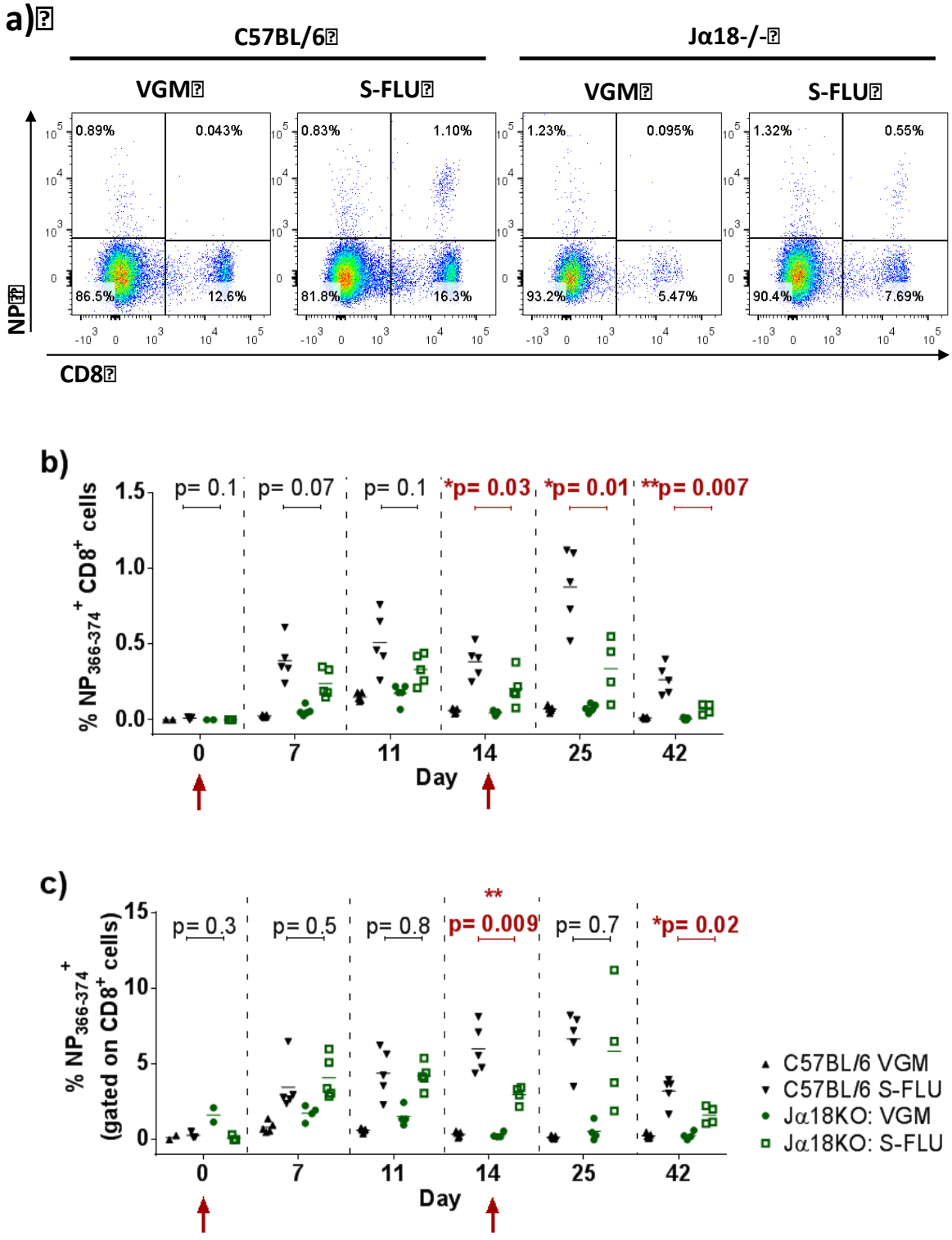


Figure 45. Reduced NP-specific CD8 T cells in the periphery of S-FLU-inoculated Jα18$^{-/-}$ mice.

*a) Example $NP_{366-374}$-tetramer staining at day 25 post- S-FLU priming. b) Summary of the percentage of $NP_{366-374}$-specific $CD8^+$ cells across the vaccination period- values given as a percentage of live cells. c) Summary of the percentage of $NP_{366-374}$-specific cells as a percentage of $CD8^+$ cells, across the vaccination period. Red arrows indicate days where S-FLU was administrated. Horizontal lines indicate sample means. Data representative of two experimental repeats, with 4-7 mice per group. Statistical analysis was performed using student's t test (p<0.05, ** p<0.01, *** p<0.001).*

5.6. iNKT cell- deficiency does not affect anti-HA antibody production following S-FLU inoculation

We next measured the antibody levels in the serum in response to S-FLU vaccination, using HA-specific ELISA. Consistent with the findings of Powell et al. wild-type mice produced anti-HA antibodies in the serum, as compared with the VGM-inoculated controls. HA-titers were also observed in *Jα18*$^{-/-}$ mice, however we observed no significant difference between the titers of the two strains at either day 14 or day 30 post-S-FLU inoculation **(Figure 46a, b)**. Importantly, the presented data does not inform us whether antibodies from either strain differ in their capacity for micro-neutralisation; this will need to be addressed in future studies if we are to gain a comprehensive understanding of the relevance of antibody response following S-FLU vaccination.

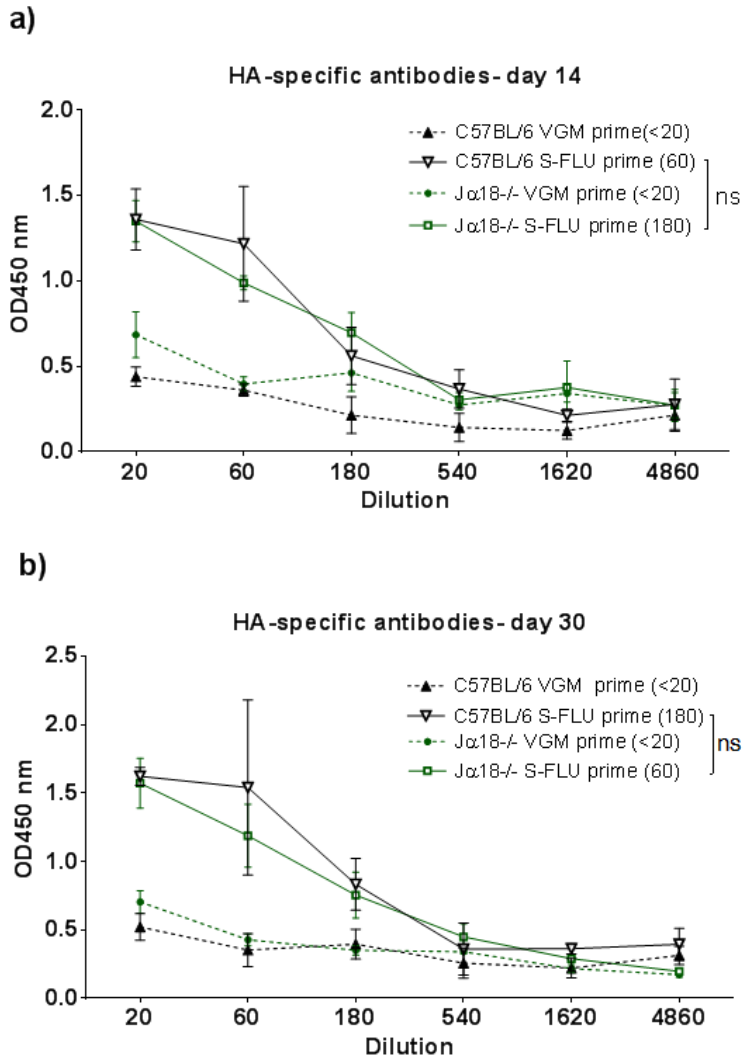


Figure 46. Comparable anti-HA antibody levels in the serum of C57BL/6 and *Jα18*$^{-/-}$ mice during S-FLU vaccination.

Serum was taken from tail vein bleeds, and analysed for anti-HA antibodies using ELISA. a) HA-titer at day 14 following initial S-FLU inoculation. b) HA-titer at day 30 following S-FLU inoculation. Titers shown (legend, value in brackets) are the last dilution to give >50% of the plateau value for ELISA. Statistical significance was evaluated using a one-way ANOVA (p<0.05, ** p<0.01, *** p<0.001). Error bars represent standard deviation.*

5.7. S-FLU inoculation shows reduced efficacy during heterotypic challenge in the absence of iNKT cells

It is known that $CD8^+$ CTLs are a key component of heterotypic immunity; extending upon our earlier results suggesting that the presence or absence of iNKT cells can affect the longevity of NP^+CD8^+ T cells following S-FLU priming **(*Figure 45*)**, we progressed to assess impact of iNKT cells on S-FLU vaccinated mice which were challenged with either homotypic (PR8- H1N1) or heterotypic (X31- H3N2) influenza virus. Viral challenge was administered 6 weeks after initial S-FLU inoculation **(Figure 41)**.

Weight analysis showed a strong protective role for S-FLU following homotypic infection, with minimal weight loss over the 5 day infection period, and no significant difference between S-FLU primed wild-type and *Jα18*$^{-/-}$ mice **(Figure 47a).** In comparison, heterotypic challenge with X31 influenza caused significant weight-loss in both wild-type and *Jα18*$^{-/-}$ mice; this was only marginally less than the weight-loss observed without vaccination (VGM-only prime) **(Figure 47b)**. Statistical analysis demonstrated that throughout the X31 challenge period, the wild-type and *Jα18*$^{-/-}$ mice are comparable in weight-loss. Interestingly however, at day 5, although not quite statistically significant ($p=0.058$), the wild-type and *Jα18*$^{-/-}$ mice were beginning to show indications of differential weight loss following infection; this observation fits with the clinical scoring assessments, where the *Jα18*$^{-/-}$ mice exhibited worsened scores of mobility, appearance, and breathing intensity later in infection, compared with the wild-type mice (data not shown).

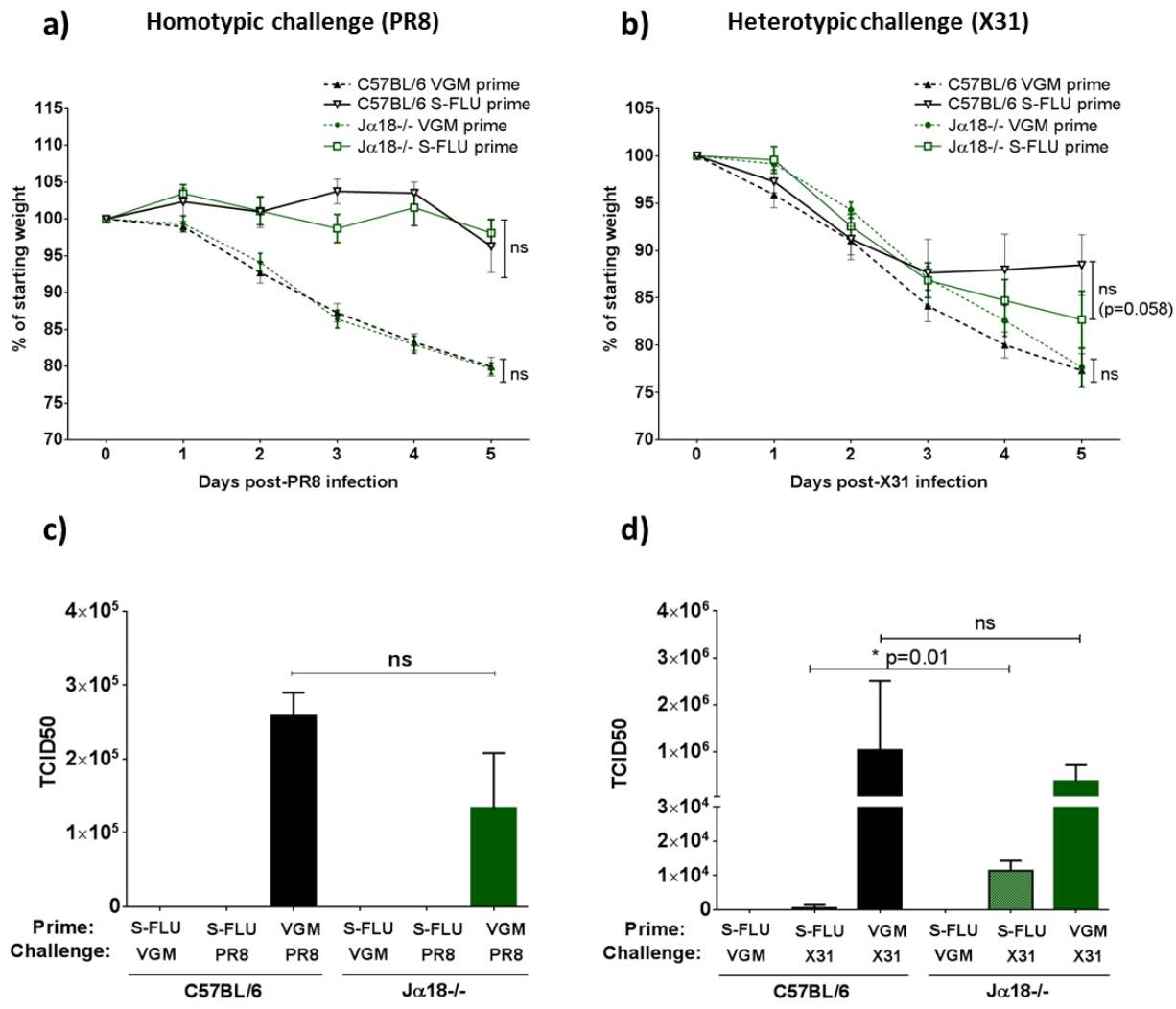


Figure 47. S-FLU inoculation shows reduced efficacy during 5 day heterotypic challenge in the absence of iNKT cells

Following 6 weeks of priming with S-FLU, mice were challenged with either a) 32 HAU of PR8 or b) 32 HAU of X31. Mice were weighed every day, and values are plotted as a percentage of the starting weight. Viral titers in lung tissue following homotypic c) or heterotypic d) challenge were calculated using TCID50 analysis (note different axis scales used). Error bars show standard deviation. Statistical significance was evaluated using a student's t test (p<0.05, ** p<0.01, *** p<0.001).*

Complimentary to the weight changes observed above, analysis of viral titer in the lungs at day 5 demonstrated complete viral clearance in both strains following homotypic challenge of S-FLU vaccinated mice **(Figure 47c)**. In comparison, there was significantly more virus in S-FLU- vaccinated *Jα18*$^{-/-}$ mice following X31-challenge, as compared with X31-challenge of S-FLU vaccinated wild-type mice (p=0.01). Interestingly, we found that when given highly lethal dose of both PR8 and X31 influenza virus without S-FLU, *Jα18*$^{-/-}$ mice exhibited equivalent weight loss and comparable viral titers to wild-type mice. This is in contrast to the greater susceptibility and viral load of *Jα18*$^{-/-}$ observed using sub-lethal viral infection [41, 127], demonstrating that the virulence of virus can affect the role of iNKT cells during influenza infection. Taken together, these observations support the findings of Powell et al., who reported that S-FLU provides heightened heterotypic immunity in wild-type mice [445]. Furthermore, these data indicate that iNKT could play a role in the heterotypic protection to influenza since Sig-HA vaccination is less efficient at controlling the viral load and clinical symptoms in the absence of iNKT cells. These results warrant further analysis, with a longer challenge period, to make sure that the weight loss is differentially regained in the wild-type and *Jα18*$^{-/-}$ mice challenged with X31 virus after vaccination with S-FLU.

5.8. Anti-HA antibody levels do not account for the increased pathogenicity following heterotypic challenge of *Jα18*$^{-/-}$ mice

To address the amount of pre-existing anti-HA antibodies for S-FLU-mediated homotypic and heterotypic immunity, and whether a difference, or reduced amount of anti-HA antibody could account for the differences in viral load and weight observed in *Jα18*$^{-/-}$ mice challenged with X31, we assessed the antibody titer in the serum and in the BALF at day 5 post-challenge, using ELISA (**Figure 48**). In both the serum and the BALF of homotypically-challenged animals we did not observe elevation of anti-HA antibody greater that the background levels found in VGM inoculated mice (**Figure 48a, c**). This is somewhat unexpected given that homotypic challenge is thought to be predominantly mediated by neutralising antibodies.

Clinical scoring of the PR8-challenged animals demonstrated that the PR8 challenge was much less potent than the X31 challenge, and this could account for the difference in antibody titers observed between these experiments. Further experimental repeats are in progress which will help to clarify these results. Since we observed protection in the homotypic model, as indicated by weight changes and viral titers, it is also possible that this result is not indicative of the actual antibody titers, and could be a technical issue within samples from this experiment, which will be addressed in future studies. In comparison, in the heterotypic challenge model, we observed clear increases in antibody titer in both the serum and in the BALF following S-FLU priming, as compared with VGM-alone priming (**Figure 48b, d**). We did not detect a significant difference between S-FLU primed wild-type and *Jα18*$^{-/-}$ mice in the heterotypic challenge model. We are in the process of assessing neutralising antibodies in these models, however, current data regarding antibody levels have suggest that antibodies are not responsible for heterotypic protection, as would fit with the suggested role of CTLs in heterotypic immunity [461, 465].

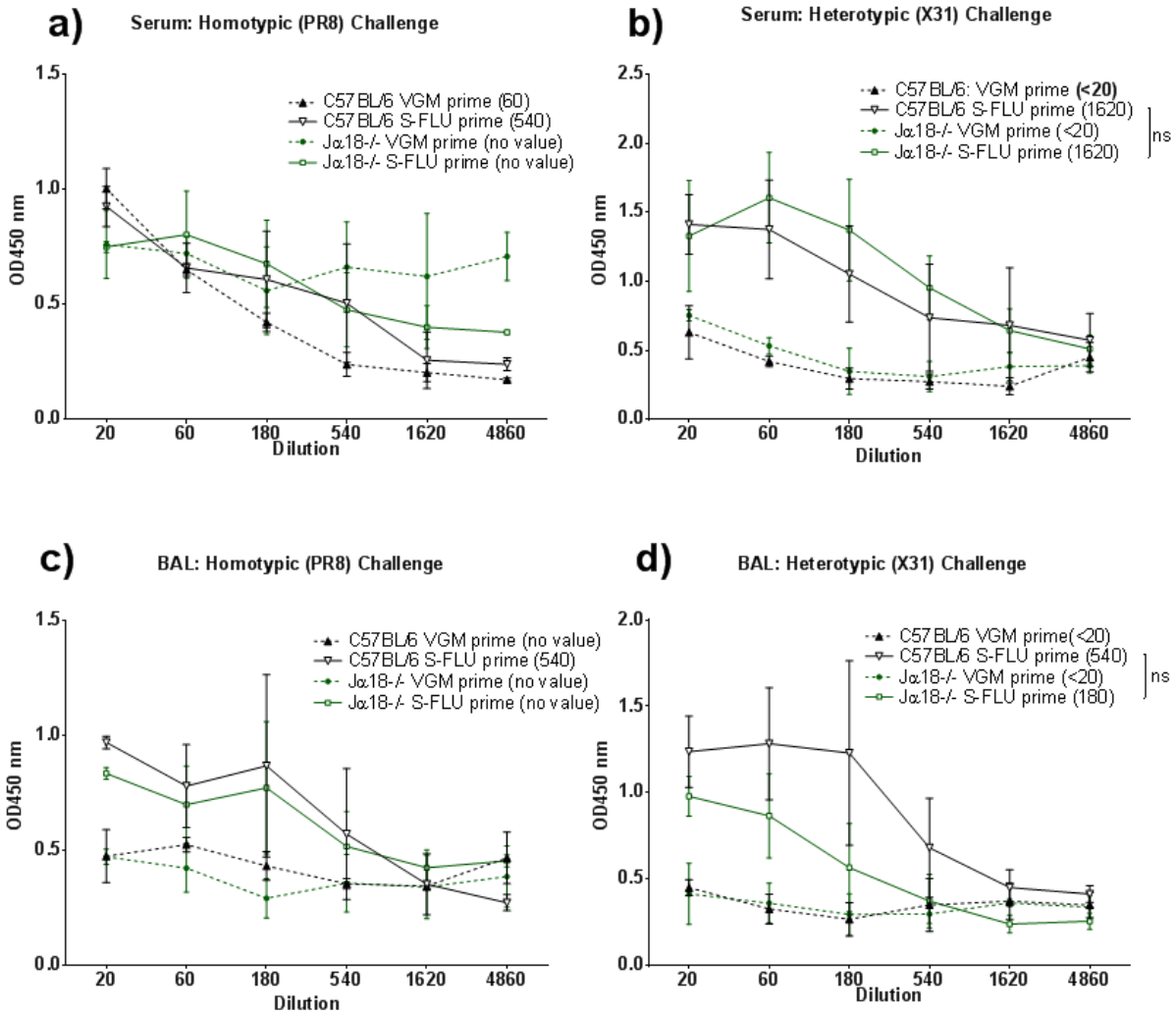


Figure 48. Differential anti-HA antibody responses do not account for the increased viral load in X31-challenged *Jα18*$^{-/-}$ mice

Following 6 weeks of priming with S-FLU, mice were challenged with either a) and c) 32 HAU of PR8 or b) and d) 32 HAU of X31. Serum and BALF were extracted and analysed for anti-HA antibodies using ELISA. Resulting antibody titers (legend, value in brackets) are given as the last dilution to give >50% of the plateau value for ELISA. Statistics were performed using the student t test. Error bars represent standard deviation.

5.9. Cellular responses following S-FLU prime and PR8 or X31- challenge

5.9.a. Homotypic (PR8) challenge

Since assessment of antibody titers suggested that altered antibody levels were not responsible for the increased viral load and weight changes observed in heterotypic challenge in *Jα18*$^{-/-}$ mice (**Figure 47**), we progressed to compare cell expansion and infiltration to the lung and BALF, and additionally the peripheral blood. Myeloid cells, in particular neutrophils and monocytes, have been strongly implicated in the increased lung pathology and worsened disease outcome in the absence of iNKT cells [41, 127], and the differential localisation of these inflammatory monocytes and neutrophils into the BALF and parenchyma tissue, respectively, prompted us to compare the effect of S-FLU in these compartments separately. Additionally, given the reduced percentage of NP^{+}CD8^{+} T cells observed in the periphery of *Jα18*$^{-/-}$ mice at later stages following S-FLU vaccination (***Figure 45***), we hypothesised that a reduced number of influenza-specific T cells, or a reduced capacity for T memory cell recall, in the absence of iNKT cells, could account for the reduced ability to clear virus, and the worsened clinical symptoms.

As expected, those mice which received S-FLU exhibited greater T cell recruitment, as shown by both CD3^{+} and CD8^{+} staining (**Figure 49a, b**). VGM-only primed mice exhibited undetectable infiltration of T cells by day 5 post-infection, consistent with the mice having not yet reached the adaptive-stage of immunity. Notably, although we did not observe a significant decrease in the percentage of NP^{+}CD8^{+} T cells in the lungs of *Jα18*$^{-/-}$ mice following challenge (**Figure 49c**), when converted to absolute cell numbers, C57BL/6 mice showed a statistically significant increase in influenza-specific T cells, as compared with S-FLU-primed *Jα18*$^{-/-}$ mice (**Figure 49f**). Further investigation into the kinetics of NP-specific T cells with other influenza-induced cells in this model is required; we hypothesise, however, that the reduced absolute number of NP-specific T cells may correlate with elevated infiltration of inflammatory monocytes, neutrophils and NK cells in the lung, which showed

robust, although just short of significant, infiltration when evaluated as absolute numbers (data not shown). Analysis of myeloid cell populations supports the observed protective role of S-FLU vaccination following homotypic challenge; a reduced infiltration of both neutrophils and inflammatory monocytes was observed following vaccination, as compared with the expansion of these cells following PR8 challenge of unvaccinated mice (**Figure 49d, e**).

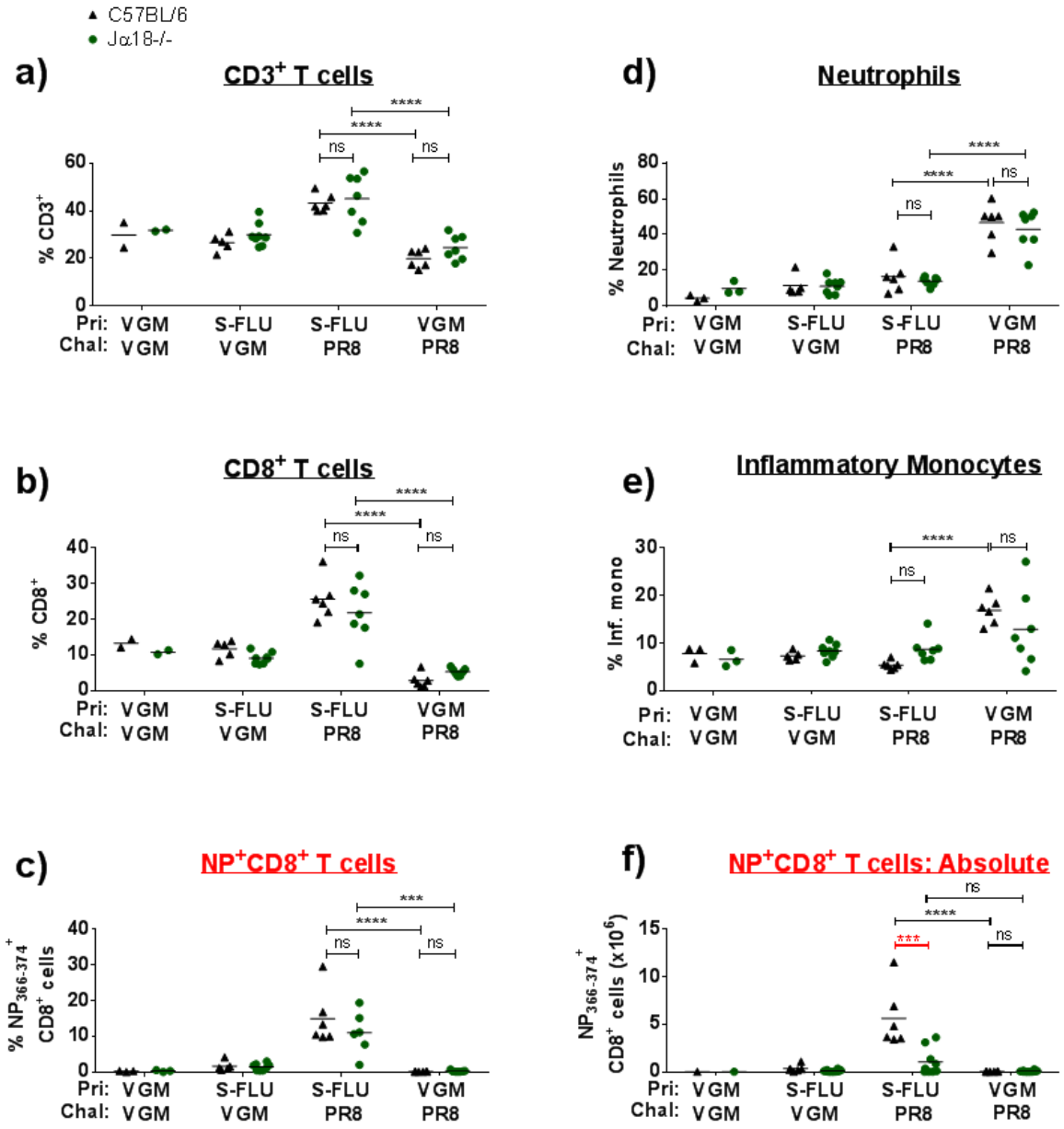


Figure 49. Reduced recruitment of NP-specific CD8⁺ T cells in the lungs of *Jα18*$^{-/-}$ mice following 5 day homotypic (PR8) influenza infection.

Lungs were processed as previously described, and analysed for infiltration using FACS. Lymphocytes are shown as a percentage of live cells. Myeloid cells are shown as a percentage of total CD3/B220/NK1.1^{-} populations. Horizontal line represents sample mean. Statistical analysis was performed using a student's t test (p<0.05, ** p<0.01, *** p<0.001, **** p<0.0005). Pr, prime; Chal, challenge; Inf. Mono, inflammatory monocytes.*

Influenza-induced cells can localise differentially across the lung tissue and in the alveolar spaces; therefore, we additionally assessed cell infiltration in the BALF, as a comparison with the lung (

Figure 50). Furthermore, in light of the initial finding that NP-specific T cells were reduced in the periphery of vaccinated mice (***Figure 45***), we subsequently assessed the peripheral blood for influenza-induced cell infiltration (

Figure 50).

In the BALF, although we found increased $CD3^+$ and $CD8^+$ cells following challenge (

Figure 50a, b), there was no significant difference in the percentage of NP-specific $CD8^+$ cells in *Jα18*$^{-/-}$ mice (

Figure 50**c**). As expected we observed greater inflammatory monocyte infiltration in the BALF following PR8-challenge of VGM-primed mice, as compared with the parenchyma tissue following challenge. S-FLU priming prior to PR8 challenge reduced the percentage of infiltrating monocytes significantly; intriguingly, however, unlike in the lung, there was still a degree of cell infiltration occurring with S-FLU priming (~20% compared with no observed monocyte infiltration in the lung) (

Figure 50**d**), indicating that local inflammation is still occurring. As in the lung, S-FLU priming exhibited completely and significantly reduced infiltration of neutrophils into the BALF, following PR8 challenge (

Figure 50**e**). The elevated lymphocyte numbers correlated with the decreased innate responders observed in the lung, and could further highlight the complex bi-directional interactions between $CD8^+$ T cells and inflammatory monocytes, in a location dependent manner.

The data obtained from the peripheral blood echoed what was observed in the lung and BALF; we observed expansion of both $CD8^+$ and $CD3^+$ cells, although to a lower extent (

Figure 50**f, g**). Interestingly, NP-specific T cells were significantly lower in *Jα18*$^{-/-}$ mice blood following S-FLU prime alone, but this difference was lost following PR8 challenge (

Figure 50**h**). We observed no significant changes in inflammatory monocytes under any treatment strategy; however, neutrophils showed greater expansion in the periphery of VGM-primed, *Jα18*$^{-/-}$ mice, as compared with VGM-primed wild-type animals (

Figure 50**i, j**).

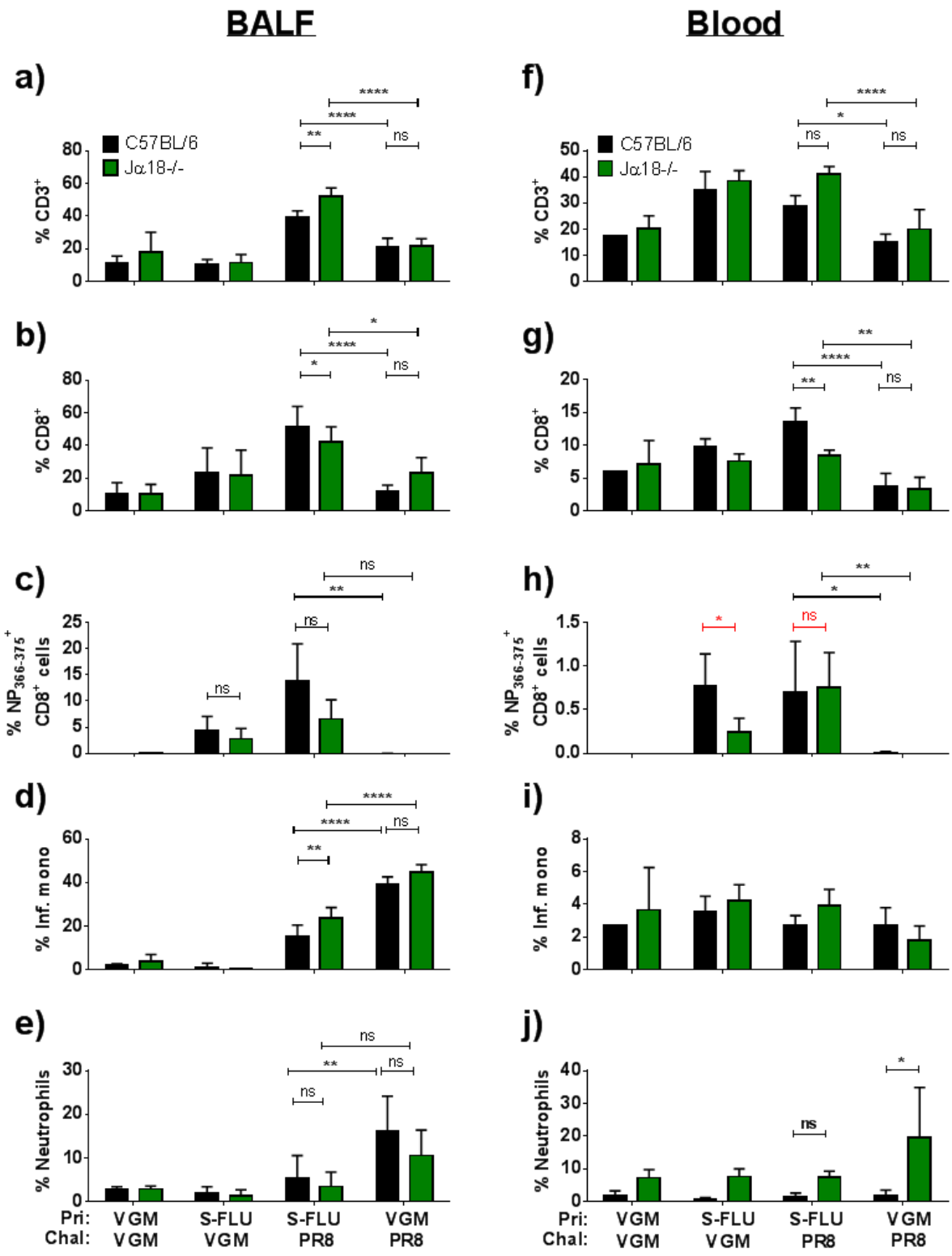


Figure 50. Analysis of cellular infiltration at day 5 in the BALF and periphery following homotypic (PR8) challenge in C57BL/6 and *Jα18*$^{-/-}$ mice.

BAL and blood was processed as previously described, and analysed for cellular infiltration using FACS. Lymphocytes are shown as a percentage of live cells. Myeloid cells are shown as a percentage of total CD3/B220/NK1.1$^-$ populations. Statistical analysis was performed using a two-way ANOVA (p<0.05, ** p<0.01, *** p<0.001, **** p<0.0005). Error bars represent standard deviation. Pr, prime; Chal, challenge.*

5.9.b Heterotypic (X31) challenge

CTLs have been widely implicated in heterotypic infection, and as such, we progressed to assess the infiltrating cell populations, both lymphoid and myeloid, following heterotypic challenge **(Figure 51**). As with homotypic challenge, we observed a clear expansion of CD3$^+$ and CD8$^+$ cells with X31 infection after S-FLU priming. We observed no difference, however, between wild-type and *Jα18*$^{-/-}$ animals (**Figure 51a, b**). The most exciting observation from this analysis was the reduction in the percent of NP-specific CD8$^+$ T cells in the lungs following X31-challenge in S-FLU primed *Jα18*$^{-/-}$ mice (**Figure 51c**). Additionally, in heterotypic challenge, we observed greater total percentages of NP-specific CD8$^+$ T cells, as compared with the percentages observed in homotypic challenge (20% compared with ~10%). Taken together, these findings agree with the reported role of CTLs in heterotypic challenge, and additionally, support the hypothesis that iNKT cells are implicated in heterotypic immunity, but are more redundant in the regulation of homotypic immunity. Finally, as with homotypic challenge, we demonstrated that S-FLU priming prevented both neutrophil and inflammatory monocyte recruitment to the lungs at day 5 following challenge (**Figure 51d, e**).

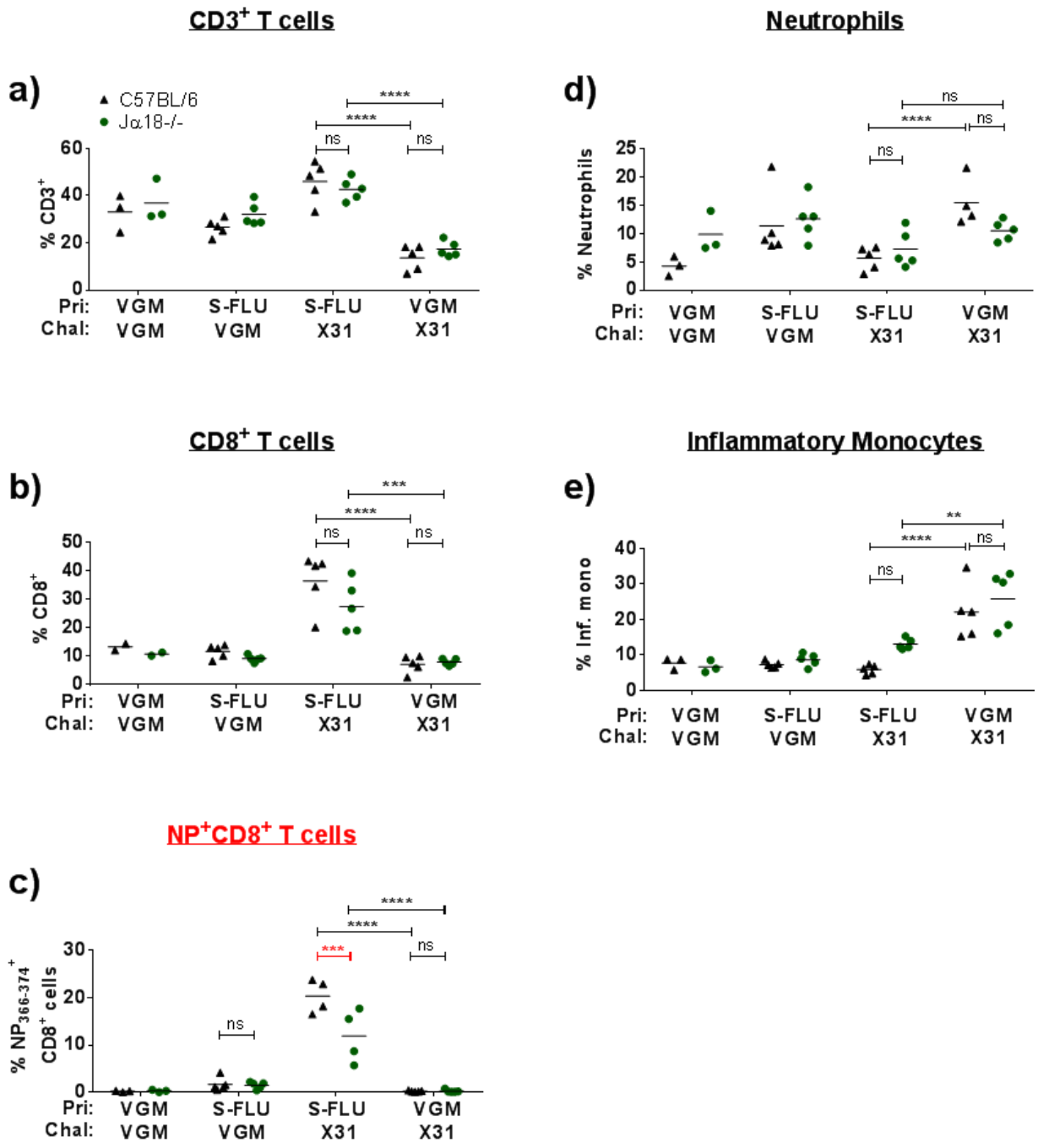


Figure 51. Analysis of cellular infiltration to the lung following 5 day heterotypic (X31) influenza challenge in C57BL/6 and *Jα18*$^{-/-}$ mice.

Lungs were processed as previously described, and analysed for infiltration using FACS. Lymphocytes are shown as a percentage of live cells. Myeloid cells are shown as a percentage of total CD3/B220/NK1.1^{-} populations. Statistical analysis was performed using

2-way ANOVA (p<0.05, ** p<0.01, *** p<0.001, **** p<0.0005). Horizontal line represents sample mean.*

To gain a complete understanding of the effect of S-FLU on heterotypic immunity, we also analysed cell populations in the peripheral blood, and BALF; analysis of the BALF was comparable with the observations in the lung, with both strains of mice exhibiting increased $CD3^+$ and $CD8^+$ cells following S-FLU vaccination, as compared to animals without prior stimulation **(Figure 52a, b**). Although both mouse strains exhibited an infiltration of NP-specific T cells into the BALF, we observed no significant difference in the percentage of NP-specific T cells in the absence of iNKT cells (**Figure 52c).** Similarly to heterotypic infection, S-FLU inoculation in both wild-type and *Jα18*$^{-/-}$ mice did not reduce infiltrating inflammatory monocytes into the BALF **(Figure 52i).** Furthermore, iNKT-deficient mice consistently exhibited increased inflammatory monocytes in X31 challenged animals with prior S-FLU priming ($P < 0.01$), or VGM-priming ($p < 0.05$), as compared with wild-type animals of the same treatments **(Figure 52).**

Unlike in the homotypic challenge, we observed only minimal changes in blood cell populations **(Figure 52f-j).** Consistent with the previously observed reduction of NP^+CD8^+ T cells in the periphery of *Jα18*$^{-/-}$ mice following S-FLU priming alone (***Figure 45***), we observed the same reduction at day 47, in mice which had been primed, but not challenged. However, upon X31 challenge, we found equivalent numbers in both strains **(Figure 52h).**

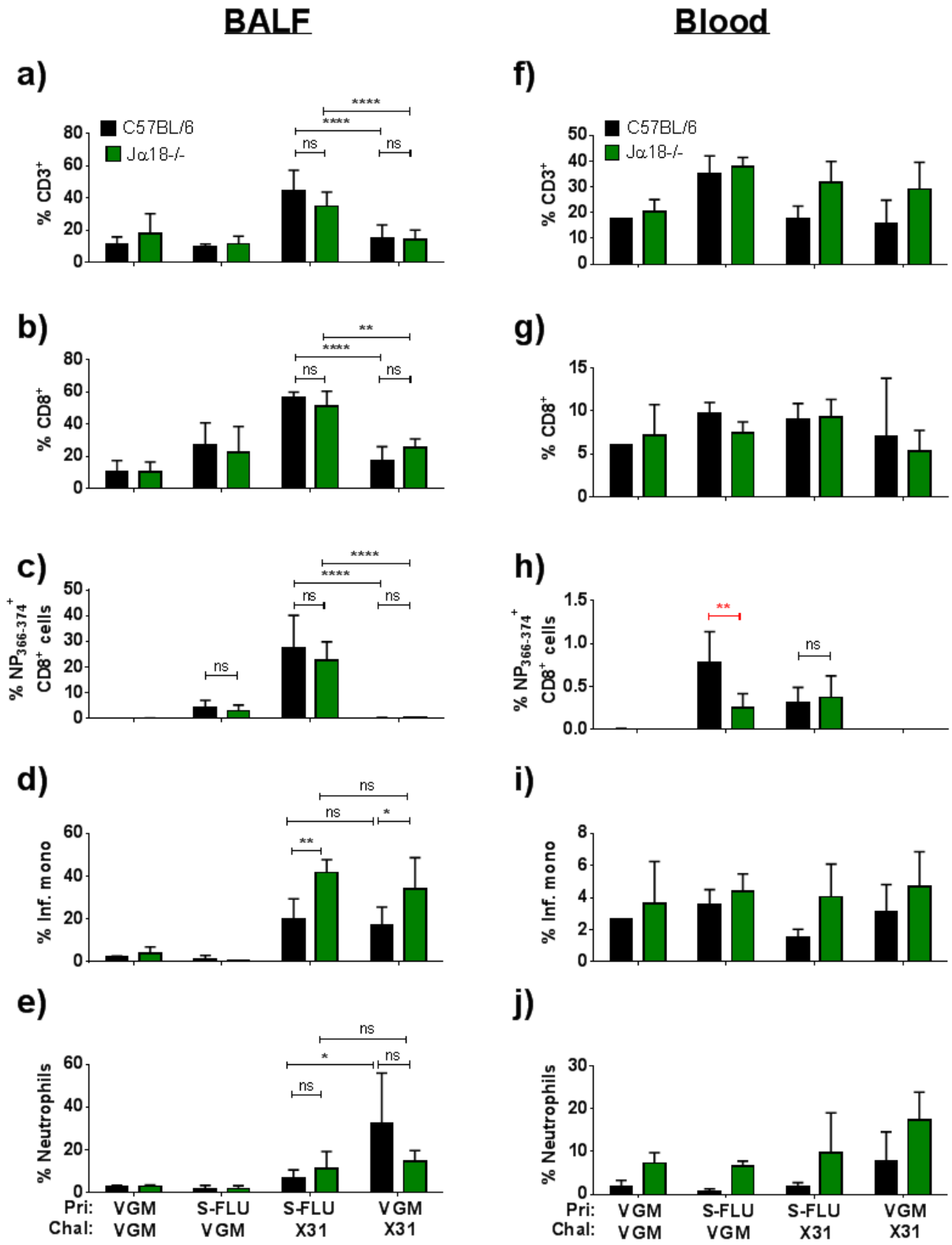


Figure 52. Analysis of cellular infiltration to the BALF and periphery 5 days following heterotypic (X31) challenge in C57BL/6 and *Jα18*$^{-/-}$ mice.

BAL and blood was processed as previously described, and analysed for infiltration using FACS. Lymphocytes are shown as a percentage of live cells. Myeloid cells are shown as a percentage of total CD3/B220/NK1.1$^-$ populations. Statistical analysis was performed using 2-way ANOVA (p<0.05, ** p<0.01, *** p<0.001, **** p<0.0005). Error bars represent standard deviation. Pr, prime; Chal, challenge.*

5.10 Discussion:

5.10.a. Chapter summary

The data presented in this chapter suggests that the protective immunity afforded by S-FLU during heterotypic infection could be largely attributed to an enhanced CTL response, a response which we now show is regulated by the presence or absence of iNKT cells.

As previously discussed, heterotypic immunity has been reported to be shorter-lived compared to homotypic immunity, with a reduced ability of $CD8^+$ T cells to maintain avidity for viral determinants for long periods of time after the initial inoculation [257]. The pseudotyped virus (S-FLU) has since been shown to offer protection against heterotypic challenge in wild-type mice, and thus represents a promising alternative influenza vaccine. Our data supports the previously observed protective role for pseudotyped influenza (S-FLU) [445]; we have shown that memory T cells are maintained until at least 42 days post-inoculation in wild-type mice which were inoculated with S-FLU, and furthermore, we demonstrated that following both heterotypic and homotypic challenge, wild-type mice exhibited significant infiltration of NP-specific $CD8^+$ T cells following homotypic and heterotypic challenge in wild-type mice, correlating with complete viral clearance and only minimal clinical symptoms or weight loss **(Figure 47)**. Conversely, in the absence of iNKT cells (in *Jα18*$^{-/-}$ mice), we observed a reduced capacity to produce and/or maintain NP^+-specific $CD8^+$ memory cells in the periphery, following vaccination. Furthermore, although S-FLU was protective against both

homotypic and heterotypic challenge in *Jα18*$^{-/-}$ mice until day 5 post-infection, upon heterotypic challenge, *Jα18*$^{-/-}$ mice exhibited reduced percentages of NP^{+}CD8^{+} T cells in the lungs. This correlated with a limited capacity to control viral load in the lung, and a marginally greater (p=0.058) weight loss in these mice following heterotypic challenge **(Figure 47).** Subsequent analysis of antibody titers, which were comparable in wild-type and *Jα18*$^{-/-}$ during vaccination and challenge **(Figure 46) (Figure 48)** suggested that iNKT cells are not essential regulators of the humoral response, or homotypic immunity; this was consistent with the comparable clinical outcomes of wild-type and *Jα18*$^{-/-}$ mice during homotypic (PR8) challenge.

Although this study is limited by lack of knowledge regarding the mechanisms modulating these differing responses to vaccination and challenge, taken together, these data strongly highlight a role for iNKT cells in modulating heterotypic immunity. There are a number of plausible explanations which could link iNKT cells with the maintenance of S-FLU-induced CTLs, which are further discussed below.

3.10.b. Sub-optimal priming of T cells in the absence of iNKT cells?

One potential hypothesis for a defective memory response in our model of *Jα18*$^{-/-}$ mice is suboptimal priming of T cells; indeed it has been shown that very weak TCR interactions are sufficient for T cell activation, but strong TCR ligation is required to sustain T-cell expansion [466]. Priming of T cells is largely down to their interactions with APCs. Guillonneau et al., have recently attempted to decipher the role of iNKT cells using α-GalCer as an adjuvant alongside inactivated influenza virus; in line with our hypothesis, the authors clearly demonstrate that α-GalCer-activation of iNKT promotes pro-survival genes, namely Bcl2, in CD8^{+} memory cells, however the authors were unable to characterise the mechanism

responsible, and it is unclear at which stage, or in which compartment iNKT cells exert their effects [297].

In view of the direct and indirect roles of iNKT cells in modulating other cells of the immune system, as well as their pleiotropic cytokine repertoire, we hypothesis that iNKT cell interactions with DCs could modulate T cell priming indirectly. Indeed, it is well known that iNKT cells can be activated during viral infection via soluble factors released by TLR-activated DCs, such as type I IFN and IL-12 [37, 82, 83], or by co-stimulatory molecules like OX40/OX40L [84], which then promote a bi-directional relationship with DCs. The subsequent iNKT-mediated maturation and elevated antigen presentation on DC's increases their ability to enhance both $CD4^+$ and $CD8^+$ T cell responses. Indeed, in agreement with such a mechanism, the up-regulation of CD70 [467] and OX40 ligand [468, 469] by mature DCs has recently been shown to be important for co-stimulating iNKT cells, and in turn promoting an antigen-specific $CD8^+$ T-cell response in tumour models.

Fitting with this hypothesis, a number of authors have highlighted the importance of DCs in influenza, whereby maturation and migration of virus-antigen-loaded APCs, such as pulmonary DCs, to the mediastinal lymph nodes (MLNs) plays a key role in the induction of the $CD8^+$ T cell response early after IAV infection [470-473]. In agreement with this, it has been shown that maintenance of tissue resident memory cells (T_{RM}) in the airways is dependent upon both the route of infection and prolonged presentation of viral antigen in the draining lymph nodes (DLN) of the respiratory tract [474]. Although utilising a model of primary infection and addressing only effector $CD8^+$ cells, Paget and colleagues suggested that defective $CD8^+$ T cell accumulation in the absence of iNKT cells stems from altered accumulation and maturation of pulmonary $CD103^+$ DCs in the mediastinal lymph nodes [284]. Finally, in light of recent data which suggests that memory T cells are highly dependent on antigen presentation by lymph-node resident $CD8\alpha^+$ DCs [475], we would hypothesis that a predominant role of iNKT cells on T cell priming would occur in the lymph

nodes during primary infection, and not at later stages following viral clearance. iNKT cells have been shown to localise in the MLN following LPS stimulation [310], thus their co-localisation with DCs would permit this bi-directional signalling. A number of groups have demonstrated that processed viral antigens continued to be presented to virus-specific CD8 T cells in the MLN of the respiratory tract up until 2 months post-influenza infection [474, 476, 477], which could alter the generation of CD8 memory cells. Early cessation, or prolonged accumulation of antigen presentation in the lymph nodes could represent a mechanism responsible for the differences we observed in CTLs, however, it is still debated whether persistent antigen presentation is required for maintenance of $CD8^+$ memory T cells [478].

The important role of DCs in T cell priming is clear from the current literature. Although it is well established that iNKT cells play a key role in regulation of DCs, further work will elucidate where these interactions may occur, and whether the interaction is directly or directly mediated.

5.10.c. Cytokine/chemokine microenvironments can modulate T cell priming

A second hypothesis for a role of iNKT cells in CTL maintenance stems from the notion that the detection of presented antigen alone is not sufficient to fully activate naïve $CD8^+$ T cells; additional cell-cell co-stimulatory signals, and specific cytokines are required, which can determine the effector and memory preferences of anti-viral T cells during the initial stages of infection (reviewed by [479]). In support of this, Sung et al. have demonstrated that chemokine guidance of central memory T cells was critical for anti-viral recall responses in lymph nodes [480]. Given the vast cytokine release, or cytokine storm observed locally in the lungs following infection, as well as the recruitment of iNKT cells into the parenchymal tissue and alveolar spaces in response to influenza infection [127, 481], it is plausible that

iNKT cell-derived signalling to effector T cells, either directly, or indirectly via DCs, could affect the differentiation of T cells locally in the lungs.

5.10.d. The effect of MDSC expansion in the absence of iNKT cells on $CD8^+$ T cell survival and differentiation to memory T cells

Our laboratory has shown that the absence of iNKT cells during influenza infection allowed for the expansion of myeloid derived suppression cells (MDSCs) in the lungs. IAV-induced MDSCs released anti-inflammatory and immuno- suppressive factors such as IL-10, iNOS and arginase, which possessed the capacity to decrease the local $CD8^+$ T cell proliferation, and thus the number of NP^+CD8^+ memory T cells [41]. Importantly, how the effect of MDSC-mediated suppression of effector T cell responses subsequently affected the efficiency of memory cells was not addressed in this model. It will be essential to address whether S-FLU inoculation affects the recruitment of iNKT cells to the lungs, and whether vaccination in the presence or absence of iNKT cells affords a differential cytokine profile in the lung and periphery, or affects the polarisation of myeloid cells, as compared with wild-type mice, which could account for the differential memory response observed.

5.11. Further questions and future objectives

This chapter highlighted both the high efficacy of the novel pseudotyped virus, S-FLU, as a vaccine against homotypic (PR8), and heterotypic (X31) influenza infection, as well as demonstrating key role for iNKT cells in mediating S-FLU-induced heterotypic immunity, which we suggest occurs via maintenance of $CD8^+$ T cell populations. We have discussed above, a selection of the most plausible explanations for our observations in this chapter, however further investigation is required if we are to fully elucidate the precise mechanisms responsible for this regulatory capacity. The key experiments we suggest need to be carried out in order to address these questions, are discussed below.

5.11.a. iNKT cells, DCs and CD8$^+$ T cells: Localisation, interaction and activation?

In the present study we were unable to examine the internal organs, such as lungs, spleen or MLN during the 42 day vaccination period, or at earlier stages of viral challenge. To investigate this role of iNKT cells will we focus on assessing the kinetics of iNKT cells, DCs (and other APCs) and CTLs during S-FLU inoculation and challenge. To do this we will assess the localisation and activation of specific DC subsets in the lung and lymph node, and compare the kinetics with iNKT cells and CTLs. In particular we will look for alterations migration, maturation and persistence of specific DCs subsets in the lymph nodes, where they have been shown to present antigen to CD8$^+$ T cells [475]. This could be achieved using confocal or FACs analysis, the localisation, accumulation and activation status of APCs both in the lungs and in the MLN following S-FLU inoculation. We would additionally assess local cytokine and chemokine milieus to determine whether differential cytokine signalling, such as IL-12 or IFN-γ are present and correlate with CD8$^+$ memory response and protection.

5.11.b. Do iNKT cells regulate the humoral response to IAV?

In contrast to the published studies utilising α-GalCer, where potent iNKT cell activation enhanced protection against infections such as influenza, by eliciting higher frequencies of memory B cells, and higher antibody responses to booster immunizations [290], our data suggested that endogenous signalling of iNKT cells during vaccination and challenge, did not effect antibody production, even during homotypic challenge **(Figure 48)**. Although iNKT cells are known to regulate B cell maturation and antibody production [482], in a CD1d-dependent manner, it is possible that without potent exogenous adjuvant activation of iNKT cells, the iNKTs alone are unable to modulate the humoral response during vaccination. We are currently addressing the neutralisation capacity of antibodies from both transgenic strains; future studies will aim to assess for functional differences of antibodies from each

strain. Furthermore, we will characterise the antibody subtypes observed in the serum versus the BALF.

3.11.c. Dose-dependent effects of S-FLU and iNKT cells?

One key observation from this work was the effect of potent viral infection on the phenotype of both wild-type and *Jα18*$^{-/-}$ mice with; indeed, we observed only minimal differences in survival and cellular infiltration in these mice with 32 HAU of PR8 (Cambridge) or X31, in comparison to the greater susceptibility of *Jα18*$^{-/-}$ infected with PR8 (MtSinai) which we previously observed (**Figure 6)**[41, 127]. Importantly, Cambridge PR8 virus, which has differences in HA which have been shown to be more severe than Mount Sinai strains of PR8, is likely a key contributor to this phenotype. At such high doses, it is likely that both PR8 and X31 caused such a strong immune response and cytokine storm, that the role of iNKT cells in heterotypic immunity is masked. We will therefore carry out dose- optimisation of both S-FLU and PR8/X31 challenge. Furthermore, utilising the iNKT agonist, α-GalCer, which has not yet been used in concert with S-FLU, may assist in identifying key immune populations, and signalling cascades, which are modulated following influenza-induced activation of iNKT cells.

3.11.d. The importance of CD8^{+} memory subsets and function during influenza infection

Finally, an important consideration in interpretation of our data is that we currently have not distinguished between subsets of CD8^{+} T memory cells in the lung, or in the periphery; following viral or bacterial infection two major populations of virus-specific memory CD8^{+} T cells remain in the circulation after viral clearance [483, 484]. Central memory T cells (T_{CM})

circulate through the secondary lymphoid tissues and enter encapsulated lymph nodes (LNs) via CD62L and CCR7. Conversely, effector memory T cells (T_{EM}) cells, which lack CD62L and CCR7 expression, preferentially circulate through non-lymphoid tissues. In addition to the above peripheral populations, a third population of $CD69^+$ and $CD103^+$ tissue resident memory cells (T_{RM}) are present following infection [222, 465, 485]; these cells populate peripheral tissues, and do not return to the circulation after viral clearance [486]. T_{RM} are suggested to be the predominant population mediating heterotypic immunity, with a reduction in numbers of T_{RM} correlating with decreased ability to control viral load [487, 488]. This population, however, is reported to stay resident in the tissue, and is not found to migrate to the periphery, and thus, is unlikely to account for the differences in $CD8^+$ T cells which we observed in the blood. Notably, the data presented here addresses only quantitatively the T cell memory populations, and unfortunately, we do not currently have information on the functional comparisons of NP-specific T cells, their ability to respond to challenge, and to exert antiviral functions.

Future studies will address the specific subsets of T cells expanded in the blood, spleen, mediastinal lymph node (MLN) and the lung, using a panel of specific T cell and activation markers. Since iNKT cells exhibit broad distributions, and variable percentages throughout the body, it is plausible that the differences observed in different tissues examined stem from localised or subset-specific regulation by iNKT cells. This analysis may help elucidate where iNKT cells elicit their regulation of the adaptive immune response. We will then need to assess the capacity of infiltrating memory cells to perform cytolytic functions (i.e. perforin and granzyme production) and the release of soluble mediators, namely IFN-γ. Indeed, the cytokine profiling of CTLs has demonstrated a hierarchy in cytokine expression throughout the acute phase of the primary and secondary response [489]. Analysis of these factors, among others, could help elucidate the cytolytic mechanisms and viral elimination capacity of influenza-specific $CD8^+$ memory cells during influenza infection in the presence or absence of iNKT cells.

Chapter 6: Thesis summary and overall conclusions

6.1. Summary

The annual influenza epidemics and recurring pandemics, which cause severe illness and death worldwide, highlight the importance of effective vaccines and therapeutics to combat this disease. In this thesis, we aimed to investigate the roles of iNKT cells during the innate and adaptive immune response to influenza A virus (IAV). We show definitively that modulation of single components of the immune system can have a profound effect on the host response, and ultimately disease outcome. In line with this, we have further highlighted the role of the cross-talk between iNKT cells and other host components, both innate and adaptive, in maintaining the delicate balance between raising an effective immune response and preventing immunopathology:

1) CD1d-mediated iNKT cell interactions determine the genetic profile and responsiveness of myeloid cells during influenza infection

Utilising *Jα18*$^{-/-}$ mice, we have confirmed a protective role of iNKT cells during IAV infection [41, 127, 292, 336, 490]. We show that worsened disease outcome correlated with accumulation of both inflammatory monocytes and neutrophils in the lungs, and exacerbated lung pathology.

One of the key findings that we report in this thesis is the profound effect of lack of CD1d expression on the genetic profiling of myeloid cells following influenza infection. These results are consistent with the hypothesis that the interaction between CD1d-restricted type I or type II NKT cells with myeloid cells modulate myeloid cells' genetic profiling. Utilising chimeric and knockout animals, we report that iNKT cells were able to exert an anti-inflammatory effect, dampening the secretion of both pro- and anti-inflammatory cytokines

and chemokines, in a CD1d-dependent manner. Excitingly, we showed that more than 100 genes were differentially regulated in *CD1d*$^{-/-}$ cells, as compared with the *CD1d*$^{+/+}$ control cells following influenza infection. Combining these results with the observations during influenza infection of *Jα18*$^{-/-}$ mice, and other viral infections [491], we propose that iNKT cells play a protective role during influenza virus-induced pulmonary inflammation, by interacting directly with innate immune responders via CD1d signalling, and counteracting IAV-induced hyper-responsiveness. The definitive question remaining following these experiments is where iNKT cells exert this modulation of gene profiling of myeloid cells during IAV infection: in the inflamed lungs, or in the bone marrow during enhanced myelopoiesis. Although additional experiments are warranted to distinguish between these two possibilities, consistent with latter possibility, the frequency of iNKT cells recruited to IAV-infected lungs is an order of magnitude lower than the frequency of myeloid cells. In agreement with this, iNKT cells infiltration peaks in IAV infected lungs 3-4 days after infection, whereas myeloid cells are very abundant already after 24 hours, and peak at day 2-3 post-infection [127, 284]. Conversely, iNKT cells are highly abundant in the bone marrow and they may be activated upon enhanced secretion of acute phase response proteins, such as SAA.

We hope that visualisation iNKT cells in the lung, spleen and bone marrow during influenza infection, may provide important insights to understand the mechanisms and location, which allows iNKT cells to modulate the activity and phenotype of myeloid cells. We have shown CD1d-mediated interactions to play a key role in defining the phenotype of cells during influenza infection; a comprehensive investigation into the kinetics and differentiation of myeloid precursor cells (GMPs and CMPs), in the bone marrow and the periphery, as well as temporal and spatial regulation of these CD1d-iNKT interactions may provide important insights into the regulatory capacity of iNKT cells during infection.

2) Complex interplay between iNKT cells, the innate, and the adaptive immune response

It has previously been shown that iNKT cells can enhance protection against secondary influenza infection (reviewed by [449]), as well as other diseases (reviewed by [286]). In order to dissect the complexity of the mechanisms by which iNKT cells modulate immune responses during viral infections, we utilised a pseudotyped influenza virus (S-FLU) vaccination model, in the absence of strong adjuvant co-administration. We have shown that *Jα18*$^{-/-}$ mice vaccinated with S-FLU [445] exhibit reduced longevity of influenza-specific CD8^{+} memory T cells in the periphery. This strongly supports the hypothesis that iNKT cells may play an important role in modulating the 'quality' of antigen specific T cells. We subsequently showed S-FLU vaccination is fully protective against homotypic and heterotypic challenge in wild-type mice. Both wild-type and *Jα18*$^{-/-}$ mice lost weight significantly following heterotypic challenge, however *Jα18*$^{-/-}$ mice showed indications of greater weight loss, and significantly poorer viral clearance compared to their wild-type controls. We found no difference in the amount of anti-HA antibody, however, further investigation into the neutralisation ability of anti-HA antibodies, either systemic, or local to the lung, may shed light on the role of this immune component during heterotypic infection in the absence of iNKT cells. In fact, it may not be surprising that iNKT cells did not have an effect in our model of homotypic challenge, since we did not use an adjuvant as shown by other groups [111, 112, 299].

Although our experiments fell short of providing mechanistic insights into this observation, it is tempting to speculate that the effect of iNKT cells on adaptive IAV specific T cell responses may be accounted for by an effect on myeloid cells, which may modulate early events during IAV specific T cell priming. Further experiments are warranted to provide conclusive results to support this hypothesis.

3) Immunoregulatory factors and the host-response to influenza

Throughout this thesis we have demonstrated the complexity of unravelling the host-immune response to influenza; we show that multiple cytokine- and cell-mediated interactions play essential roles in fine-tuning each aspect of the immune response, and minor alterations to the induction or prevalence of individual components of host signalling can substantially impact upon whether innate and adaptive immune cells adopt a pro- or anti-inflammatory phenotype, and thus can determine the outcome of the disease. Indeed, histological and pathological analysis in several species, suggests that influenza-induced mortality is associated with pulmonary immunopathology, rather than uncontrolled viral dissemination (reviewed by [140]). The cytokines present in the lungs, and the gene profiles of wild-type and CD1d$^{-/-}$ cells infiltrating the lung during influenza infection further highlight this complex cascade. This was demonstrated with the observation that CX3CR1-CX3CL1 signalling plays an important role in cell migration in wild-type mice, but by utilising CX3CR1$^{-/-}$: *Jα18*$^{-/-}$ double knockout mice, we established that CX3CR1 expression was not implicated in migration of myeloid cells during exacerbated influenza infection.

Furthermore, we have now identified infiltrating inflammatory monocytes and neutrophils as a source of the SAA isotype, SAA3, and later extending these results to show that SAA stimulation of neutrophils can modulate IL-10 release, as well as the expression of CX3CL1. Pathway mapping of genes upregulated in CD1d$^{-/-}$ chimeras highlighted the capacity of single factors to impact on many signalling pathways. Indeed, we observed significant upregulation of SAA, IL-6, IL-10, FPR and TLR4; combined these genes can impact a number of signalling pathways, such as chemotaxis [352, 353], survival, adhesion and activation [330]. It is clear that each element of the immune response which we have analysed, in particular iNKT cells, SAA and CX3CR1-CX3CL1, can exhibit dual effects, which can shift the balance between pro- and anti-inflammatory immune responses. Such dichotomy between these

factors is likely to maintain the fine balance in the inflammatory response, which is required to control viral replication without causing excessive tissue damage.

6.2. Overall Conclusions

Taken together, these data highlighted the complexity of the cellular and soluble factor signalling occurring during the innate and adaptive immune response, which is particularly striking during lethal infection, where attenuation or dysfunction of multiple factors is commonly observed. We suggest that gaining a more comprehensive understanding of the signalling components and cellular interactions present during the influenza-induced immune response, which may regulate this balance between host-protection and tissue pathology, with have therapeutic potential not only for the treatment of against influenza infection, but also for other sterile or non-sterile inflammatory diseases.

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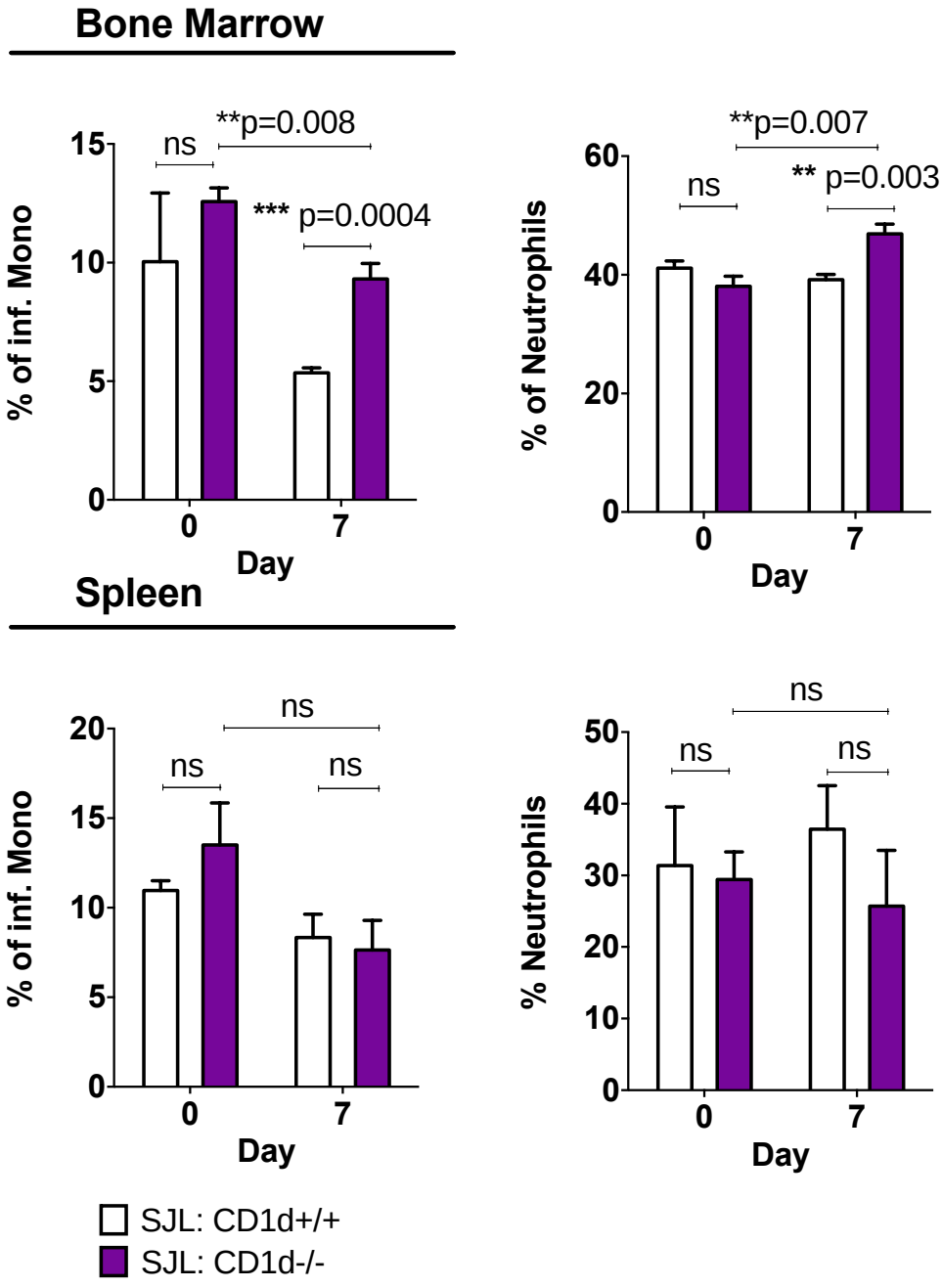
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Appendix

Appendix 1. Comparison of the expansion of inflammatory monocytes and neutrophils in the bone marrow and spleen of influenza-infected chimeric mice.

Graphs summarising the relative expansion of the inflammatory monocytes or neutrophils within the bone marrow (a) (b) or spleen (c) (d) of influenza infected mice. Statistics were performed using a two-tailed student's t test.



Appendix 2. RNA quality control analysis.

The RIN value is determined by an algorithm generated by the Agilent Bio-analyser, which evaluates the electrophoretic trace of RNA to give a value for integrity. The electrophoretic trace of the RNA can be affected by RNA degradation and contamination of solvents; the algorithm assigns a score ranging from 1-10, with a score of 10 representing in-tact, contamination free RNA, which is present at a concentration high enough to provide accurate results. RIN numbers greater than 7 are suitable for using on gene arrays. Additionally, all RNA samples had a 260/280 value between 1.9 - 2.1 (data not shown).'DPI' represents days post infection.

Treatment	CD45.2 Strain	Cell Type	KO status	RIN
Naïve	B6-1	Monocyte	WT (CD45.2)	9
Naïve	B6-1	Monocyte	WT (CD45.1)	8.9
Naïve	B6-1	Neutrophil	WT (CD45.2)	7.4
Naïve	B6-1	Neutrophil	WT (CD45.1)	8.2
Naïve	B6-2	Monocyte	WT (CD45.1)	9.3
Naïve	B6-2	Monocyte	WT (CD45.2)	9.3
Naïve	B6-2	Neutrophil	WT (CD45.2)	8.4
Naïve	B6-2	Neutrophil	WT (CD45.1)	8
Naïve	B6-3	Monocyte	WT (CD45.2)	9.2
Naïve	B6-3	Monocyte	WT (CD45.1)	9.6
Naïve	B6-3	Neutrophil	WT (CD45.2)	8.3
Naïve	B6-3	Neutrophil	WT (CD45.1)	8.8

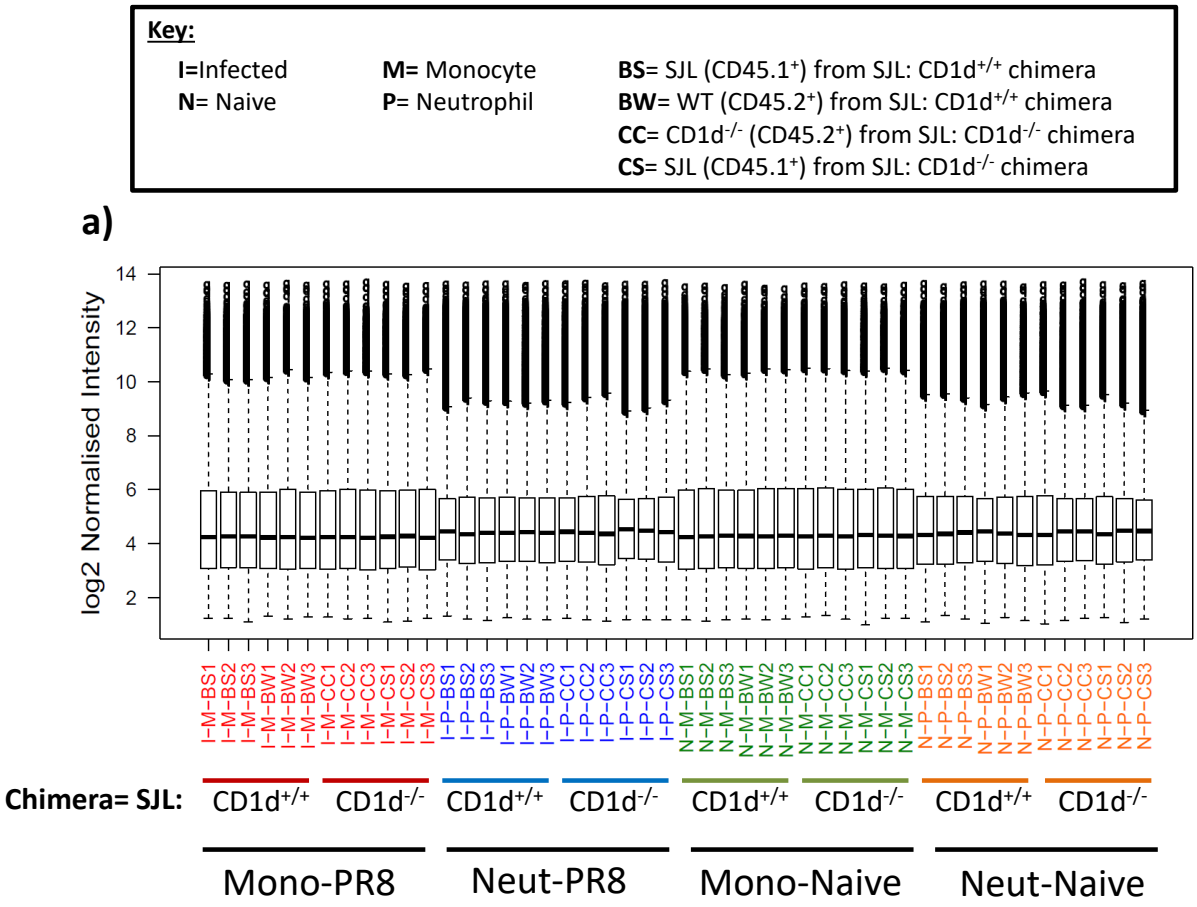
Treatment	CD45.2 Strain	Cell Type	KO status	RIN
Naïve	CD1d-1	Monocyte	CD1dKO (CD45.2)	9.3
Naïve	CD1d-1	Monocyte	WT (CD45.1)	9.4
Naïve	CD1d-1	Neutrophil	CD1dKO (CD45.2)	8.1
Naïve	CD1d-1	Neutrophil	WT (CD45.1)	7.7
Naïve	CD1d-2	Monocyte	WT (CD45.1)	8.9
Naïve	CD1d-2	Monocyte	CD1dKO (CD45.2)	8.8
Naïve	CD1d-2	Neutrophil	CD1dKO (CD45.2)	8.4
Naïve	CD1d-2	Neutrophil	WT (CD45.1)	9.1
Naïve	CD1d-3	Monocyte	CD1dKO (CD45.2)	8.8
Naïve	CD1d-3	Monocyte	WT (CD45.1)	8.6
Naïve	CD1d-3	Neutrophil	CD1dKO (CD45.2)	7
Naïve	CD1d-3	Neutrophil	WT (CD45.1)	8.6

7 DPI	B6-2	Monocyte	WT (CD45.2)	8
7 DPI	B6-2	Monocyte	WT (CD45.1)	7.9
7 DPI	B6-2	Neutrophil	WT (CD45.2)	7.3
7 DPI	B6-2	Neutrophil	WT (CD45.1)	7.7
7 DPI	B6-4	Monocyte	WT (CD45.2)	8.4
7 DPI	B6-4	Monocyte	WT (CD45.1)	7.3
7 DPI	B6-4	Neutrophil	WT (CD45.2)	7
7 DPI	B6-4	Neutrophil	WT (CD45.1)	7.3
7 DPI	B6-5	Monocyte	WT (CD45.2)	8.4
7 DPI	B6-5	Monocyte	WT (CD45.1)	8.2
7 DPI	B6-5	Neutrophil	WT (CD45.2)	7.1
7 DPI	B6-5	Neutrophil	WT (CD45.1)	7.5

7 DPI	CD1d-2	Monocyte	CD1dKO (CD45.2)	8.1
7 DPI	CD1d-2	Monocyte	WT (CD45.1)	7.5
7 DPI	CD1d-2	Neutrophil	CD1dKO (CD45.2)	7.2
7 DPI	CD1d-2	Neutrophil	WT (CD45.1)	7.3
7 DPI	CD1d-3	Monocyte	CD1dKO (CD45.2)	8.2
7 DPI	CD1d-3	Monocyte	WT (CD45.1)	8.5
7 DPI	CD1d-3	Neutrophil	CD1dKO (CD45.2)	6.9
7 DPI	CD1d-3	Neutrophil	WT (CD45.1)	7.3
7 DPI	CD1d-4	Monocyte	CD1dKO (CD45.2)	8.3
7 DPI	CD1d-4	Monocyte	WT (CD45.1)	8.3
7 DPI	CD1d-4	Neutrophil	CD1dKO (CD45.2)	7.4
7 DPI	CD1d-4	Neutrophil	WT (CD45.1)	7.9

Appendix 3. Comparable chip Log2 intensities across samples.

Expression intensities following RMA normalisation and summarisation are illustrated as box plots. Plot shows consistent chip intensities across the samples. Dendrogram was generated by Stephen Taylor, CBRG.



Appendix 4. Complete list of genes which were significantly expressed in CD1d$^{-/-}$ neutrophils

Table showing the all genes observed in CD1d$^{-/-}$ neutrophils isolated from the lungs of PR8 challenged mice, as compared with CD1d$^{+/+}$ neutrophils. Values are adjusted to p<0.05 and includes both up and down signalling events with LogFc >1.5.

Gene assignment	Log2FC	Adjusted P value
Edn1	4.05	0.033
Saa3	4.01	0.042
Klhl6	3.41	0.028
Atp6v0d2	3.28	0.047
Bcl2l15	2.91	0.031
Entpd3	2.88	0.033
Lipg	2.85	0.035
Ifnb1	2.83	0.036
Rab15	2.82	0.042
Prnp	2.67	0.047
Fmnl2	2.61	0.041
Il12a	2.60	0.042
Lyrm4	2.58	0.046
NA	2.56	0.047
Msh3	2.54	0.033
Tm4sf19	2.52	0.047
Sdhaf1	2.51	0.044
Gm19951	2.48	0.035
Chi3l3	2.47	0.043
Rps6ka2	2.40	0.036
Dpep2	2.37	0.034
Syne1	2.34	0.038
Gm20134	2.32	0.033
Mt1	2.32	0.033
Crispld2	2.32	0.040
Kcnma1	2.31	0.049
Mrpl52	2.30	0.040
Dhfr	2.28	0.046
Inhba	2.27	0.033
Hilpda	2.24	0.033
Ptpn22	2.24	0.038
1700017B05Rik	2.21	0.043
Cldn1	2.19	0.035
Dock10	2.17	0.040

Gm4955	2.17	0.046
Cxcl3	2.17	0.041
NA	2.15	0.033
Gpnmb	2.14	0.040
F10	2.13	0.040
Mreg	2.13	0.033
Id2	2.13	0.040
Rasd1	2.10	0.034
Gns	2.07	0.040
Chka	2.06	0.044
NA	2.03	0.033
NA	2.03	0.040
Rcan1	2.02	0.045
Slc7a5	2.00	0.040
NA	2.00	0.040
Igfbp6	1.99	0.043
Mmp19	1.97	0.049
Aoah	1.96	0.045
Fnip2	1.95	0.044
AI607873	1.95	0.033
Apoc2	1.94	0.040
NA	1.94	0.045
Gm4832	1.94	0.033
Il15ra	1.93	0.044
Serpina3n	1.92	0.033
NA	1.91	0.044
Pyhin1	1.90	0.040
Fam20c	1.89	0.043
Olfr433	1.89	0.041
Gm14446	1.89	0.047
Nos2	1.89	0.047
Ngp	1.86	0.033
Ecm1	1.85	0.046
Gm5431	1.85	0.042
NA	1.85	0.033
NA	1.85	0.050
NA	1.84	0.040
Tnfrsf26	1.82	0.042
Slc31a2	1.82	0.036
Ralgds	1.80	0.043
Gde1	1.77	0.047
Gpr97	1.76	0.046
Pts	1.76	0.040
Cd69	1.76	0.040

NA	1.74	0.040
NA	1.72	0.028
Arid5b	1.71	0.045
Fam188a	1.70	0.043
Gas2l3	1.69	0.047
Acsl1	1.69	0.039
NA	1.69	0.039
Sla	1.69	0.034
Ddx28	1.69	0.036
NA	1.68	0.037
Cbr3	1.68	0.042
Cpeb4	1.66	0.042
Gm19624	1.65	0.046
NA	1.65	0.045
Bcl2l1	1.64	0.047
Creb5	1.63	0.039
NA	1.63	0.040
NA	1.62	0.034
Pik3ip1	1.62	0.043
NA	1.61	0.049
Itgb3	1.60	0.044
F7	1.58	0.046
NA	1.56	0.043
Cish	1.55	0.034
Tbrg1	1.54	0.041
Usp18	1.54	0.043
NA	1.53	0.044
Meig1	1.52	0.033
Dhx58	1.52	0.047
Gatsl3	1.50	0.046
Hibadh	1.48	0.047
Plcxd2	1.47	0.048
Gm11827	1.47	0.033
Rgs1	1.47	0.047
Fkbp5	1.47	0.047
Trim56	1.45	0.044
Traf1	1.45	0.050
Gm885	1.44	0.048
D730005E14Rik	1.42	0.036
NA	1.42	0.036
Whamm	1.41	0.046
Pml	1.39	0.048
Gem	1.38	0.043
Dpep2	1.36	0.034

Gba	1.36	0.047
Plk2	1.36	0.042
Galnt6	1.35	0.040
Abca1	1.35	0.040
Dph1	1.32	0.049
Nt5c2	1.32	0.050
Hpgds	1.31	0.047
NA	1.31	0.033
Klhdc4	1.30	0.033
Nt5c3	1.29	0.043
Fndc3a	1.28	0.049
Pcgf5	1.28	0.044
Trim30c	1.26	0.049
Map4k4	1.25	0.047
Sema6b	1.25	0.040
Cd164	1.25	0.044
Sphk1	1.22	0.046
Daxx	1.20	0.049
Lcn2	1.18	0.040
Cxcl1	1.17	0.041
Snora33	1.16	0.040
Azin1	1.16	0.046
Apod	1.12	0.047
Ogfr	1.11	0.047
AA467197	1.10	0.044
Qsox1	1.09	0.040
Gstm1	1.05	0.046
Mmp8	1.04	0.044
Tmem219	1.03	0.046
Tifa	1.00	0.043
Rnd1	1.00	0.041
Ppbp	-1.01	0.040
Cklf	-1.01	0.034
St8sia4	-1.01	0.024
Abca7	-1.02	0.033
Slc16a3	-1.03	0.041
Lbh	-1.03	0.042
Fam129a	-1.03	0.028
Pqlc3	-1.03	0.022
Foxq1	-1.04	0.036
Mir1931	-1.04	0.034
Mapk3	-1.04	0.033
Palm	-1.04	0.033
Ngly1	-1.04	0.028

Pot1b	-1.05	0.033
Lyz2	-1.05	0.040
Rasgrp4	-1.05	0.033
Lst1	-1.06	0.033
NA	-1.07	0.044
Myo1f	-1.10	0.033
Dynlt1b	-1.10	0.033
Cmtm7	-1.10	0.033
Clec12a	-1.10	0.028
Mxi1	-1.11	0.049
Ltb	-1.11	0.033
Tinf2	-1.12	0.033
Myo1g	-1.13	0.040
Dynlt1b	-1.13	0.033
LOC100861999	-1.13	0.028
Ccpg1	-1.13	0.033
Srpk2	-1.15	0.033
Plcg2	-1.15	0.033
Vrk1	-1.17	0.033
Dynlt1b	-1.17	0.033
Tpcn1	-1.17	0.040
Stat4	-1.17	0.040
Fcrl1	-1.17	0.033
Mxi1	-1.18	0.033
Rab3d	-1.18	0.033
Parp8	-1.18	0.048
Aga	-1.18	0.033
Cnn2	-1.20	0.033
Tmem43	-1.21	0.033
Map3k15	-1.21	0.033
Gm2a	-1.21	0.021
Rassf3	-1.23	0.020
Crip1	-1.23	0.022
Fosb	-1.24	0.033
Padi4	-1.24	0.044
Mfsd6	-1.28	0.028
Cdc42ep3	-1.30	0.028
Calcrl	-1.31	0.021
NA	-1.32	0.021
Cd101	-1.33	0.047
Ogt	-1.35	0.040
Mrps15	-1.36	0.024
Pcbd2	-1.37	0.034
Itga4	-1.40	0.033

Rasgrp2	-1.41	0.033
Mir3074-2	-1.41	0.022
Pacs1	-1.41	0.021
Prkar2b	-1.42	0.033
Tmem181a	-1.43	0.033
Slc9a9	-1.44	0.040
Stap1	-1.44	0.019
NA	-1.44	0.034
Nbeal2	-1.45	0.028
Rnf13	-1.46	0.021
Man2b1	-1.47	0.033
Far1	-1.49	0.021
Vnn3	-1.49	0.033
Ear2	-1.52	0.021
Mapk14	-1.52	0.033
Ighm	-1.53	0.040
NA	-1.53	0.019
Tspan2	-1.53	0.033
Pi16	-1.54	0.024
Anxa6	-1.56	0.033
Gzmb	-1.62	0.044
NA	-1.63	0.040
NA	-1.79	0.033
Map4k2	-1.82	0.021
Ptplad2	-1.84	0.028
Cd300lb	-1.86	0.022
Tmem123	-1.89	0.022
Atp1a3	-1.90	0.019
Kif23	-2.13	0.021
Dck	-2.14	0.019
NA	-2.96	0.033

Appendix 5. Complete list of differentially expressed genes in CD1d$^{-/-}$ inflammatory monocytes.

Table showing the all genes observed in CD1d$^{-/-}$ inflammatory monocytes isolated from the lungs of PR8 challenged mice, as compared with CD1d$^{+/+}$ inflammatory monocytes. Values are adjusted to p<0.05 and includes both up and down signalling events with LogFc >1.5.

Gene Assignment	Log2FC	Adjusted P Value
Ifnb1	5.27	7.11E-06
Cxcl3	4.49	9.40E-07
Il1r2	4.22	2.57E-06
Il6	3.71	1.97E-05
1100001G20Rik	3.57	1.42E-06
Lcn2	3.56	1.86E-07
Fabp7	3.50	1.42E-06
Mmp8	3.48	9.07E-06
Edn1	3.35	5.21E-06
Inhba	3.34	1.41E-06
Scrg1	3.26	9.89E-06
S100a8	3.25	9.41E-06
Mrap	3.12	9.41E-06
Lrg1	3.07	1.42E-06
Slc7a11	3.03	1.97E-05
Mertk	2.88	2.04E-04
Gpnmb	2.87	2.57E-06
Slc7a10	2.72	9.89E-06
Cd33	2.64	2.82E-05
Vcan	2.62	9.89E-06
Fpr1	2.59	1.51E-05
Gm19951	2.58	2.19E-05
Hamp	2.58	1.66E-03
Dhfr	2.46	5.21E-06
Tnfrsf23	2.43	1.21E-04
Tlr3	2.42	9.29E-05
Dpep2	2.40	4.51E-06
Lrp12	2.40	5.88E-06
Mmp19	2.38	2.19E-05
Clec4d	2.34	1.25E-05
Il1a	2.33	5.17E-05
Clec5a	2.33	1.25E-04
Ecm1	2.32	6.10E-06
Rsad2	2.31	9.89E-06
Kctd19	2.28	1.87E-04

AU022793	2.28	2.85E-05
Lipg	2.26	5.21E-06
Saa3	2.25	5.21E-06
Nos2	2.23	5.21E-06
Ms4a8a	2.23	6.39E-05
Mt2	2.21	5.21E-06
Gab1	2.21	6.15E-05
Htra4	2.16	1.40E-05
Tm4sf19	2.14	3.31E-05
St7	2.13	3.68E-04
Gm14446	2.10	8.68E-06
Pnp2	2.10	5.04E-04
P2ry14	2.09	1.40E-05
Tnfaip6	2.08	9.89E-06
Siglec1	2.05	1.85E-05
Cxcl2	2.04	3.18E-05
Ctsk	2.01	1.85E-05
2400009B08Rik	2.01	6.72E-05
Fkbp5	1.97	1.85E-05
Slpi	1.97	8.08E-04
Fpr2	1.96	5.68E-05
Gm14023	1.95	4.85E-05
Cmpk2	1.94	3.99E-05
Ddit4	1.93	1.08E-04
Rhov	1.93	4.77E-05
Atp6v0d2	1.93	6.15E-05
Car13	1.90	4.33E-05
Adam8	1.89	2.21E-05
Mx2	1.89	1.40E-05
Serpinb2	1.87	7.10E-04
Rilpl1	1.84	1.24E-04
Fnip2	1.82	5.17E-05
Cd38	1.82	5.21E-06
Serpina3n	1.82	4.62E-04
Kcnma1	1.82	3.58E-03
1300002K09Rik	1.82	9.46E-06
Ttll9	1.81	6.70E-05
Cd300lf	1.80	1.05E-04
Clec4n	1.80	9.95E-05
NA	1.79	1.42E-03
Arg2	1.79	9.95E-05
Mx1	1.78	6.15E-05
P2ry13	1.78	4.49E-05
Ccr1	1.76	6.39E-05
Chi3l3	1.74	3.90E-04

Ctsl	1.73	4.51E-06
Emp1	1.73	9.49E-06
Pde7b	1.72	1.73E-04
Sh3pxd2b	1.72	2.90E-04
Spon1	1.71	1.44E-03
NA	1.71	3.99E-05
Fmnl2	1.70	2.71E-04
Stfa3	1.70	9.51E-03
I830012O16Rik	1.69	1.57E-04
Clec4e	1.69	3.73E-05
Siglece	1.67	7.05E-05
NA	1.67	1.51E-05
Ifit1	1.66	1.32E-05
Ms4a4a	1.66	6.39E-05
Dmxl2	1.66	1.10E-04
Dhrs9	1.65	2.62E-04
2610039C10Rik	1.65	4.33E-05
Slc27a1	1.65	5.21E-06
Trim30c	1.65	5.58E-04
Cd80	1.64	9.39E-05
NA	1.64	1.85E-05
Pla2g7	1.64	1.97E-05
Il15ra	1.64	2.19E-05
F7	1.63	1.71E-04
Gm10872	1.62	4.02E-04
Creb5	1.62	9.33E-04
NA	1.62	7.03E-03
Niacr1	1.62	1.97E-05
LOC100505155	1.62	2.28E-04
Emp1	1.62	6.72E-05
Stfa2l1	1.61	8.93E-03
Tfec	1.61	6.70E-05
Crispld2	1.60	1.73E-04
C3ar1	1.60	8.13E-04
P2ry12	1.60	4.74E-04
Npy	1.59	1.41E-04
NA	1.59	1.85E-05
Oasl1	1.59	8.03E-05
Slc7a8	1.58	4.14E-04
Dab2	1.58	9.95E-05
NA	1.57	7.27E-05
P2rx7	1.57	2.36E-05
Irg1	1.57	3.93E-04
Cd63	1.56	7.11E-05
NA	1.56	4.17E-04

NA	1.55	2.06E-03
Nt5c3	1.55	9.07E-06
Adamtsl4	1.55	2.18E-05
Prnp	1.54	5.27E-04
Msr1	1.54	3.35E-04
Slc31a2	1.53	5.27E-05
Apol9b	1.52	1.54E-03
Tmem171	1.52	1.16E-04
Mt1	1.52	1.55E-04
Jdp2	1.51	2.33E-04
Gde1	1.51	1.33E-04
F13a1	1.51	2.90E-03
Fgd6	1.51	7.11E-06
Gm16894	1.51	1.99E-04
Plekhn1	1.50	3.90E-04
NA	-1.50	7.26E-03
NA	-1.50	1.55E-04
NA	-1.51	1.25E-03
NA	-1.51	1.25E-03
Cd8b1	-1.51	1.55E-04
Serpinb10	-1.51	3.94E-05
NA	-1.52	9.60E-04
Gatm	-1.52	1.74E-03
NA	-1.52	9.11E-04
NA	-1.53	2.14E-02
NA	-1.54	7.84E-03
Ncr1	-1.54	2.34E-04
Ccnb2	-1.54	3.74E-04
NA	-1.55	2.32E-04
Cd226	-1.55	4.48E-04
Trav9d-3	-1.55	1.65E-02
NA	-1.56	3.60E-03
Zfp526	-1.56	8.64E-06
40787	-1.57	2.64E-04
Ltb	-1.57	1.80E-04
NA	-1.57	9.83E-04
NA	-1.57	3.66E-02
Slc46a3	-1.58	1.12E-04
Tcf7	-1.58	1.97E-05
Lilra5	-1.59	4.96E-04
A430107P09Rik	-1.60	2.06E-03
Trav3n-3	-1.60	2.06E-03
NA	-1.60	2.09E-02
Dapl1	-1.60	5.14E-04
NA	-1.61	3.93E-04

Hist1h2ak	-1.63	1.06E-04
Cd8a	-1.63	7.72E-05
NA	-1.64	2.54E-03
Klrg1	-1.64	1.02E-03
Cd96	-1.64	1.73E-04
Trav7-4	-1.64	8.86E-03
NA	-1.64	3.25E-04
Syne1	-1.65	1.47E-03
Cd27	-1.66	1.13E-04
NA	-1.66	2.60E-03
NA	-1.67	2.09E-03
NA	-1.67	8.76E-03
Scd2	-1.68	2.38E-04
Tcrb-J	-1.69	1.26E-02
Klrd1	-1.69	6.39E-05
NA	-1.69	5.55E-04
NA	-1.70	1.14E-04
Ighm	-1.70	3.11E-04
NA	-1.71	6.78E-03
Zdhhc2	-1.71	1.70E-04
NA	-1.72	3.17E-03
NA	-1.73	1.50E-04
NA	-1.75	4.50E-03
NA	-1.75	1.40E-02
Hist1h2bk	-1.76	3.99E-05
LOC100862491	-1.76	9.95E-05
NA	-1.76	1.47E-03
Eno3	-1.76	6.15E-05
NA	-1.77	1.00E-02
NA	-1.78	2.19E-05
Cd209a	-1.78	1.34E-02
Syne1	-1.79	4.20E-03
Cxcr6	-1.79	2.38E-04
Gbp1	-1.79	7.28E-03
Cmah	-1.80	8.26E-04
G0s2	-1.81	6.40E-05
Ccl24	-1.82	3.57E-05
Cx3cr1	-1.83	2.13E-04
Hspa1a	-1.83	2.38E-05
Samd3	-1.84	1.14E-04
Adrbk2	-1.85	5.15E-05
NA	-1.85	3.42E-02
BC035044	-1.85	2.36E-04
Cd3g	-1.86	1.14E-04
NA	-1.86	1.27E-03

Spn	-1.87	1.65E-05
NA	-1.88	3.31E-05
NA	-1.88	3.49E-03
S1pr1	-1.89	1.49E-05
NA	-1.90	1.42E-03
Gm6637	-1.91	2.40E-04
Ear1	-1.94	2.46E-03
Slc12a2	-1.96	1.26E-03
NA	-1.96	4.75E-02
Snord35b	-1.97	4.62E-04
Hpgd	-1.99	7.29E-04
Pglyrp1	-2.02	2.57E-06
Ccl8	-2.06	2.27E-05
Fam55d	-2.07	1.06E-04
NA	-2.10	1.87E-02
Hspa1b	-2.10	8.68E-06
NA	-2.11	1.19E-02
NA	-2.13	1.54E-03
NA	-2.17	3.57E-05
NA	-2.19	9.07E-06
NA	-2.19	9.07E-06
NA	-2.20	3.73E-05
Cd7	-2.28	4.67E-05
LOC100861812	-2.29	6.39E-05
NA	-2.29	1.84E-05
NA	-2.29	1.84E-05
Klre1	-2.30	2.98E-04
NA	-2.31	1.81E-02
NA	-2.31	1.57E-03
NA	-2.37	3.73E-03
NA	-2.37	5.89E-03
Hsph1	-2.37	4.57E-04
Trbv13-2	-2.44	3.74E-04
NA	-2.49	6.85E-04
Treml4	-2.54	2.57E-06
Igj	-2.55	8.04E-04
NA	-2.55	1.73E-02
Hspa1b	-2.65	5.21E-06
NA	-2.71	4.35E-02
Hist1h2ac	-2.80	6.17E-04
NA	-2.80	3.98E-02
Ace	-3.18	2.02E-06
Emr4	-3.36	4.44E-06
Retnlg	-3.45	1.08E-04