Mechanisms of Induction and Modulation of the Pro-inflammatory Cytokine Interleukin-1β

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Interleukin (IL)-1β is a powerful pro-inflammatory cytokine with important roles in directing both innate and adaptive immunity. As a result, its production is tightly controlled, with the synthesis of an inactive form (pro-IL-1β) and the requirement of a second signal. This induces the formation of the inflammasome, a macromolecular complex which mediates the maturation of IL-1β into the bioactive cytokine. Given its significance, it is important to identify mechanisms of IL-1β induction and modulation.

Firstly, we describe serum amyloid A (SAA), an acute phase protein with immunomodulatory properties, as a novel inducer of IL-1β. Using cells from genetically modified mice, the molecular mechanisms responsible were dissected, demonstrating the receptors TLR2 and NLRP3 as required for this effect. By instilling SAA into mice, we also show that SAA is able to induce IL-1β production *in vivo*.

Invariant natural killer T (iNKT) cells have also been shown to be important modulators of immunity, mediating both pro- and anti-inflammatory responses. iNKT cells are non-conventional T lymphocytes which recognise glycolipid in the context of CD1d, with the ability to interact with immature antigen presenting cells in an autoreactive manner. We link the regulatory ability of iNKT cells with IL-1β production, showing that a low activation signal leads to the induction of an IL-13-dominated cytokine profile, as well as weak engagement of the CD40-CD40L pathway. We show for the first time that through these mechanisms, iNKT cells are able to dampen the secretion of IL-1β upon subsequent stimulation of dendritic cells.

We hypothesise that this effect of iNKT cells is important in controlling inflammatory responses *in vivo*, and demonstrate exacerbated IL-1β production and inflammation during influenza virus infection of iNKT cell-deficient animals. This novel anti-inflammatory property of iNKT cells may be harnessed in the therapeutic intervention of inflammatory disorders.

For my family at home, and my family away from home

For Mum and Dad,

and for Carmen.

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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.

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Contents

Frequently used abbreviations

α-GalCer	α-galactosylceramide
APC	antigen presenting cell
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
BAL	broncho-alveolar lavage
BALF	broncho-alveolar lavage fluid
BCR	B cell receptor
BHA	butylated hydroxyanisole
BMDC	bone-marrow derived dendritic cell
BMDM	bone-marrow derived macrophage
CAPS	cryopyrin-associated periodic syndromes
CARD	caspase activation and recruiting domain
CD	cluster of differentiation
CD40L	CD40 ligand
DAMP	danger-associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalitis
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FACS	fluorescence-activated cell sorting
FPR	formyl-peptide receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
IAV	influenza A virus
IFN	interferon
Ig	immunoglobulin
IKK	inhibitor of κB kinase
IL	interleukin
IL-1Ra	IL-1 receptor antagonist
IL-1RAP	IL-1 receptor associated protein
iNKT	invariant natural killer T
iNOS	inducible nitric oxide species
IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
IκB	inhibitor of κB
LPS	lipopolyssacharide
LRR	leucine-rich repeat
m.o.i.	multiplicity of infection

MAPK	mitogen-activated protein kinase
MdDC	monocyte-derived dendritic cell
MDSC	myeloid-derived suppressor cell
MSU	monosodium urate
NF-κB	nuclear factor-κB
NLR	nucleotide-binding domain and leucine-rich repeat-containing receptor
PAMP	pathogen-associated molecular pattern
PR8	influenza A virus A/Puerto Rico/8/34
PRR	pattern recognition receptor
PYD	pyrin domain
RNA	ribonucleic acid
ROS	reactive oxygen species
SAA	serum amyloid A
sCD40L	soluble CD40 ligand
$sCD40L_1$	monomeric soluble CD40 ligand
$sCD40L_m$	multimeric soluble CD40 ligand
SEM	standard error of the mean
TCR	T cell receptor
TGF	tumour growth factor
Th	T helper
TLR	toll-like receptor
TNF	tumour necrosis factor
TRAF	tumour necrosis factor receptor-associated factor
TRIF	toll/IL-1R domain-containing adaptor inducing interferon-β
WT	wild-type

CHAPTER 1

Introduction

Chapter 1: Introduction

I. The physiological response to infection

Multicellular organisms need to efficiently gather information from their surroundings and respond appropriately in order to survive. This is especially important in the interaction between host and pathogen, where detection of infection needs to be rapidly communicated into an effective immune response. In vertebrates, the immune system is generally divided into two branches – innate and adaptive immunity [1].

The evolutionarily more ancient innate immune system is the first line of defence against pathogenic invasion, relying on detection of pathogen-associated molecular patterns (PAMPs) to distinguish between self and non-self and launch an attack which is non-specific. In contrast, adaptive immunity generates a highly specific response to the microbe detected, although this process takes a number of days before pathogen-specific cells accumulate in sufficient numbers.

As such, cells of the immune system are classified according to whether they fall within the innate or adaptive arm. Innate immune cells recognise PAMPs using germline-encoded pattern-recognition receptors (PRRs), and these include cells of the myeloid lineage such as monocytes/macrophages, dendritic cells (DCs) and granulocytes, encompassing neutrophils, eosinophils and basophils. In addition to PAMPs, damage to host cells, in the form of danger-associated molecular patterns (DAMPs), can also be detected by PRRs. Activation of PRRs in these cells triggers a cascade of signalling pathways to initiate a rapid, non-specific inflammatory response which controls pathogen spread and promotes clearance of infection. Initially, this is mediated through the secretion of anti-microbial molecules, as well as pro-inflammatory cytokines and chemokines. If not properly regulated, this inflammatory response can cause extensive damage to host tissues, leading to immunopathology. In addition, monocytes, macrophages and DCs facilitate the presentation of pathogen-derived antigens to cells of the adaptive immune system. As such, they are termed antigen presenting cells (APCs), and have critical roles in the initiation of adaptive immunity.

The adaptive immune system consists mainly of T and B lymphocytes which express highly diverse antigen-specific receptors. B cells are responsible for mediating humoral immunity by producing antibodies, or immunoglobulins (Ig), whilst T cells are divided according to coreceptor expression –

helper T cells ($CD4^+$) which coordinate and modulate the immune response, and cytotoxic T cells ($CD8^+$) which kill virus-infected and tumour cells. $CD4^+$ helper T cells are further classified based on function – Th1 (associated with cell-mediated immunity), Th2 (humoral immunity) and Th17 (interleukin (IL)-17 producing, associated with chronic inflammatory responses), with other novel subsets also suggested. T and B cell receptors (TCRs and BCRs) are generated by genetic rearrangement of fragments encoding TCR and BCR chains during differentiation, in a process called V(D)J recombination. In this way, a highly varied repertoire of antigen-specific T and B cells are generated. After recognising their antigen, a specific T or B cell clone then rapidly proliferates and begins to differentiate into effectors which are then responsible for clearing infection. Due to their specificity, adaptive immune cells are also responsible for establishing immunological memory, where later re-exposure to these antigens will induce a more rapid and robust anamnestic response.

Whereas B cells recognise antigen in their native conformation, T cells exclusively recognise processed antigen (in the form of peptides) in the context of major histocompatibility complex (MHC). T cells therefore rely on APCs to sample antigens from their environment and present peptides in the context of MHC. Consequently, APCs play a critical role in initiating the adaptive response. By secreting cytokines and expressing costimulatory molecules on the cell surface, APCs are also responsible for shaping the immune response by polarising T cells into specific subsets.

II. Activating the immune system – innate immune recognition

Discrimination between self and non-self forms the basis of pathogen recognition. In the case of the innate immune system, this is achieved by the engagement of PRRs by PAMPs and DAMPs (summarised in **Fig. 1.1**). This is important in the infectious non-self and danger models of innate immunity proposed by Janeway and Matzinger respectively, who provided explanations to the observation that recognition of antigen by T and B cells alone could not elicit an effective response [2, 3]. T and B cells also require a second signal, in the form of costimulation from APCs, to drive full activation. Upregulation of these costimulatory molecules is critically dependent on PAMP and DAMP recognition by PRRs on APCs, emphasising the significance of immune recognition by innate cells in driving the immune response.

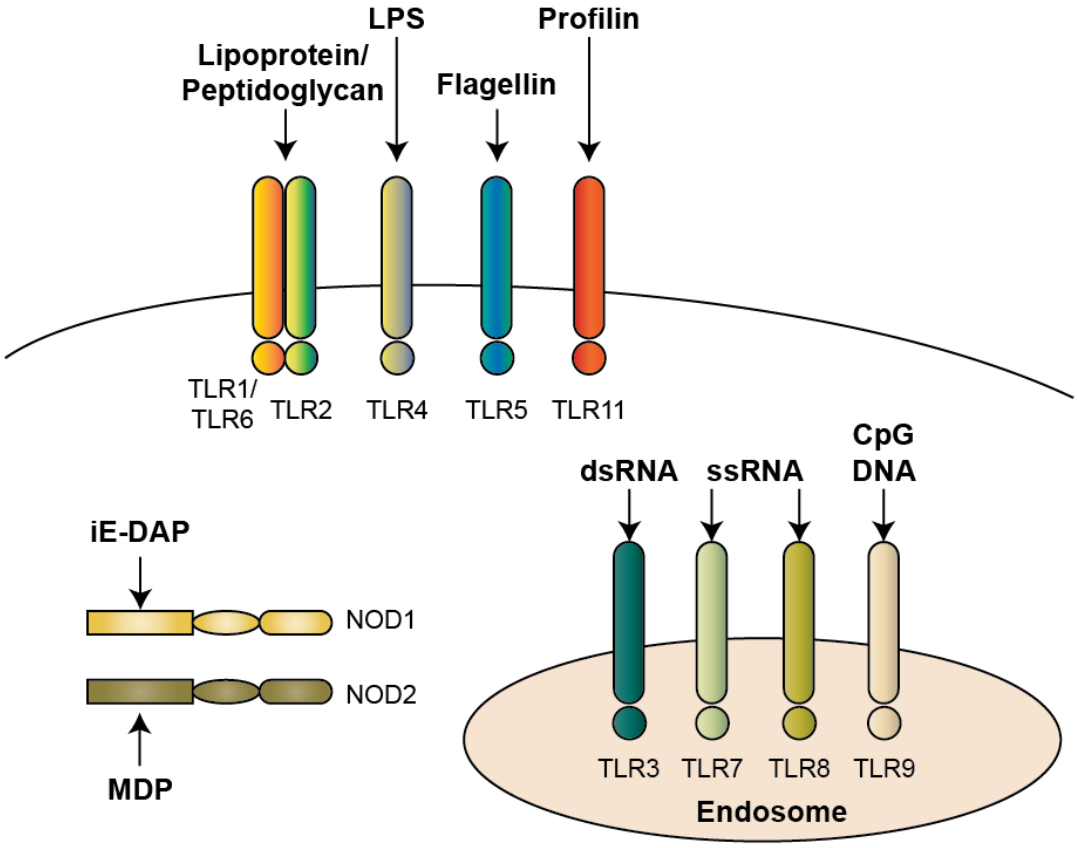

Fig. 1.1 Pattern recognition receptors. TLRs and NLRs reside on the cell membrane, endosomal membrane and cytoplasm to sense various PAMPs.

1. The TLRs

Toll-like receptors (TLRs) are a family of PRRs considered to be key in mediating innate recognition. TLRs are named because of their homology to Toll, first discovered in *Drosophila melanogaster* to regulate embryogenesis via the engagement of its ligand Spätzle [4]. Subsequent studies have also identified the importance of this pathway in *Drosophila* immunity, specifically in the production of anti-microbial peptides during bacterial and fungal infection [5] and, more recently, in antiviral immunity [6]. Toll activation leads to a signalling cascade culminating in activation of the transcription factor Dorsal, a homologue of the mammalian nuclear factor (NF)-κB [7].

The search for a mammalian homologue of the Toll proteins then began, with Medzhitov and Janeway identifying a protein which, when overexpressed, led to the production of pro-inflammatory cytokines [8]. This protein was later cloned and identified, by genetic ablation, to respond to bacterial lipopolysaccharide (LPS) or endotoxin, and is known today as TLR4 [9, 10, 11].

So far, 10 TLRs have been identified in humans, and 12 in the mouse, with their ligands and signalling pathways elucidated by overexpression and genetic ablation studies [12]. Of note, TLR2 binds to lipoproteins by forming heterodimers with TLR1 or TLR6, and TLR5 to bacterial flagellin. Whilst most of the TLRs reside on the cell surface to bind extracellular PAMPs, TLR3, 7, 8 and 9 are found in the endosomes, where they recognise RNA (TLRs 3, 7 and 8) or unmethylated CpG DNA (TLR9), important in the response against viral infection.

The TLRs have a conserved structure, consisting of a leucine-rich repeat (LRR) important in ligand recognition, and a Toll/IL-1 receptor (TIR) intracellular domain through which signalling occurs via protein-protein interactions with signalling adaptors. The TIR domain can interact with different combinations of adaptor molecules, such as myeloid differentiation primary response gene (MyD)88, which leads to the formation of a signalling complex including IL-1 receptor associated kinases (IRAKs) and TNF receptor associated factor (TRAF)6, which are important in transmitting signals downstream of the TLRs. As such, *Myd88*$^{-/-}$ animals show significant immune defects, and are highly susceptible to infection with intracellular pathogens such as *Mycobacterium tuberculosis* and *Leishmania major* [13, 14], via an inability of DCs from these animals to efficiently sense these microbes and induce production of the pro-inflammatory cytokine interleukin (IL)-12.

2. The NF-κB signalling pathway

TLR signalling through MyD88 leads to the activation of transcription factors which drive expression of genes associated with the pro-inflammatory response. Of these, one of the best characterised is the NF-κB family of transcription factors. There are five transcription factors in this family: p65 (RelA), RelB and c-Rel, together with NF-κB1 (p105) and NF-κB2 (p100), which are subsequently proteolytically processed into p50 and p52 respectively [15]. The NF-κB proteins typically function as dimers, with gene specificity dependent on the composition of the dimer.

MyD88-dependent signalling leads to activation of the canonical pathway of NF-κB activation, mainly generating p65-containing heterodimers which move into the nucleus to drive the transcription of pro-inflammatory genes such as *IL1B*, *TNFA* and *IL6* [16] **(Fig. 1.2)**. In resting cells, NF-κB dimers are sequestered in the cytoplasm by IκB (inhibitor of κB) proteins. Downstream of MyD88 signals, the IKK (IκB kinase) complex, which includes NEMO (NF-κB essential modifier) and IKKβ, phosphorylates the inhibitory IκB proteins, leading to their ubiquitinylation and subsequent degradation by the proteosome [17]. This releases NF-κB dimers and unmasks their nuclear localisation signal, allowing them to move into the nucleus for pro-inflammatory gene transactivation.

A second, non-canonical pathway of NF-κB activation is also present, mostly generating a different heterodimer, RelB-p52 and driving activation of a different set of genes [18]. A key step in this pathway is the processing of NF-κB2 (p100) into p52, mediated downstream of NIK (NF-κB-inducing kinase) and IKKα. Activation of signalling downstream of certain receptors, including lymphotoxin (LT)-β receptor, CD40 and other receptors in the tumour necrosis factor (TNF) receptor superfamily, has been reported to preferentially activate this pathway [15]. However, many studies also indicate that the canonical and non-canonical pathways are mechanistically linked, with perturbations in one able to affect the other. In addition, ligand binding of many receptors can result in activation of both pathways.

Downregulation of NF-κB signalling following activation occurs via three mechanisms; the re-expression of IκB proteins in the cell, the upregulation of deubiquitinases, such as A20, which modulate signalling components upstream of IκB, and the dissociation of signalling complexes by the induction of dominant-negative adaptors [19]. IκBα resynthesis is driven by NF-κB itself [20], setting up an autoregulatory loop. Newly synthesised IκBα enters the nucleus and associates with DNA-

bound NF-κB dimers, leading to their export back into the cytoplasm [21]. This leads to reduced NF-κB signalling by sequestering p50 and p65 subunits in the cytosol in an inactive state. Like IκBα, the expression of A20 (or TNFAIP3) is also driven by NF-κB signalling [22]. A20 is an ubiquitin editor which can modify the activity of various signalling proteins in the NF-κB pathway, playing an important role in regulating inflammation and immunopathology [23, 24, 25]. A20 acts on multiple proteins upstream of IκB degradation, including RIP1, RIP2, TRAF6 and NEMO, removing K63-polyubiquitin chains which are important for their signalling, and adding K48-polyubiquitin chains to mark them for proteosomal degradation [19, 26, 27]. In this way, A20 inhibits NF-κB signalling by attenuating transmission of the signal upstream of the activation of the IKK complex.

Whereas most TLRs require MyD88 to mediate their signalling, TLR3 signals exclusively through another adaptor, TRIF (TIR-domain containing adaptor inducing interferon-β) [12]. This can lead to NF-κB activation via an alternative pathway, and also the activation of the transcription factor interferon response factor (IRF)3, required for driving the expression of type I interferons (IFN), molecules important in the antiviral response. Interestingly, as well as utilising MyD88, TLR4 is also able to signal through TRIF; in fact LPS-induced NF-κB activation is only completely abolished in *Myd88*$^{-/-}$*Trif*$^{-/-}$ double knockout mice [28].

As well as NF-κB, TLR signalling also activates various other pathways, including mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK) and p38, also important in pro-inflammatory signalling. One important transcription factor downstream of these proteins is activator protein (AP)-1, which, as well as acting independently, can cooperate with NF-κB to drive transcription of certain pro-inflammatory genes such as *IL8*, *CCL5* and *GMCSF* [29].

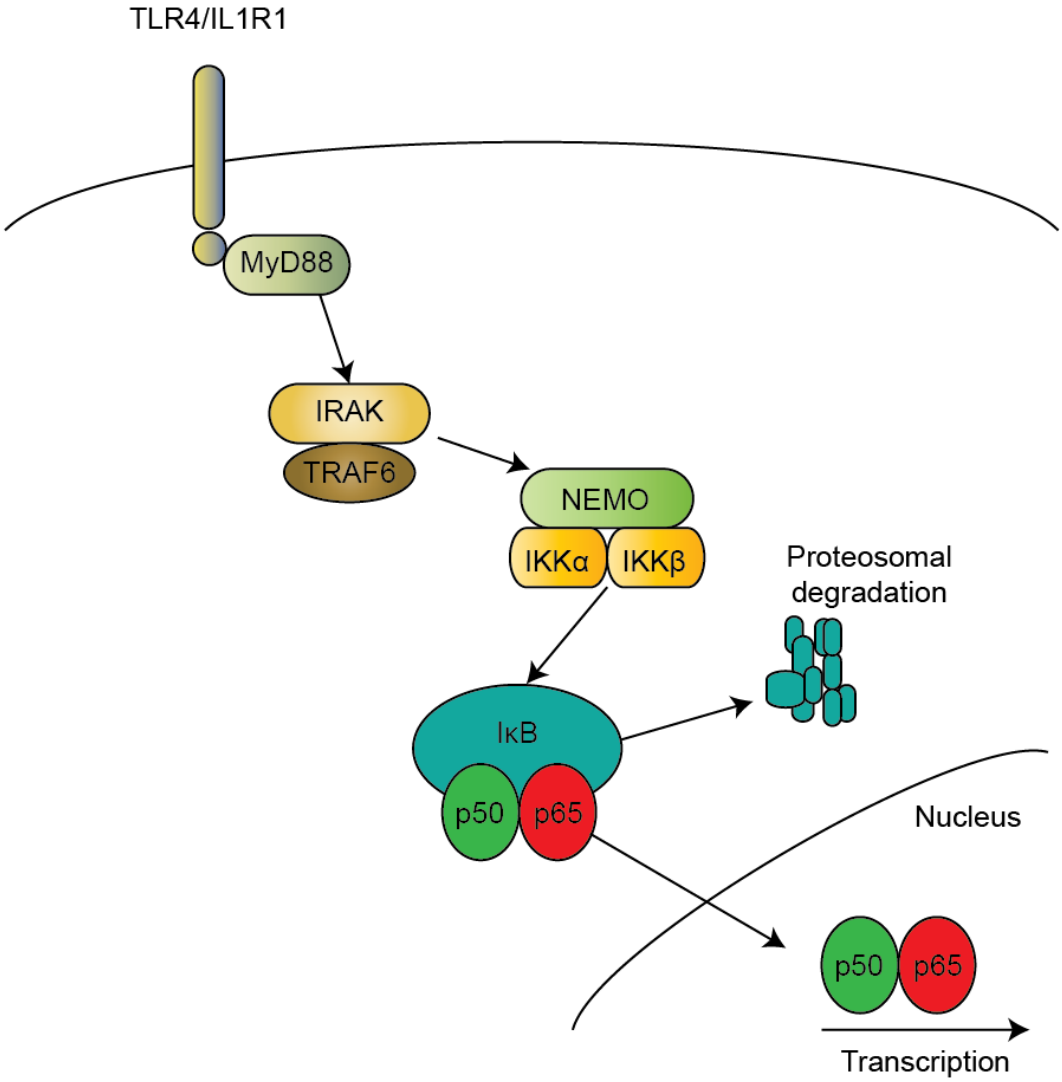

Fig. 1.2 Canonical pathway of NF-κB activation. TLR signalling culminates in the degradation of IκB proteins, which releases p50/p65 heterodimers to translocate into the nucleus and drive pro-inflammatory gene transcription.

3. The NLRs

The nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs) are cytoplasmic PRRs which are becoming increasingly important in our understanding of the innate immune response. Thus far, 23 members of this family have been identified in humans, with at least 34 in the mouse [30]. However, the function and ligands for most of these proteins remain elusive. The NLRs share a conserved tripartite structure within the family. The C-terminal part of the protein contains a leucine-rich repeat which detects PAMPs, followed by a central NACHT domain, important in self-oligomerisation following activation. The N-terminal portion of these proteins is more variable, with some NLRs containing a caspase recruitment domain (CARD) and others expressing a pyrin domain (PYD), acidic transactivating domain or a baculovirus inhibitor repeat (BIR) domain. All these motifs are responsible for signalling from the NLR, with each domain important for mediating protein-protein interactions with signalling adaptors. The NLRs can be divided into two groups based on the signalling pathway they stimulate. The activation of the first group leads to the formation of a macromolecular complex named the inflammasome, responsible for the maturation of certain cytokines in the IL-1 family. These NLRs will be considered later. The second group, the non-inflammasome NLRs, function in a fashion more akin to other PRRs in inducing pro-inflammatory signalling.

3.1 NOD1 and NOD2

NOD1 and NOD2 were among the first NLRs to be characterised, with both recognising products derived from the bacterial cell well. NOD1 is activated by the dipeptide γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) found in gram-positive bacteria [31], whereas NOD2 recognises muramyl dipeptide (MDP), a constituent of the cell wall in nearly all bacteria [32, 33]. Activation of NOD1 and NOD2 initiates signalling cascades which are qualitatively similar to those of the TLRs [30]. Oligomerisation upon ligand binding recruits receptor-interacting protein (RIP)2, via homotypic interactions between CARD domains on NOD1/2 and RIP2. This leads to activation of both NF-κB and MAPK signalling. NF-κB activation occurs via the ability of RIP2 to promote K63-polyubiquitylation of NEMO and recruitment of transforming growth factor-β activated kinase (TAK)1, pre-requisites for activating the IKK complex to degrade IκBα. In addition, NOD1 and NOD2 are able to initiate MAPK pathway signalling (specifically p38, extracellular signal-regulated kinase (ERK) and

JNK) via less well understood mechanisms, although this also involved RIP2, and possibly the adaptor CARD9. In this way, NOD1 and NOD2 activation culminates in NF-κB and MAPK pathway activation, which cooperate in inducing the expression of pro-inflammatory genes.

Whilst NOD1 and NOD2 activate similar transcription factors to TLRs, their agonists appear to induce lower levels of cytokines then those of TLRs. The reasons of this disparity are unclear, although triggering of TLRs in conjunction with NOD1 or NOD2 results in a synergistic effect, producing much stronger responses than either alone [34, 35].

The importance of NOD1 and NOD2 in the immune system has been highlighted by genetic linkage studies. NOD1 polymorphisms have been linked with the development of atopic hypersensitivities such as eczema and asthma [36, 37]. NOD2, in particular, has garnered great interest due to its genetic association with Crohn's disease, an autoinflammatory disease affecting the intestines [38, 39]. The molecular mechanism which links NOD2 with this disease is still unclear. However, perhaps surprisingly, the common polymorphisms associated with excessive intestinal inflammation are loss-of-function mutations, leading to reduced NF-κB activation and cytokine production when NOD2 is stimulated. Investigators have attempted to corroborate this finding with several hypotheses, including the decrease in NOD2-dependent secretion of anti-microbial defensins in Paneth cells, defective recognition and clearance of the bacterial flora and dysregulation of other TLR-mediated signalling pathways in the absence of functional NOD2 [30].

3.2 NLRs with regulatory properties

As well as activating PRRs to initiate and perpetuate the pro-inflammatory response, several NLRs have been described to perform modulatory roles. These NLRs typically have not been described to bind microbial ligands in their anti-inflammatory action, so it remains to be elucidated whether these proteins are constitutively active, or activated by binding to a self or exogenous ligand.

NLRP6 was initially described to be important in the production of IL-18 in maintaining intestinal homeostasis [40]. Recently, its role as a negative regulator of pro-inflammatory signalling downstream of TLRs was described. *Nlrp6*$^{-/-}$ cells were shown to induce stronger canonical NF-κB activation, as well as ERK signalling, in response to LPS [41]. Physiologically, this translated to increased

resistance to infection by several bacterial species, including *Salmonella typhimurium* and *Escherichia coli*, via the increased recruitment of neutrophils and monocytes.

Like NLRP6, NLRP12 has also been reported to downregulate NF-κB signalling [42]. However, this NLR was shown to control the non-canonical NF-κB pathway, by decreasing levels of NIK, which phosphorylate IKKs. As a result of constitutively active and elevated NIK and processing of NF-κB2, *Nlrp12*$^{-/-}$ animals were highly susceptible to colon inflammation and colitis-associated cancer.

A bioinformatics screen for TRAF-binding NLRs recently identified NLRC3 as a modulator of canonical NF-κB signalling [43]. NLRC3 levels were shown to decrease in macrophages upon LPS stimulation, with *Nlrc3*$^{-/-}$ cells inducing higher levels of *Il1b*, *Tnf* and *Il6* transcription at early time points after stimulation. In addition, NLRC3-deficient mice were reported show more severe septic shock upon LPS injection.

Finally, NLRX1 was also shown to downregulate canonical NF-κB signalling [44]. Dissociating from TRAF6 after activation, NLRX1 binds to the IKK/IκB complex to reduce IKK phosphorylation and activation. NLRX1 knockdown using shRNA expression *in vivo* led to increased susceptibility to septic shock, highlighting its role in regulating TLR signalling.

4. Other families of PRRs

As well as TLRs and NLRs, another major class of receptors on the cell surface is the C-type lectin-like receptors (CLRs), a large family of carbohydrate-binding proteins which primarily bind to their ligand in a calcium-dependent manner [45]. This diverse group of receptors encompasses receptors in the Ly49 family which regulate NK cell activation, endocytic receptors on the DC cell surface such as DC-SIGN and DEC-205 which are important for antigen uptake for processing and presentation, as well as a subgroup including Dectin-1, Dectin-2 and Mincle, which function as PRRs. Upon ligation, canonical and non-canonical NF-κB activation is induced, largely via the adapters Syk and CARD9. As a result of their ability to recognise carbohydrates, these receptors are especially important in immunity against pathogens with complex sugars in their cell walls, such as fungi [46], parasites [47] and certain bacteria such as *Mycobacterium* [48].

In addition to these membrane-bound PRRs, another class of nucleic acid sensors are found in the cytosol. As such, they are particularly important in antiviral immunity. These include retinoic acid-

inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5), RNA helicases which mediate signalling culminating in NF-κB activation and pro-inflammatory gene transcription via TRAF6, in addition to IFN induction via IRF3 and IRF7 [49, 50]. These receptors which recognise dsRNA have also been shown to activate inflammasome signalling, both in NLRP3-independent and dependent ways [51]. In addition, there are DNA sensors present in the cytoplasm, such as RNA polymerase III and the interferon-inducible protein IFI16 (which has been shown to induce an inflammasome during herpesvirus infection) [52]. As yet, the exact nucleic acid species recognised by each receptor is still unclear, although it is becoming increasing apparent that this class of sensors is also important in immunity to intracellular bacteria and parasites, as well as viruses.

III. The inflammatory response

One of the key features of innate immunity is its ability to rapidly mount an inflammatory response to pathogenic insult or tissue damage. Historically, it was shown that five cardinal features accompany the physiological response to injury or infection – *dolor* (pain), *calor* (heat), *rubor* (redness), *tumor* (swelling) and *functio laesa* (loss of function). This was termed 'inflammation', and our insight into this complex process was only understood at the cellular level in the 1830's owing to the invention of microscopy.

After activation of PRRs, innate immune cells rapidly secrete cytokines and chemokines which culminate in vasodilation and the recruitment of myeloid cells at the site of infection [53]. At the early stages, one characteristic of the acute inflammatory response is the accumulation of neutrophils at the infection site. Via their ability to phagocytose microbes and produce enzymes with power anti-microbial activity (such as myeloperoxidases and proteinases), neutrophils are among the first line of defence in containing the infection and preventing pathogen dissemination to other sites of the body [54].

The ability of neutrophils to externalise powerful proteolytic enzymes is one mechanism which can lead to immunopathology. This results from an exacerbated inflammatory response, where the activation of innate immune cells becomes dysregulated. The failure to properly regulate the inflammatory response can be due to a breakdown in regulatory networks and/or the inability of phagocytes to clear the pathogen. This perpetuates activation signals and leads to chronic phases of inflammation, which differs from the acute phase by mononuclear cells such as monocytes/macrophages becoming the main cellular constituents of the inflamed area [53]. Immune regulation is therefore key in maintaining the balance between promoting microbial clearance and preventing immunopathology.

1. The acute phase response

The acute inflammatory response is also characterised by a series of systemic reactions to infection. This acute phase response clinically presents with fever, an increase in blood leukocyte numbers and the concentration of pro-inflammatory mediators in the blood, activation of the complement system, and elevated levels of several plasma proteins, termed the acute phase proteins [55]. The half-life of

these proteins is generally short, making them useful diagnostic tools in assessing inflammation. The acute phase response is typically strongest when induced by bacterial infection, owing to the induction of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 by myeloid cells. During the acute phase, profound changes in cellular metabolism occur in the liver, with these cytokines stimulating the synthesis of the acute phase proteins by hepatocytes [55]. The two proteins whose levels increase most dramatically are C-reactive protein, which binds and opsonises dead cells and microbes to facilitate complement activation, and serum amyloid A (SAA).

2. Serum amyloid A

During infection, SAA levels in the blood plasma can increase to over 1000 times that of resting levels, peaking three days after infection and reaching the mg/ml range [56]. Its significantly elevated synthesis during the acute phase is largely mediated by hepatocytes, but myeloid cells such as monocytes and macrophages have also been shown to secrete SAA [57, 58]. The term SAA describes a family of proteins – with SAA1 and SAA2 being induced during the acute phase and SAA4 expressed constitutively. A fourth member, SAA3, is also present, but as a pseudogene. During the acute phase, SAA1 and SAA2 production are stimulated by IL-6 and IL-1, via IL-6 and NF-κB responsive elements in the promoter and enhancer region [59].

SAA is an apolipoprotein which associates with high-density lipoprotein (HDL) in the serum. During the acute phase response, its composition changes dramatically, with up to 80% of HDL consisting of SAA [60]. This has profound effects on lipid flux and processing, especially in immune cells. Studies with THP-1 cells showed preferential binding to SAA-containing HDL molecules, enhancing their uptake [61]. This has been associated with increased uptake of cholesterol in macrophages during chronic inflammation, promoting the formation of foam cells which are prevalent in atherosclerotic lesions.

SAA has also been shown to have a role in immune modulation, by its ability to induce cytokine production from leukocytes. These include both pro- and anti-inflammatory cytokines, and include IL-1β, IL-10, IL-12, TNF-α and G-CSF [56]. As well as cytokine production, SAA has been shown to induce leukocyte recruitment. The ability of SAA to induce a wide variety of cytokines and cellular responses may be partly due to its ability to bind a large number of cell surface receptors. Of particular relevance to the immune system are TLR2, TLR4, FPR2 and RAGE. Signalling downstream

of all these receptors can lead to pro-inflammatory NF-κB signalling, suggesting SAA is part of a self-amplifying loop which enhances and prolongs the inflammatory response. However, SAA has also been shown to induce anti-inflammatory cytokines such as IL-10 and TGF-β downstream of these receptors [62, 63]. The ability of SAA to bind multiple receptors, together with the ability of these receptors to engage different signalling pathways depending on the cell type, contributes to the considerable plasticity in SAA-mediated immune modulation (summarised in **Fig. 1.3**).

As well as increasing during infection, SAA levels have been observed to be elevated during various

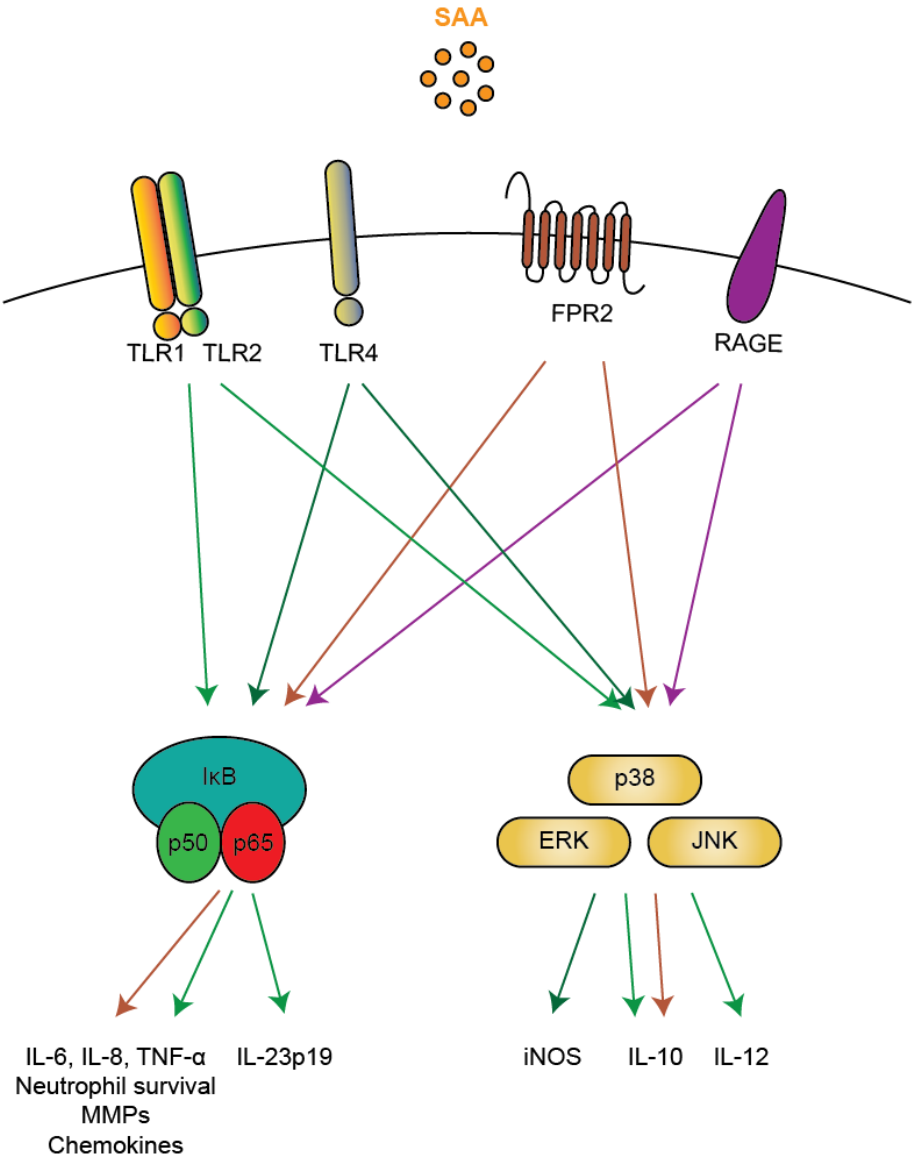

Fig. 1.3 Signalling induced by SAA. SAA engages multiple receptors on the cell surface, culminating in the activation of several intracellular signalling pathways, including NF-κB and MAPK. The expression of various cytokines, chemokines and factors associated with immunomodulation have been described downstream of these pathways. Adapted from Malle *et al.* 2009

cancers [56], such as melanoma, consistent with carcinogenesis being closely allied with inflammation. This gives SAA the potential to be used as a biomarker during cancer diagnosis. The role played by SAA in the tumour microenvironment is still unclear, although a recent report from our laboratory highlighted increased serum SAA in melanoma patients, correlating with the stage of progression, and its ability to elicit IL-10 secretion from neutrophils [62]. Therefore, one mechanism by which SAA can promote tumour persistence is by downregulating the tumour-specific response. The exact outcome resulting from SAA interaction will most likely depend on the cell type, receptor(s) engaged and microenvironment involved.

3. Interleukin-1β

Even several decades before the formal identification of IL-1α and β, the pyrogenic action of these two inflammatory proteins were already demonstrated. In 1948, Paul Beeson showed that injection of lysed neutrophils into rabbits could induce fever [64], with Wood *et al.* demonstrating, 20 years later, that the pyrogen is a 10-20kDa protein [65]. Various other properties of this as yet unidentified pyrogen were observed in the next decade, including the potentiation of mitogenic effects in T cells (with the molecule named Lymphocyte Activating Factor) [66] and the ability to induce the release of other molecules, including prostaglandins and SAA [67]. However, the first published isolation of IL-1 was in 1981, when a 17kDa protein, purified from synovial cells and termed catabolin, was shown to induce resorption of cartilage proteoglycan [68]. Two IL-1 proteins were then cloned in 1984 (mouse IL-1α and human IL-1β), before the human IL-1α and β sequences were published a year later, in 1985 [69, 70, 71].

The discovery of IL-1α and β cemented the idea that inflammation is a process induced and controlled by endogenous soluble factors, and that these pro-inflammatory molecules would have important medical applications. Today, our understanding of both these pro-inflammatory factors has informed the design of novel therapies for diseases such as rheumatoid arthritis and psoriasis. Due to the attention the inflammasome has received in the last decade, IL-1β has enjoyed a renaissance in the field of immunology.

3.1 Induction of IL-1β production

The importance of IL-1β in the immune system is highlighted by the steps required for its production. IL-1β, like some other cytokines in the IL-1 family, requires two steps for its production [72]. The first results in the induction of an inactive form of the cytokine, pro-IL-1β. A subsequent, second signal is then required for activating cleavage of this pro-form into the bioactive cytokine, with subsequent secretion from the cell **(Fig. 1.4)**.

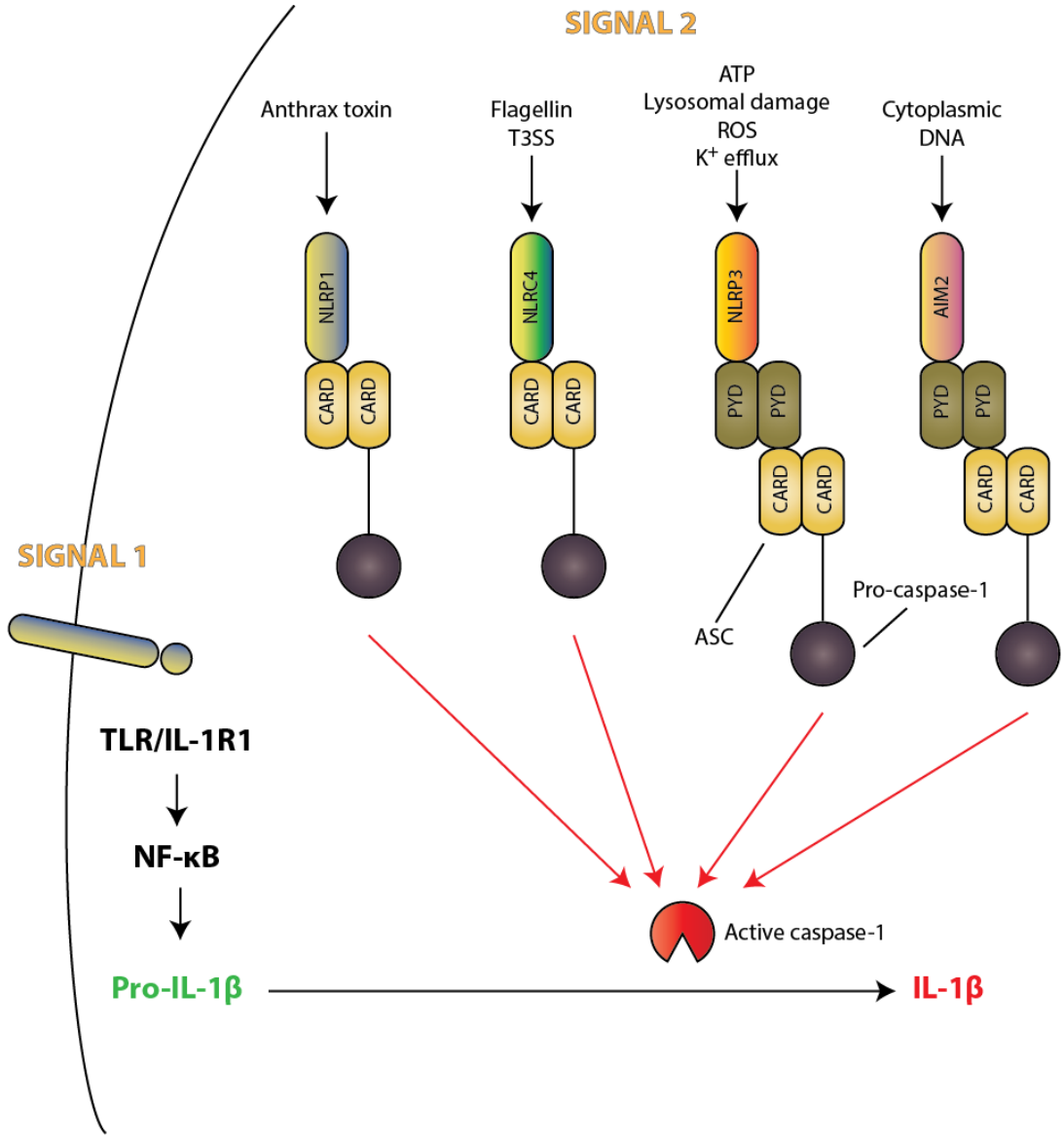

Fig. 1.4 Induction of IL-1β maturation by the inflammasomes. Signal 1 from TLR/IL1R1 signalling leads to the induction of pro-IL-1β. A second signal engages cytoplasmic NLRs and leads to the formation of the inflammasome, which activates caspase-1. Caspase-1 is responsible for cleaving pro-IL-1β into its active form and mediating its secretion from the cell.

3.2 IL-1β transcription

Transcription of the *Il1b* gene is dependent on the activation of NF-κB, with primarily the canonical p50/p65 (or p50/RelA) heterodimer driving transcription [73, 74]. As such, most microbial and self-derived stimuli which activate the canonical NF-κB pathway are able to provide signal 1 for pro-IL-1β synthesis. Biochemical and structural analyses of the *Il1b* promoter and enhancer regions have also identified various other transcription factors which can bind, but whether some of these interactions are physiological are debatable. However, unlike most other cytokines, the *Il1b* regulatory regions spread over several thousand base pairs upstream of the transcriptional start site, suggesting strict control and modulation [75]. Kinetic analysis of *Il1b* transcription carried out in myeloid cells showed two distinct phases of transcription factor association. Stimulation was followed by rapid recruitment of the NF-κB protein p65 to the enhancer region of *Il1b*, initiating rapid transcription which was driven by the combined activity of p65 and IRF8. This was followed by a second phase where phosphorylated PU.1 and IRF4 mediated sustained, but more moderate, transcription [76]. This and other reports firmly point to activation of p65 as a crucial event in driving *Il1b* transactivation. Indeed, modulation of NF-κB activity with pharmacological agents such as glucocorticoids correlates with *Il1b* gene transcription [77, 78].

The *Il1b* gene is one of the most rapidly transcribed genes after stimulation, with transcript levels increasing before those of other cytokines, including IL-12p40, IL-4 and IFN-β [79, 80, 81]. This has been attributed to the enhancer of *Il1b* having a poised chromatin architecture which remains unchanged after transactivation of the gene, with PU.1 pre-bound to the DNA [82]. This configuration was shown to be induced during monocyte differentiation, with the promoter opening during monopoiesis [83]. These observations suggest that the chromatin structure at the *Il1b* enhancer is available for rapid transcription, with this architecture imprinted onto IL-1β-producing cells during their differentiation.

Modulation of IL-1β transcription by exogenous factors occurs at several levels. Most cytokine-induced negative regulation is exerted at the level of NF-κB signalling, with IL-4, IL-13 and IL-10 shown to be able to reduce IκBα degradation and p65 translocation into the nucleus [84, 85]. In addition, epigenetic changes and transcription factor binding at the promoter can also influence the transcriptional activity of the gene. During endotoxin tolerance, a refractory period following strong

pro-inflammatory stimulation, chromatin remodelling occurs at the *Il1b* promoter, with increased levels of H3K9 methylation leading to repression of transcription [86]. This structure has been shown to be induced by RelB, raising the possibility that an initial phase of NF-κB activation may imprint this conformation on the *Il1b* promoter [87]. In addition, the enhancer is constitutively occupied by p50 homodimers, which lack the transactivation domain present on p65 required for driving transcription. Conversely, IκBβ, a member of the IκB family of proteins, was recently found to prolong IL-1β transcription *in vivo* [88]. Unlike IκBα which shuttles between the cytoplasm and nucleus, hypophosphorylated IκBβ remains nuclear [89]. One mechanism of shutting off NF-κB signalling involves the chaperone activity of newly resynthesised IκBα, which dislocates NF-κB proteins from DNA and accompanies them out of the nucleus [90]. By associating with p65 bound on the DNA, IκBβ prevents the binding of IκBα and thus prolongs its transactivating activity [91, 92].

3.3 IL-1β translation

Interestingly, several reports have pointed to the dissociation between transcription and translation of the *Il1b* mRNA. Whilst microbial substances and IL-1 itself induce both transcription and translation, some non-TLR ligands, including the complement component C5a, hypoxic conditions and low level CD40 signalling induce the upregulation of *Il1b* mRNA without significant translation in pro-IL-1β protein [93]. In these cases, most of the *Il1b* transcript is degraded, owing to an instability element in the coding region [94, 95]. This separation of transcription and translation adds another layer of regulation in the production of IL-1β.

3.4 The inflammasomes

Following accumulation pro-IL-1β in the cell, a second signal is then required to cleave this protein into the mature cytokine. This is mediated by recognition of PAMPs and DAMPs by certain members of the NLR family. These PRRs have been under intense scrutiny due to their ability to activate the assembly of a multi-protein complex known as the inflammasome. The inflammasome is responsible for the proteolytic cleavage of inactive precursors of IL-1 family members IL-1β and IL-18, as well as IL-1α in some reports [96]. Inflammasome assembly leads to the autocatalytic activation of caspase-1, the enzyme generally responsible for the cleavage event, from its inactive form. In addition, caspase-1 is also important for the secretion of the processed cytokines by a non-classical secretion pathway, as these proteins do not contain a conventional signal peptide [97]. Signal 1 (NF-κB activation) and

signal 2 (inflammasome activation) are also intimately linked, as inflammasome activation fails to occur in the absence of a good quality signal 1. This was attributed to the requirement for NF-κB, in addition to inducing pro-IL-1β, to prime the inflammasome by upregulating the expression of its components [98].

The idea of an inflammasome was first described in a biochemical study by Tschopp and colleagues [99], who identified NLRP1 as a protein capable of associating with caspase-1. To date, the NLRs NLRP1, NLRP3, NLRP6 and NLRC4 have been described to be able to form inflammasomes. Despite identification of stimuli which activate of each of these receptors, the exact identity of the molecule(s) which interact with the LRR domain is unknown. In addition, inflammasome formation has also been demonstrated by absent in melanoma (AIM)2 and melanoma differentiation-associated protein (MDA)5, non-NLR family members which bind cytosolic dsDNA.

3.4.1 NLRC4

NLRC4 (previously known as Ipaf) contains a CARD domain and is therefore able to directly interact with caspase-1. Like TLR5, NLRC4 is activated by bacterial flagellin, although it is unique in that it recognises intracellular flagellin [100, 101]. An elegant study also showed that the rod components of the bacterial type III secretion system can also trigger NLRC4, via the recognition of a conserved sequence motif found in both rod proteins and flagellin [102]. Recently, various reports have increased our understanding of how NLRC4 is activated. Two independent studies concluded that NAIP2, NAIP5 and NAIP6, other members of the NLR family, are the receptors which physically associate with the rod protein PrgJ (NAIP2) and flagellin (NAIP5 and 6). These NLRs then associate with NLRC4 to activate its inflammasome [103, 104]. However, whereas NAIPs 1 to 7 are expressed in the mouse, only one is present in humans, and this NAIP does not respond to flagellin or PrgJ [103]. The laboratory of Vishva Dixit has also identified the presence of a single, conserved serine residue on NLRC4, which is phosphorylated in the presence of NLRC4 activators by protein kinase C (PKC)δ [105]. These reports extend our understanding of how NLRC4 inflammasome activation is triggered.

In vivo models have identified NLRC4 as an important sensor in generating protective immunity against intracellular, flagellated bacteria. *Nlrc4*$^{-/-}$ animals show much greater susceptibility to infection by *Salmonella* and *Legionella* [106, 107]. An essential role of NLRC4 was recently highlighted in the intestine, through its activity in discriminating between commensal and pathogenic bacteria in the gut

[108]. The authors found that intestinal phagocytes such as macrophages and DCs were refractory to TLR stimulation but constitutively expressed pro-IL-1β, and that NLRC4 was the only active inflammasome in these cells. The ability of these cells to distinguish between flora and pathogen comes from the requirement of NLRC4 ligands to be present in the cytosol, as only pathogenic bacteria would inject bacterial proteins into target cells. These studies emphasise the importance of the NLRC4 inflammasome in maintaining anti-bacterial immunity.

3.4.2 NLRP3

NLRP3 (previously NALP3 and cryopyrin) is the most promiscuous of the inflammasome-forming NLRs, with many different molecules and pathogens shown to induce its activation, ranging from adenosine triphosphate (ATP), silica and alum to influenza virus, *Candida albicans* and bacterial pore-forming toxins [109]. NLRP3 expression has been shown to be limited to myeloid cells including monocytes, macrophages and DCs [110]. Unlike NLRC4 which has a CARD domain at the N-terminal end, NLRP3 has PYD domain and therefore recruits caspase-1 via the adaptor apoptosis-speck containing (ASC). The considerable diversity of NLRP3 activators has led investigators to question the identity of the ligand which is directly engaged, as yet unclear. NLRP3 activation has broadly been divided into three signalling pathways, involving lysosomal damage, oxidative stress and ion imbalance in the cell. Nevertheless, it should be noted that most NLRP3 stimuli are capable of inducing more than one of these interlinked pathways.

Particulate activators of NLRP3 include monosodium urate (MSU) [111], silica, asbestos [112] and alum [113, 114]. MSU is an endogenous molecule released from dying cells, building up in the inflammatory condition gout, whereas silica and asbestos are environmental insults which, once inhaled, are associated with chronic inflammation. Alum is the most commonly used vaccine adjuvant, consisting of aluminium phosphate or aluminium hydroxide suspensions. Once these particulates are phagocytosed by myeloid cells, activation of NLRP3 has been shown to be dependent on rupture of the phagolysosomes. This process releases the proteinase cathepsin B into the cytosol, with inhibition of this enzyme able to abolish NLRP3 activation.

Secondly, inhibition of reactive oxygen species (ROS) generation has been demonstrated to dampen NLRP3 activation, with pharmacological inhibition of NADPH oxidase able to inhibit IL-1β cleavage induced by ATP, MSU, silica and asbestos. The source of ROS required for these processes is likely

to be both cellular and mitochondrial, with reports implicating a role for NADPH oxidase and mitochondrial activity [112, 115]. Thioredoxin-interacting protein (TXNIP) has recently been identified as a link between oxidative stress and inflammasome activation, with a rise in intracellular ROS able to displace TXNIP from its interaction partner thioredoxin and allow TXNIP to directly bind to the LRR of NLRP3 [116].

The third mechanism for NLRP3 activation involves a change in the ionic balance of the cell, with potassium (K^+) efflux shown to be essential in this process [117]. Pore-forming toxins derived from bacteria such as *Salmonella* and *Listeria* are able to insert into the plasma membrane and allow K^+ ions to flow out of the cell [118]. In addition, stimulation with ATP engages the puronergic P2X7 ion-gated channel on the cell surface, resulting in K^+ efflux and the formation of a pannexin-1 pore [119]. The role of K^+ efflux is supported by the abrogation of these responses in high extracellular K^+ concentration, thereby abolishing the ion gradient. In addition to K^+, perturbing the balance of other cationic species, such as H^+, can also lead to NLRP3 activation. This was observed during influenza virus infection, with the viral protein M2 forming a proton-selective pore in the Golgi apparatus and ionic efflux from the Golgi essential in NLRP3 stimulation [120].

However, the exact molecular mechanism linking low K^+ concentration with NLRP3 activation remain unclear, and low intracellular K^+ concentrations alone are unable to directly activate the NLRP3 inflammasome [121]. In addition, the induction of ROS by other agents, such as cytokines, without the activation of NLRP3 must be explained, perhaps by the requirement for a specific ROS molecule or localisation. These reports indicate that intracellular K^+ and ROS, whilst required, are not sufficient to activate NLRP3. This suggests that there may be considerable redundancy and crosstalk between these pathways.

As a result of the range and nature of its stimuli, NLRP3 has been described as a sensor of immune danger signals. In particular, MSU and ATP are released from dying cells, implying that NLRP3 is an indirect sensor of cell death. Furthermore, NLRP3 has also been shown to be activated by necrotic cells and ER stress, as well as oxidised DNA during apoptosis [122, 123, 124, 125]. This gives NLRP3 the ability to respond to a range of DAMPs, as well as initiate sterile inflammatory responses.

The physiological importance of NLRP3 has been clearly demonstrated in *Nlrp3*$^{-/-}$ deficient mice, as well as patients harbouring mutations in the *NLRP3* gene. Gain-of-function mutations in these patients

lead to one of three autoimmune diseases, known collectively as the cryopyrin-associated periodic syndromes (CAPS) – familial cold autoinflammatory syndrome, Muckle-Wells syndrome and neonatal-onset multisystem inflammatory disease [126]. These debilitating diseases are characterised by episodic or fluctuating systemic inflammation and fever, with mononuclear cells from CAPS patients able to spontaneously secrete IL-1β *ex vivo*. In addition, proof of IL-1β as the causative agent of these syndromes has come from the use of IL-1 receptor antagonists as treatments [127]. Furthermore, the diverse range of stimuli which can activate NLRP3 has implicated it in many different pathologies. These include gout (with the involvement of MSU) [111], Alzheimer's disease (fibrillar amyloid-β) [128], atherosclerosis (cholesterol crystals) [129] and diabetes (islet amyloid polypeptide and glucose signalling leading to ROS generation) [116, 130], as well as many infections including influenza [131], tuberculosis [132], candidiasis [46] and gonorrhoea [133].

3.4.3 Other inflammasomes – NLRP1, NLRP6 and AIM2

Despite its association with caspase-1 reported in the first paper describing the inflammasome [99], the activity of NLRP1 is less well-described. This is perhaps due to the scarcity of activators which have been found. Whereas only one *NLRP1* gene exists in humans, three highly polymorphic *Nlrp1* genes have been described in the mouse. One of these, *Nlrp1b*, encodes a receptor which responds to the lethal toxin of *Bacillus anthracis* [134]. Macrophages from mice expressing sensitive forms of this allele are extremely sensitive to caspase-1-dependent cell death [135]. In human cells, however, NLRP1 has not been shown to recognise lethal toxin, although there are reports that it can activate caspase-1 in response to MDP [136, 137].

A recent report has identified NLRP6 as important in mediating caspase-1-dependent responses *in vivo* [40]. This study found that a NLRP6- and ASC-containing inflammasome was important in the production of bioactive IL-18 from the intestinal epithelium, which prevents the colonisation of specific bacterial species associated with colitis. Interestingly, the authors also reported the ability of this "dominant" colitogenic flora to be transmitted from *Nlrp6*$^{-/-}$ to WT mice during cohousing, suggesting a role for the inflammasome and IL-18 during the acquisition of the intestinal microbiota in preventing the establishment of these pathogenic species. This adds to the described role of NLRP6 as an inhibitor of NF-κB signalling [41].

The AIM2 inflammasome is the best characterised inflammasome not to involve an NLR. AIM2 senses dsDNA via its C-terminal HIN domain, with oligomerisation leading to recruitment of ASC and, subsequently, caspase-1 via its N-terminal pyrin domain [138, 139]. AIM2 was described to be important in mediating immunity against intracellularly replicating bacteria such as *Francisella tularensis*, via detection of bacterial DNA [140]. In addition, *Listeria monocytogenes* has also been reported to be recognised by AIM2, with the NLRP3, NLRC4 and AIM2 inflammasomes all cooperating to respond to this pathogen [141].

3.4.5 Caspase-1-independent IL-1β maturation

Although inflammasome activation is required for the cleavage of pro-IL-1β into its bioactive form in response to many stimuli, caspase-1-independent IL-1β maturation has also been described. Neutrophil serine proteases have been suggested to cleave IL-1β *in vivo*, although their exact specificities and mode of activation remain to be clarified. However, it has been observed that *Casp1*$^{-/-}$ mice show a similar phenotype to wild-type mice in neutrophil-dominated inflammatory diseases such as gout [93]. In this model, neutrophil cell death leads to the release of proteases which cleave extracellular IL-1β. The exact proteases responsible are yet to be identified, but may include cathepsin G, chymase, neutrophil elastase and proteinase A. Biochemical and *in vitro* evidence also point to neutrophil-derived proteinase 3 as a candidate IL-1β cleaving enzyme, with multiple studies reporting its ability to cleave pro-IL-1β, as well as rescue IL-1β cleavage when caspase-1 activity is inhibited [142, 143, 144, 145].

Increasing evidence also suggests that other caspases are able to cleave IL-1 family cytokines into their bioactive form. Caspase-8 was demonstrated to cleave IL-1β downstream of TLR3 and TLR4 activation, with TRIF signalling essential to drive caspase-8 activity [146]. FAS-ligand, a potent inducer of caspase-8-dependent apoptosis, has also been reported to induce IL-1β cleavage in a caspase-1-independent manner [147]. In addition, caspase-8 is able to cleave caspase-1 [148], bringing about the possibility that caspase-8 may enhance NLR activity by activating caspase-1. However, there are no reports indicating that active caspase-8 forms inflammasome complexes.

In contrast, both caspase-4 and caspase-11 have recently been reported to associate into inflammasomes. Caspase-4 was shown to be required for pro-IL-1β maturation in human keratinocytes and macrophages, with caspase-4 found to be associated with the NLRP3 and AIM2

inflammasomes [149]. Alteration of the active site of caspase-4 revealed that caspase-1 cleavage in this model was dependent on caspase-4, thus indicating caspase-4 may have a role in mediating caspase-1 cleavage, or at least in enhancing its autoproteolytic activity. Caspase-11 was recently reported to be important due to the discovery that *Casp1*$^{-/-}$ mice which have been used in inflammasome studies are, in fact, doubly deficient in caspase-1 and caspase-11 activity [150]. This occurred because the *Casp1*$^{-/-}$ strain was generated in a 129 background, which harbours a natural mutation in the *Casp11* gene. Subsequent backcrossing onto other strains has failed to eliminate this mutated gene as the *Casp1* and *Casp11* genes are under strong genetic linkage. This study showed that certain IL-1β-inducing stimuli, such as *E. coli* and cholera toxin, induce the formation of a non-canonical inflammasome which includes both caspase-11 and caspase-1. Caspase-11 was important in activating caspase-1 and therefore IL-1β cleavage under these conditions. In addition, a requirement for caspase-11 but not caspase-1 was shown for induction of cell death in response to these stimuli. However, other stimuli such as ATP were shown to be solely dependent on caspase-1, forming a canonical NLRP3 inflammasome which does not include caspase-11. These studies begin to suggest that, depending on the stimulus encountered, inflammasomes with different compositions are formed. Further study will elucidate whether these "non-canonical" inflammasomes can be regulated differently and to what extent can contribute to IL-1β-mediated immunity.

3.5 IL-1β-induced signalling

A functional receptor for IL-1β was identified and cloned in 1988, with the realisation that both IL-1α and IL-1β signal through this receptor [151]. Signalling through this receptor, IL-1R1, was found to be dependent on the recruitment of a second protein, named IL-1R accessory protein (IL-1RAP) [152]. Details of the IL-1R signalling pathway were only elucidated almost a decade later, with the discovery that the IL-1R proximal signalling complex includes the proteins IRAK, MyD88 and TRAF6 [153, 154, 155]. Upon binding of IL-1α or IL-1β, IL-1RAP is recruited to the IL-1R1, juxtaposing two TIR domains and allowing the association of MyD88, IRAK4 and TRAF6 to the heterodimer. The ensuing signalling pathway is identical to that downstream of most TLRs, culminating in the canonical activation of NF-κB and pro-inflammatory gene transcription to initiate or perpetuate the inflammatory cascade. Given the dependency of pro-IL-1β expression on NF-κB, this sets up a self-amplifying loop, where IL-1 induces IL-1.

With their potent pro-inflammatory effects, signalling as well as production of IL-1α and IL-1β are tightly regulated. IL-1R2 was discovered to act as a decoy receptor, with a similar extracellular region as IL-1R1 [156]. However, IL-1R2 has a truncated cytoplasmic domain, such that it can bind IL-1α and IL-1β without inducing signalling. In addition, IL-1R2 can recruit IL-1RAP, thereby sequestering it away from the functional IL-1R1. IL-1R2 expression was found mainly on cells which produce IL-1, suggesting it has an important role in breaking the self-amplifying loop in IL-1 production. In addition, the IL-1 receptor antagonist (IL-1Ra) was found to be induced by stimuli which also induce IL-1 production, as well as by IL-1 itself [157]. In addition, anti-inflammatory cytokines such as IL-4, IL-13 and IL-10 have also been shown to stimulate its expression. IL-1Ra blocks IL-1-induced signalling by binding to IL-1R1 without allowing the recruitment of IL-1RAP. This occupies the receptor but blocks signalling activity. Moreover, IL-1Ra has been shown to bind IL-1R2 poorly, avoiding the possibility of these two mechanisms negating each other.

Our understanding of these pathways has been reflected in the use of various therapies targeting IL-1-induced signalling. Recombinant IL-1Ra, known in the clinic as anakinra, is increasingly being used for IL-1-dependent autoinflammatory diseases, including systemic-onset juvenile idiopathic arthritis and refractory adult Still's disease, both characterised by systemic inflammation [158]. Further evidence that blocking IL-1R1 signalling can break the positive-feedback loop set up by IL-1 production comes from the observation that a single injection of anakinra in gout patients can lead to remission of symptoms for up to a year. Other IL-1-blocking molecules, such as the IL-1 Trap (extracellular domains of IL-1R1 and IL-1RAP which bind and inactive IL-1) and anti-IL-1β monoclonal antibodies (such as XOMA052) are being shown to have high efficacy in trials for the treatment of type 2 diabetes, atheroschlerosis and even malignancies such as multiple myeloma.

3.6 The role of IL-1β in inflammation

A role for IL-1β in the acute inflammatory response has been observed, perhaps unwittingly, since the 1920s, when blood from malaria patients was shown to be pyrogenic when injected into syphilis patients, in a treatment called fever therapy. Since these early observations, the molecular mechanisms linking IL-1β and the induction of inflammation have gradually been elucidated, deepening our understanding of the interplay between this pivotal cytokine and the inflammatory response.

Humans injected with 1-10ng/kg of bioactive IL-1β subsequently present with fever, increased blood neutrophils, acute-phase proteins, nitric oxide and other cytokines and chemokines, including IL-6, which has been shown to be particularly sensitive to IL-1β *in vivo* [159]. These dramatic effects, observed upon administration of a relatively low concentration of IL-1β, has led to the hypothesis that tissue acute inflammatory responses to pathogens rely on locally high concentrations of IL-1β. Indeed, in several IL-1β-dominated diseases, such as CAPS, refractory adult Still's disease and systemic-onset juvenile idiopathic arthritis, no measurable IL-1β is detected in the blood, although all three of these diseases are treatable with molecules targeting IL-1β signalling [93].

The similarities between IL-1R1 signalling and TLR signalling suggest that IL-1β functions within an amplifying loop, with IL-1β production important in conveying PAMP signals to cells which cannot directly recognise microbes. The effects of IL-1β on local non-haematopoietic cells such as endothelial cells are important in inducing the expression of adhesion molecules such as ICAM and VCAM [158], which slow the movement of circulating immune cells near the area of infection. In addition, IL-1β has been shown to directly induce the production of various chemokines. These effects, in combination, drive the recruitment of leukocytes to the site of infection. The induction of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) by IL-1β increases local levels of prostaglandin E2, platelet activating factor and nitric oxide, molecules important in mediating the hallmarks of the inflammatory response – fever, vasodilation leading to tissue oedema and swelling, a decrease in blood pressure and increased sensitivity to pain. IL-1β-deficient mice fail to raise an acute-phase response, and have no fever or increased circulating IL-6 upon administration of turpentine (an agent that induces systemic inflammation) [159]. These mice also have reduced inflammation in a variety of models, including zymosan-induced peritonitis and disseminated *Candida albicans* infection [93]. In addition, alum-induced peritonitis was shown to be abrogated in *Nlrp3*$^{-/-}$ mice, specifically identifying IL-1β as the cytokine responsible for inducing the influx of inflammatory cells into the peritoneum [160].

IL-1 signalling also has effects on the bone marrow, the site where haematopoietic cells are derived. IL-1-mediated effects were shown to be specific to the granulocyte/macrophage lineage. Reactive neutrophilias upon infection or injection of inflammation-inducing agents, such as alum, are accompanied by a programme of emergency granulopoiesis in the bone marrow. This induces rapid

proliferation of granulocyte/macrophage progenitors, as well as other haematopoietic stem cells, replenishing progenitor numbers when large numbers of cells differentiate and leave the bone marrow towards the site of infection. Mice deficient in IL-1R1 signalling were shown to have a defect in this process, implicating IL-1 in the bone marrow as important for mounting reactive neutrophilias [161]. Furthermore, IL-1 was shown to act indirectly on haematopoietic progenitors, as bone-marrow chimera experiments showed the IL-1R1 expression was required on radio-resistant, non-haematopoietic cells. These data suggest that cells in the bone marrow stroma respond rapidly to IL-1α and IL-1β during acute inflammation, and induce the proliferation of progenitors and differentiation of neutrophils which move to the inflammatory site.

As well as the response to infection, inflammation, especially in a chronic state, has been implicated in the development of cancer [162]. Whilst chronic inflammation has been associated with increased risk of carcinogenesis, during cancer the tumour environment has various trademarks of inflammation and wound healing, including angiogenesis, cell renewal and tissue remodelling. For example, infection with *Helicobacter pylori*, which leads to chronic inflammation, is associated with gastric cancer [163]. Similar links have been reported between hepatitis viruses and liver cancer, and *Schistosoma* infection with bladder cancer. The role of inflammation is further highlighted by the observation that, in certain cancer models, acquisition of dominant oncogenes by itself is unable to induce cancer spontaneously. A second insult which causes tissue injury (and therefore an inflammatory response) is required. As tumour development and progression is a chronic process, requiring the accumulation of multiple genetic defects to overcome checkpoints in cell division, differentiation and death, carcinogenesis has been observed to be a feed-forward loop, with inflammatory cells and cytokines in the tumour microenvironment contributing to this self-amplifying process.

The central role of IL-1β in inflammation therefore has significant implications in cancer. Importantly, IL-1β is known to induce inflammatory and healing responses by recruiting inflammatory cells, inducing chemokine and cytokine production, and stimulating tissue remodelling responses in immune and stromal cells, as well as activating angiogenic pathways [164]. The ability of IL-1R1 signalling to activate various pro-inflammatory pathways, including NF-κB, also links IL-1β activity to potentiating tumorigenesis. The ability of IL-1 to induce IL-1 via NF-κB signalling is also important in establishing and maintaining an inflammatory milieu which promotes tumour development and progression. IL-1β

was directly implicated in the induction of gastric carcinoma in a model where constitutive bioactive IL-1β expression was driven under the control of a gastric promoter [165]. IL-1β-deficiency was also associated with reduced hepatic metastasis (and VCAM-1 expression) in a B16 melanoma model [164]. In addition to solid tumours, IL-1β has been associated with lymphoid malignancies, with IL-1R1 blockade an effective therapy in halting the progression of multiple myeloma to the malignant stage [166]. This has been attributed to the attenuation of a cytokine loop, where IL-1β potently stimulates the production of IL-6 from bone marrow stromal cells, a factor which is critical in supporting the proliferation of myeloma cells. In addition to inducing cytokine production, IL-1β has been shown to be important in the recruitment of myeloid-derived suppressor cells (MDSCs) to the tumour microenvironment. These are immature cells of the myeloid lineage which inhibit the anti-tumour response, with much heterogeneity observed in terms of cell type and mechanism of suppression, depending on the tumour model. These range from IL-10 secreting neutrophils [62] to arginase-expressing tumour-infiltrating macrophages and tolerogenic DCs [167]. Many reports link IL-1R1 signalling to the accumulation of MDSCs at the tumour site, with a similar phenotype in inflammasome-deficient animals confirming the role of IL-1β specifically. IL-1β produced by the NLRP3 inflammasome was also identified as being able to decrease anti-tumour vaccine efficacy, via the accumulation of MDSCs [168]. However, none of these reports resolve whether IL-1β is important in the generation of MDSCs or their recruitment, or both.

IL-1β therefore has important roles in establishing innate immunity by inducing an inflammatory response. With important roles in inducing pro-inflammatory cytokines and chemokines, cellular recruitment and tissue remodelling, IL-1β also has the potential to establish a carcinogenic environment which supports the development and progression of tumours.

3.7 IL-1β as a bridge between innate and adaptive immunity

In addition to its key role in inducing the pro-inflammatory response, it has become clear that IL-1β is also important in establishing effective adaptive immunity. IL-1β acts as a mediator to transmit PRR signals from the innate immune system to the adaptive arm, generating antigen-specific T and B cell responses.

Even before IL-1β was isolated, various groups in the 1970's described an unidentified molecule that was able to potentiate the mitogenic effects of phorbol esters (eg. PHA) on T cells [66]. This molecule

was named Lymphocyte Activating Factor and subsequently discovered to be two molecules, IL-1α and IL-1β, which signal through the same receptor, IL-1R1. More recently, IL-1β was shown to enhance $CD4^+$ T cell responses *in vivo* when continuously administered via the implantation of IL-1β-releasing osmotic pumps [169]. IL-1β was shown to induce expansion and survival of both naïve and memory T cells, acting directly as the effect persisted even in animals which only expressed IL-1R1 on T cells. Interestingly, mice which received IL-1β had increased $CD4^+$ T cell responses across all subsets tested (Th1, Th2 and Th17), suggesting that IL-1β can directly act to expand both naïve and memory T cells regardless of polarisation.

Despite this non-specific effect on T cell expansion, IL-1β has been linked to the development of Th17 T cells, as well as the maintenance of IL-17 and IL-22-producing memory cells. This is due to a failure of *Il1r1*$^{-/-}$ mice to develop Th17 responses outside of the gut [93]. These mice are also resistant to Th17-dominated diseases, such as experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis [170]. IL-1R1 expression has been shown to be enhanced in the Th17 subset when compared to Th1 and Th2 cells, with signalling through this receptor inducing the expression of IRF4 and RORγt, two transcription factors essential in Th17 development [171]. Interestingly, this effect was already observed a few days after antigen administration, suggesting a role for IL-1 signalling in the early stages of T cell specification. In *in vitro* experiments, addition of exogenous IL-1β can also enhance the polarisation of Th17 responses in a naïve $CD4^+$ T cell-DC coculture system, with IL-1R1 blockade inhibiting Th17 development. Exogenous IL-1β was also shown to be able to potentiate Th17 cell survival in the absence of TCR stimulation.

The role of IL-1β in inducing vaccine-specific antibody responses has also been under scrutiny, since alum, the most commonly used vaccine adjuvant, is an activator of the NLRP3 inflammasome. Alum predominantly induces Th2 responses, with production of vaccine-specific immunoglobulins a common readout for vaccine efficacy. However, despite its widespread use, its mechanism of action remains unclear. IL-1 signalling can strongly increase B cell proliferation in response to mitogenic stimuli such as CD40 ligation [172]. In addition, the ability of IL-1β to upregulate costimulatory molecules such as CD40L and OX40 on $CD4^+$ T cells may drive further B cell proliferation, as well as antibody class switching. Indeed *Il1a*$^{-/-}$*Il1b*$^{-/-}$ double-knockout mice have been shown to have defective T cell-dependent antibody responses [173]. Recent reports have also identified alum's ability to induce uric

acid (another inflammasome activator) production as a mechanism for activating inflammatory DCs in the draining lymph nodes [174]. Interestingly, depletion of $CD11c^+$ DCs and monocytes completely abolished adjuvanticity, implicating a mechanism where alum and uric acid act on myeloid APCs, possibly by inflammasome activation, to prime the adaptive response. *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Casp1*$^{-/-}$ animals were shown to be less able to develop antigen-specific IgG1 and IgE upon intraperitoneal injection of alum-adsorbed ovalbumin, implicating IL-1β generated by the NLRP3 inflammasome in inducing humoral responses to alum-adjuvanted vaccines [113, 175]. However, these results were not reproduced by some other groups, who found no link between NLRP3 and antibody responses to alum-containing vaccines [176, 177].

3.8 IL-1β in establishing protective immunity against infection

Work on identifying pathogens which activate the inflammasome has also inevitably led to the discovery that IL-1β is indispensable for controlling infection *in vivo*, with defects in limiting pathogen spread observed in inflammasome-deficient mice. These include control of infection by intracellular, flagellated bacteria such as *Salmonella typhimurium* and *Legionella pneumophila* in *Nlrc4*$^{-/-}$ mice [100, 101, 106], as well as susceptibility to infection by *Candida albicans* and *Schistosoma mansoni* in *Nlrp3*$^{-/-}$ animals [46, 47]. Interestingly, failure to control *S. mansoni* infection was associated with a decrease in liver immunopathology, but also reduced antigen-specific T cell responses.

However, most of these reports do not distinguish between effects attributed to the pro-inflammatory effects of IL-1β in inducing the innate response, or its ability to stimulate adaptive immunity. Nevertheless, a dual-role of IL-1β in immunity to infection is apparent – the induction of sufficient levels of IL-1β to initiate protective inflammation as well as to prime the adaptive response. Conversely, failure to regulate IL-1β production may lead to exacerbated inflammation and immunopathology. This duality in the role of IL-1β in infection is illustrated by studies into its effects during influenza virus infection. Whilst important for mounting $CD4^+$ and $CD8^+$ T cell influenza-specific responses and decreasing viral load, IL-1R1 signalling also increased early infiltration of myeloid cells and immunopathology in the lung [131, 178]. Production of IL-1β therefore needs to be carefully regulated, to induce sufficient inflammation to control pathogen dissemination and prime adaptive immunity, but avoid excessive tissue damage.

IL-1β was also shown to be important in anti-tumour immunity after chemotherapy through the ability of the NLRP3 inflammasome to sense cell death [179]. Dying tumour cells release ATP, which signals through the P2X7 receptor to activate the NLRP3 inflammasome. This was found to be important in priming IFN-γ production from $CD8^+$ T cells in the draining lymph node. The authors also made a correlation with breast cancer patients, with those carrying a loss-of-function allele of *P2RX7* progressing more rapidly to metastatic disease. The ability of NLRP3 to detect tumour cell death therefore has implications in immunity following anti-cancer therapy.

IV. Modulation of the immune response

During the immune response, activation signals must be countered by inhibitory signals to prevent exacerbated inflammation, immunopathology and the breakdown of self tolerance. The immune system has evolved multiple mechanisms to control the response to infection, including the production of anti-inflammatory cytokines which counteract pro-inflammatory signalling, the requirement of costimulation for full activation of adaptive immunity and the generation of populations of regulatory cells which dampen the immune response. Genetic and pharmacological manipulation of these pathways has highlighted the importance of immune modulation to maintain the balance between protective immunity and exacerbated responses.

1. The CD40-CD40L pathway in antigen presenting cells

One of the best characterised interactions at the DC-T cell interface is that between CD40 and CD40L (or CD154). CD40 is a member of the TNF receptor superfamily and engagement of CD40 is a requirement to license the DC to mature, increasing antigen presentation and cytokine production [180]. Importantly, DC maturation is also associated with the upregulation of costimulatory molecules such as CD80 and CD86, which provide costimulation to T cells. This “signal 2”, in addition to cognate antigen recognition, allows T cells to become fully activated, with the absence of co-stimulation leading to tolerance. APCs therefore have an important role in determining whether an interacting T cell will become activated. CD40 expression is best described in APCs such as DCs and macrophages, as well as B cells, whereas CD40L is upregulated rapidly on the surface of activated T cells. Furthermore, the CD40L-CD40 interaction between $CD4^+$ T cell and B cell is a critical stage in the generation of T-dependent antibody responses, with CD40 signalling in B cells leading to proliferation, germinal centre formation and immunoglobulin class switching [181].

Upon engagement of CD40, various signalling adaptor molecules are recruited to the cytoplasmic tail of the receptor to initiate multiple signalling pathways. Significantly, CD40 signalling can lead to both canonical and non-canonical NF-κB activation via various TRAF proteins, culminating in the production of cytokines and chemokines which allow the DC to activate and skew the response of the interacting T cell [182]. In addition, non-canonical NF-κB signalling also induces the expression of the anti-apoptotic factor Bcl-xL in mice [183, 184] and Bcl-2 in humans [185], increasing the lifespan of DCs

and leading to prolonged TCR-MHC-peptide interactions which strengthen T cell activation. Furthermore, activation of MAPK signalling leads to the production of IL-12p40, important in promoting Th1 immunity [186]. However, both *in vivo* and *in vitro* experiments have suggested that CD40 signalling alone is insufficient to induce IL-12p35, the limiting subunit of the active IL-12p70 heterodimer [187, 188]. Instead, stimulation with TLR ligands in combination with CD40L can have a synergistic effect on IL-12p70 production, as well as the upregulation of costimulatory molecules. The emerging paradigm is that DCs require both TLR stimulation and CD40 signalling *in vivo* to achieve full maturation and the potential to fully activate T cells. This would mean that DCs only become fully licensed in the presence of PAMPs or DAMPs, a mechanism crucial in distinguishing between self and non-self.

Various reports have suggested that the interaction between T cell and immature DC (in the absence of PRR signalling and CD40-mediated maturation) will lead to the induction of T cell anergy, an important mechanism for maintaining peripheral tolerance to self-antigens [189, 190]. There is also evidence to suggest that interaction of immature DCs with T cells can lead to a regulatory phenotype, with antigen presentation by *Cd40*$^{-/-}$ or *Relb*$^{-/-}$ DCs capable of inducing IL-10-producing T cells *in vivo*.

The strength of signalling through the CD40 pathway has also been shown to determine whether a pro- or anti-inflammatory response is initiated. *In vivo*, the strength of signalling would depend on a number of factors – the level of CD40 expression on the DC (dependent on maturation), the level of CD40L expression on the T cell (dependent on activation) and the longevity of the interaction between DC and T cell (a result of cognate antigen recognition). *In vitro*, many of these experiments were performed using recombinant, soluble CD40L, which is produced as a monomer. Cross-linking monomers to produce trimers (the biological state of the molecule [191]) or higher order multimers greatly enhances signalling activity, as multiple CD40 molecules are clustered together [192]. This, together with agonistic anti-CD40 antibodies, provided investigators with the necessary tools to investigate the influence of CD40 signal strength on the outcome of the interaction. In macrophages, weak CD40 signalling was found to induce ERK activation, resulting in production of the anti-inflammatory cytokine IL-10 [193]. However, stronger CD40 signalling activated the p38MAPK pathway and induced IL-12 production. In another study, the strength and persistence of the CD40 signal was reported to have differential effects on the resulting DC phenotype [194]. Whereas a weak

and transient signal induced DC migration but little cytokine production, a strong and long-lasting CD40 signal inhibited migration but induced the production of IL-12p70. Physiologically, this was suggested to model the response of a weakly engaged DC to migrate to the lymph nodes to further survey naïve T cells, whereas strong engagement between an activated T cell and mature DC leading to a prolonged interaction with pro-inflammatory cytokine production and reinforced T cell activation.

Increasing evidence therefore suggests that the CD40-CD40L pathway in APCs is important in modulating the immune response, with earlier work suggesting that CD40 ligation leads to an activation signal and a lack of CD40 engagement resulting in inhibition. However, more recent reports have extended this idea to propose that the strength of CD40 signals, by differentially activating intracellular pathways, determines whether the outcome is pro- or anti-inflammatory.

2. Invariant Natural Killer T Cells

Natural Killer T (NKT) cells are a subset of non-conventional T cells, so named because of their expression of an αβ TCR and CD3 together with receptors associated with NK cells. Invariant (i)NKT cells express a semi-invariant TCR, arising from the canonical rearrangement of the Vα14-Jα18 segments in mice, and Vα24-Jα18 in humans [195]. These α chains are paired with a limited set of β chains, Vβ8.2, Vβ7 and Vβ2 in mice, and Vβ11 in humans. The requirement of iNKT cell development for these gene segments has been demonstrated by their genetic manipulation – deletion of the Jα18 segment results in an iNKT cell-deficient animal, whereas transgenic overexpression of Vα14 leads to an increased frequency of iNKT cells [196]. In humans, iNKT cells may be $CD4^+$, $CD8^+$ or double negative, whereas the $CD8^+$ subset is not found in mice. iNKT cells (also called Type I NKT cells) form around 80% of the NKT cell pool, with the rest made up of Type II, or non-invariant, NKT cells [197]. Type II NKT cells have a broader usage of the TCR repertoire, but also recognise lipid presented on CD1d, although their specificities differ from those of iNKT cells. For example, Type II NKT cells are not reactive towards α-galactosylceramide (α-GalCer), but can recognise sulfatide presented on CD1d molecules [198]. Currently, we have a much better understanding of iNKT cells, owing to the diversity in the TCR repertoire of type II NKT cells. A commonly used tool to study type II NKT cell biology *in vivo* has been the comparison of the phenotypes of *Cd1d*$^{-/-}$ mice (which lack all NKT cells) with *Jα18*$^{-/-}$ mice (which only lack iNKT cells).

2.1 iNKT cell recognition of lipid antigens

The observation that iNKT cell development was dependent on β2-microglobulin [199], but not MHC class I or class II [200], led to suggestions that iNKT cells recognise ligands bound onto the non-polymorphic, class I-like molecule CD1d [201]. This was later confirmed by the lack of iNKT cells in *Cd1d*$^{-/-}$ mice, which also suggested a positive selection stage in iNKT cell development [202].

iNKT cells recognise glycolipid antigens loaded onto CD1d by virtue of hydrophobic channels in the CD1d antigen binding site [203, 204]. This leaves the polar head of glycolipids protruding from the CD1d surface, and this is the configuration which is recognised by the invariant TCR [205, 206]. Despite the non-polymorphic nature of CD1d, many different glycolipids have been shown to be presented and recognised by iNKT cells, with biochemical studies identifying molecular structures which lead to stronger iNKT cell activation [207]. Glycolipids derived from various microbes, once processed by the APC, have also been shown to be able to activate iNKT cells. One ligand which can potently activate iNKT cells is α-GalCer, derived from the marine sponge *Agelas mauritianus* [208]. The discovery of this agonist has greatly contributed to our understanding our iNKT cell biology, giving us a tool to potently activate iNKT cells and analyse their responses *in vivo* and *in vitro*. The development of CD1d-tetramers loaded with α-GalCer has also made it possible to unambiguously identify iNKT cells [209].

In addition to exogenous glycolipids, iNKT cells also respond to self lipid presented onto CD1d. This is apparent especially in the ability of pathogens which do not contain glycolipids, such as viruses, to induce CD1d-dependent iNKT cell activation [210]. The exact identities of the self ligands remain unclear, although there were suggestions that isoglobotrihexosylceramide (iGb3) was responsible for iNKT cell thymic selection and activation [211]. However, iGb3-deficient mice were later found to have functional iNKT cells [212]. Another possible candidate, lysophosphatidylcholine (LPC), has also been described [213, 214]. This unique property endows iNKT cells with a level of basal autoreactivity, meaning iNKT cells will react to basal levels of self lipid (presented on CD1d) on an immature APC [215]. This is a weak interaction which leads to a specialised activation state [216], and may account for the activated/memory phenotype of iNKT cells observed *in vivo* (CD69^{+}, CD62L^{low}, CD44^{high}, CD122^{high}) [217].

2.2 Development of iNKT cells

The development of iNKT cells in the thymus has been studied in the mouse, with iNKT cells appearing slightly after conventional T cells. Both iNKT cells and conventional T cells derive from a common progenitor, with iNKT cells diverging from conventional T cell development at the $CD4^+CD8^+$ double positive stage [218, 219]. Rearrangement of the canonical Vα14-Jα18 gene segments is believed to be a random event; this is supported by two observations. The late appearance of iNKT cells is consistent with these gene segments being inaccessible to recombination until 24-48 hours before birth [220]. In addition, the frequency of the earliest iNKT cell progenitor in the thymus is also consistent with the rate of occurrence of the Vα14-Jα18 rearrangement [221]. Positive selection of iNKT cells occurs upon recognition of self lipid complexed to CD1d, presented on the surface of $CD4^+CD8^+$ double positive thymocytes [222]. These cells then downregulate CD8 expression and start to express markers such as CD69, before expanding and acquiring CD44 expression [223]. Most iNKT cells leave the thymus at an immature stage and acquire the expression of NK markers in the periphery (such as NKG2D, Ly49 markers and NK1.1 in C57BL/6 only). However, a small subset remains resident and matures in the thymus, although the exact role of these cells is currently unclear.

2.3 iNKT cell distribution *in vivo*

The use of α-GalCer-loaded CD1d tetramers has allowed investigators to study the distribution of iNKT cells *in vivo*. In mice, the highest frequency is found in the liver, where they represent around 30% of the T cell population [224]. At other sites, they are significantly rarer, making up around 3% in the spleen and 0.2% in the blood. These numbers are lower in humans, although much variation has been documented [225]. Despite the seemingly low frequencies, it must be noted that iNKT cells have a semi-invariant TCR and are therefore still much more numerous than a naïve antigen-specific T cell.

The *in vivo* distribution and chemokine receptor expression of iNKT cells also reflects their effector memory phenotype. Human iNKT cells express high levels of CCR5, CXCR3 and CCR6 which allow them to migrate into inflamed tissues and sites of infection, but few express CCR7, which retains cells in the lymph nodes [226]. Almost all iNKT cells also express CXCR6, which, together with its chemokine CXCL16, was shown to regulate iNKT cell homing to the liver and spleen [227].

Under steady state conditions, iNKT cells have been shown to circulate within tissues and form short-lived contacts with CD1d-expressing APCs. This was demonstrated using microscopy in the liver, where iNKT cells were observed to migrate along hepatic sinusoids and touch Kupffer cells [228].

However, injection of iNKT cell agonists, or activation of APCs by infection with *Borrelia burgdoferi*, induced arrest and the formation of more stable iNKT cell-APC conjugates. Interestingly, iNKT cell arrest could also be induced by the injection of IL-12 and IL-18, cytokines which induce iNKT cell activation, suggesting this is due to iNKT cell activation, regardless of whether cognate interaction occurs. These observations were confirmed in an *in vitro* study using human cells, which compared the downstream effects of basal autoreactivity and α-GalCer-induced activation [216].

2.4 Activation of iNKT cells

iNKT cells were originally identified as a population which could rapidly produce IL-4 upon anti-CD3 injection in mice. However, later studies have shown that, upon activation, iNKT cells are able to secrete a large panel of pro- and anti-inflammatory cytokines, such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IFN-γ, GM-CSF and TNF-α [217]. The ability to release both IFN-γ and IL-4 upon α-GalCer stimulation has become a hallmark of iNKT cells, with preformed mRNA in resting cells allowing rapid cytokine production following activation [229]. In addition, iNKT cells express high levels of granzyme B, perforin and FasL, endowing them with cytolytic ability. *In vitro* experiments have shown that iNKT cells can kill α-GalCer-pulsed APCs in a CD1d-dependent manner [230].

Whilst strong activation of iNKT cells by α-GalCer leads to a broad cytokine profile, stimulation with α-GalCer analogues, which vary in their affinity to CD1d and may therefore alter the strength of iNKT cell activation, has been shown to preferentially induce the production of Th1 or Th2 cytokines. These data raise the possibility that iNKT cell phenotype and function may depend on the strength and type of signal received. Indeed, it has been demonstrated that different strengths of signals through the semi-invariant TCR impart different activation states on iNKT cells. Wang and colleagues showed that basal autoreactivity results in a specialised activation state, characterised by ERK signalling in the absence of calcium flux [216, 231]. This induced the secretion of IL-13 and GM-CSF, but negligible levels of IFN-γ and IL-4. However, addition of exogenous lipid such as α-GalCer resulted in measurable calcium flux and release of IFN-γ, IL-4 and IL-2, as well as increased levels of IL-13 and GM-CSF.

In addition to this hypothesis, it has been suggested that iNKT cells may be segregated into functionally distinct subtypes, a “Th1-like” and a “Th2-like” subset. While the iNKT cell population is defined by its TCR specificity, it is highly heterogeneous in the expression of other cell markers,

including the co-receptors CD4 and CD8. Like their conventional T cell counterparts, human $CD4^+$ iNKT cells have been suggested to produce more cytokines, whereas the human $CD8^+$ cells are more cytolytic [217]. In addition, *in vitro* studies have shown that human $CD4^+$ iNKT cells produce both Th1 and Th2-type cytokines upon stimulation, whilst $CD4^-$ iNKT cells are skewed towards the Th1 cytokines [232, 233]. Furthermore, signalling through the CD4 co-receptor has been implicated in strengthening activation signals, potentially altering the signal strength in different iNKT cell subsets upon recognition of the same glycolipid-CD1d complex [234, 235].

The microenvironment, cytokine milieu and context of signals delivered to iNKT cells are likely to be other factors in determining the outcome of iNKT cell activation. iNKT cells can be activated in a CD1d-independent manner by APC-derived cytokines such as IL-12 and IL-18. As well as allowing iNKT cells to respond to microbes which do not express CD1d ligands, IL-12 and IL-18 can synergise with CD1d signals to increase the level of iNKT cell activation. Salio and colleagues have demonstrated that TLR stimulation of APCs results in both upregulation of self-lipid-CD1d complexes and the secretion of IL-12, with blockade of both signals required to inhibit IFN-γ release from iNKT cells [236]. Whereas IL-12 has been shown to drive both IFN-γ and IL-4 expression by iNKT cells, IL-7 was suggested to promoted IL-4 secretion [217].

2.5 The dual role of iNKT cells

Consistent with their ability to produce both Th1 and Th2 cytokines upon activation, iNKT cells have been associated with both pro- and anti-inflammatory functions *in vivo*. As with their pleiotropic cytokine production, the reasons for this are still unclear. However, reports have indicated that the ability of iNKT cells to modulate the immune response is dependent both on their cytokine secretion and their ability to crosstalk with multiple different cell types.

Due to the ability of iNKT cells to recognise both endogenous and exogenous lipid, their role has been described for many bacterial, viral and parasitic infections. This is especially significant in infections where microbes express glycolipids which can be presented onto CD1d, directly activating iNKT cells. *Borrelia burgdoferi*, the causative agent of Lyme's disease, was shown to express two glycolipids (BbGL-1 and BbGL-2) which could be loaded onto CD1d [237]. Infection with this bacterium induces iNKT cell activation (upregulation of CD25 and CD69) in WT animals. Significantly, infection of *Cd1d*$^{-/-}$ mice results in increased bacterial burden and the development of severe arthritis of the knee,

indicating a protective role for iNKT cells. In addition, the cell well of *Sphingomonas* species includes various glycolipids which can activate iNKT cells [238]. Infection of *Jα18*$^{-/-}$ mice with these species leads to decreased clearance from the liver and lungs by up to 1000-fold. Similar data have also been published describing the role of iNKT cell recognition of antigens derived from *Streptococcus pneumoniae* [239] and *Helicobacter pylori* [240]. In addition to direct recognition, it has been noted that some LPS-positive bacteria which are not directly recognised, including *Salmonella* and *Pseudomonas aeruginosa*, can also be controlled by iNKT cells *in vivo*. In many of these cases, TLR-dependent signalling and IL-12 were shown to synergise with the recognition of a self lipid, implicating the role of iNKT cell activation by APC-derived IL-12 in immunity to these pathogens [236, 241].

Study of iNKT cell biology in the context of responses to model antigens has also shed light on their ability to enhance adaptive immunity, a characteristic which has been harnessed in the development of novel vaccination strategies. Expression of CD40L on iNKT cells gives them the ability to mature APCs and increase their ability to stimulate conventional T cell responses. Signalling through this pathway has been reported to be important in enhancing both $CD4^+$ and $CD8^+$ T cell responses to ovalbumin adjuvanted with α-GalCer [242]. Recently, other costimulatory pathways such as CD70-CD28 and OX40-OX40L were also reported to play a role in enhancing $CD8^+$ responses. In addition, iNKT cells can provide B cell help to enhance humoral immunity, inducing B cell maturation, higher antibody titres and expansion of the B cell memory pool [243], via CD40L expression and possibly the secretion of IL-4. This was harnessed by targeting α-GalCer presentation on CD1d to B cells, inducing B cell-iNKT cell crosstalk. This resulted in B cell proliferation and differentiation into plasma cells which produced large amounts of immunoglobulin [244]. Another report also highlighted the ability of iNKT cells to provide cognate help to B cells via CD40L expression and IFN-γ secretion, required for class switching to IgG2a, IgG2c and IgG3 [245]. iNKT cell expression of CD40L has also been shown to be important in rescuing the suppressive activity of MDSCs, both in an influenza model and in human melanoma [62, 210]. iNKT cells are therefore able to restore effective immune responses to viruses or tumours. The ability of iNKT cells to crosstalk with many different cell types may thus be key in their ability to enhance many aspects of adaptive immunity.

Despite their reported ability to enhance immunity, experiments using animal models for autoimmunity have also suggested that iNKT cells also play an anti-inflammatory role *in vivo*. Studies in type 1

diabetes (T1D) revealed activation of iNKT cells with α-GalCer or Complete Freund's Adjuvant to be able to prevent the onset of disease [246, 247]. This was supported by evidence showing protection from T1D in non-obese diabetic (NOD) mice with an increased number iNKT cells (Vα14 transgenic) [248]. The ability of iNKT cells to crosstalk with plasmacytoid DCs via the OX40-OX40L pathway has also been documented, eliciting type I IFN in the pancreas and dampening inflammation [249]. Other reports also point to iNKT cells as being able to recruit regulatory T cells, as well as secrete IL-10 in the pancreatic draining lymph node [250]. iNKT cells have also been reported to be beneficial in other autoimmunities, such as collagen-induced arthritis, colitis and EAE [251, 252, 253]. Activation of iNKT cells can delay onset and reduce severity of the disease, with transgenic enrichment of iNKT cells preventing EAE induction [254]. As well as in autoimmunity, iNKT cells have also been shown to play an anti-inflammatory role in infection. This has been demonstrated in severe influenza A virus infection, where iNKT cell-deficiency resulted in increased immunopathology and mortality due to increased influx of inflammatory myeloid cells into the lungs [230]. In addition, a model of *Legionella pneumophila* infection yielded similar results, with iNKT cells shown to reduce the inflammatory infiltrate [255]. Again, this was shown to be associated with reduced pulmonary tissue damage, showing an anti-inflammatory, yet protective, role of iNKT cells.

iNKT cells are therefore involved in both pro- and anti-inflammatory responses, both via the variety of cytokines which they secrete and their ability to crosstalk with many different cell types. However, the factors which regulate this plasticity are still unclear. Nevertheless, it is likely that the microenvironment in which iNKT cells are activated, as well as the strength of signalling, will play an important role in determining whether they are immunogenic or tolerogenic.

V. Thesis objectives

The pro-inflammatory cytokine IL-1β is a key molecule in inflammation. With the discovery of the inflammasome, the molecular mechanisms involved in its production are beginning to be clarified, with the number of identified stimuli which can induce IL-1β synthesis and cleavage continuing to grow. The importance of IL-1β in inflammation means that the discovery of novel pathways which induce and modulate the production of this cytokine is vital in the development of clinically successful therapies to target exacerbated inflammation. To this end, we investigated the roles of SAA and iNKT cells, given their abilities to modulate the immune response.

In this thesis, we initially describe SAA as a novel, non-microbial inducer of IL-1β synthesis and cleavage. We then identify the PRRs responsible for recognising SAA and the signals required which culminate in pro-IL-1β production and activation of the inflammasome. By administering SAA *in vivo*, we also examine the physiological significance of SAA-induced IL-1β, and its effects in mediating the innate response via the recruitment of inflammatory cells.

Having described this inducer of IL-1β production, we then proceed to examine a novel, cell-mediated mechanism for the modulation of IL-1β. We demonstrate the ability of iNKT cells to crosstalk with APCs, before showing that this interaction leads to the modulation of IL-1β production by DCs. Using *in vitro* assays, we dissect the mechanism of this modulation, identifying the cell surface molecules and cytokines mediating this effect, before testing the relevance of this observation *in vivo*. Utilising an influenza virus infection model, we compare the inflammatory responses of wild-type and iNKT cell-deficient mice, in order to elucidate the physiological role of iNKT cells in modulating the response to infection.

A deeper understanding of the cellular and molecular mechanisms involved in inducing and modulating IL-1β production may aid in the development of novel vaccination strategies and treatments for debilitating inflammatory diseases.

CHAPTER 2

Materials and Methods

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2.1 Materials

2.1.1 Cell culture and cellular assay reagents

2.1.1a Cells and viruses

Cells/Viruses	Source
Human peripheral blood	NHS National Blood Service
THP-1 cell line	ATCC
Influenza A/PuertoRico/8/34 (PR8) virus	Health Protection Agency

2.1.1b Tissue Culture Reagents

Reagent	Supplier
1x Phosphate Buffered Saline (PBS)	Lonza
RPMI-1640 (with L-Glutamine and $NaHCO_3$)	Gibco
IMDM	Gibco
OptiMEM	Gibco
Foetal Calf Serum (FCS)	Sigma
Human AB Serum	PAA
L-Glutamine (200mM in 0.85% Azide) (100x)	Sigma
Penicillin/Streptomycin (5000U/ml) (100x)	PAA
Non-essential amino acids (NEAA) (100x)	Gibco
Sodium Pyruvate (100mM) (100x)	Gibco
HEPES (1M) (100x)	Gibco
β-mercaptoethanol (50mM) (1000x)	Gibco
Lymphoprep	Nycomed

PMA	Sigma
Ionomycin	Sigma
PHA	Sigma
Trypan Blue	Gibco
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA)	Sigma
RBC lysis buffer	Qiagen
Collagenase type-2 filtered	Worthington

2.1.1c Composition of Tissue Culture Media

Medium	Composition
Complete medium (R10)	RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS, Penicillin/Streptomycin, L-Glutamine, Non-essential amino acids, HEPES, 1mM sodium pyruvate and 0.05μM β-mercaptoethanol.
R0	Same as R10, but without FCS.
iNKT cell medium	IMDM supplemented with 5% (v/v) heat-inactivated human AB serum, Penicillin/Streptomycin, L-Glutamine, Non-essential amino acids, HEPES, 1mM sodium pyruvate, 0.05μM β-mercaptoethanol, supplemented with 3% (v/v) IL-2.

2.1.1d Cytokines and cellular assay reagents

Reagent	Source/Catalogue No.	Supplier
Human IL-2	H558L-IL2 cell supernatant containing approx. 1000 U/ml human IL-2	N/A
Human IL-4 (for DC differentiation)	ILT4 cell supernatant, containing 1000 U/ml human IL-4	N/A
Human GM-CSF	300-03	Peprotech
Human M-CSF	300-25	Peprotech
Human IL-4 (for cellular assays)	200-04	Peprotech
Human IL-13	200-13	Peprotech
Human IFN-γ	300-02	Peprotech

Mouse GM-CSF	315-03	Peprotech
Mouse M-CSF	L929 cell supernatant containing mouse M-CSF	N/A
Recombinant Human apo-SAA (SAA)	300-13	Peprotech
LPS	0111:B4	Invivogen
Pam_3Csk_4	tlrl-pms	Invivogen
ATP	A1852	Sigma
Polymyxin B	tlrl-pmb	Invivogen
Butylated hydroxyanisole (BHA)	B1253	Sigma
KCl	P5405	Sigma
NaCl	S5886	Sigma
α-GalCer	-	Kindly donated by G. Besra, University. of Birmingham
Carboxyfluorescein succinimidyl ester (CFSE)	C34554	Molecular Probes
Recombinant soluble CD40L	ALX-522-015-2010	Enzo Life Sciences
Enhancer for ligands	ALX-804-034-C050	Enzo Life Sciences
NP peptide (ASNENMETM)	-	Kindly donated by J-P. Jukes, University of Oxford
Ovalbumin peptide (SIINFEKL)	S7951	Sigma

2.1.2 Immunoblotting reagents

2.1.2a Solutions for SDS-PAGE

Solutions	Composition
0.5 M Tris-Cl pH 6.8	6g Trizma Base (Sigma, M_W=121.14g/mol) was dissolved in 60 ml ddH_2O and pH adjusted to 6.8 using 1M HCl; the solution was made up to 100ml with ddH_2O and stored at 4°C.

1.5 M Tris-Cl pH 8.8	90.75g Trizma Base (Sigma, M_W=121.14g/mol) was dissolved in 250ml ddH_2O and the pH adjusted to 8.8 using 1M HCl; the solution was made up to 500ml with ddH_2O and stored at 4°C.
10% APS (w/v)	1g Ammonium persulphate (Sigma) was dissolved in 10ml cold ddH_2O (4 °C); the solution was stored as 200µl aliquots at –20 °C. After thawing, a sample was stored at 4 °C for a maximum of 1 week.
10 % (w/v) SDS	50g SDS (Sigma) was dissolved in 200ml ddH_2O and warmed up to 68°C while stirring. The solution was then made up to 500 ml with ddH_2O and filtered.

2.1.2b Materials for immunoblotting

Material	**Composition**
Wash buffer for immunoblotting	0.1% PBST (PBS+0.1% (v/v) Tween-20)
Antibody dilution buffer for immunoblotting	0.1% PBST + 2.5% (w/v) dried skimmed milk powder. For primary antibody solutions 0.01% (w/v) sodium azide was added.
Blocking buffer for immunoblotting	0.1% PBST + 5% (w/v) dried skimmed milk powder.
10x Running Buffer for SDS-PAGE	1920mM Glycine, 250mM Tris Base (all from Sigma) dissolved in ddH_2O.
1x Running Buffer for SDS-PAGE	1x Running Buffer with 0.1% (w/v) SDS.
1x Transfer Buffer for immunoblotting	1× Running Buffer with 20% (v/v) Methanol.
5x Protein Loading Buffer (LB), reducing.	From Pierce, contains: Proprietary pink tracking dye in 0.3M Tris-HCl, 5% SDS, 50% glycerol, 100mM dithiothreitol (DTT).
10x Cell Lysis buffer	From Cell Signaling, contains: 20 mM Tris-HCl (pH 7.5), 150 mM NaC, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin. Prior to use, supplemented with 1mM PMSF and Protease Inhibitor Cocktail from Roche.
Bradford assay reagent	BioRad
Protein Ladder	10-250kDa, from BioRad
Hybond-P PVDF membrane	GE Healthcare
Paraformaldehyde (PFA)	Sigma
ECL solution	Thermo Scientific
X-ray film	Fujifilm

2.1.2c Composition of SDS gels

Resolving (lower) gel

Components	7%	10%	12%	15%
Distilled H_20	5.1ml	4.1ml	3.4ml	2.4ml
1.5M Tris-HCl, pH 8.8	2.5ml	2.5ml	2.5ml	2.5ml
20% (w/v) SDS	0.05ml	0.05ml	0.05ml	0.05ml
30% Acrylamide (w/v) (National Diagnositcs)	2.3ml	3.3ml	4.0ml	5.0ml
10% APS	0.05ml	0.05ml	0.05ml	0.05ml
TEMED	0.005ml	0.005ml	0.005ml	0.005ml
Final Volume	10.005ml	10.005ml	10.005ml	10.005ml

Stacking (upper) gel (6%)

Components	Volume
Distilled H_20	3.075ml
1.5M Tris-HCl, pH 8.8	1.25ml
20% (w/v) SDS	0.025ml
30% Acrylamide (w/v) (National Diagnositcs)	0.67ml
10% APS	0.025ml
TEMED	0.005ml
Final Volume	5.05ml

2.1.2d Antibodies for Immunoblotting

Antibody	Isotype	Catalogue No.	Supplier
Primary Antibodies			
Anti-human IL-1β	Rabbit	2022	Cell Signaling
Anti-human caspase-1	Rabbit	D7F10	Cell Signaling

Anti-human IκBα	Mouse	4814	Cell Signaling
Anti-human A20	Rabbit	4625	Cell Signaling
Anti-mouse IL-1β	Goat	AF401	R&D systems
Anti-mouse caspase-1	Rabbit	sc-514	Santa Cruz Biotech
Anti-actin	Rabbit	A 2066	Sigma
Anti-GAPDH (C-terminal), polyclonal	Rabbit	AHP996	AbD Serotec
Secondary Antibodies			
Anti-Goat-IgG-HRP	-	HAF109	R&D systems
Anti-Mouse-IgG-HRP	-	HAF018	R&D systems
Anti-Rabbit-IgG-HRP	-	7074	Cell Signaling

2.1.3 ELISA reagents

2.1.3a Buffers for ELISA

Buffer	Composition
ELISA coating buffer	0.1 M $NaHCO_3$ (Sigma) in ddH_2O, pH 9, sterile filtered.
ELISA stop solution	2M sulphuric acid.
Substrate solution for ELISA	1x TMB (3,3′,5,5′-Tetramethylbenzidine) from Sigma cat. 040M1646.
ELISA blocking buffer	PBS containing 10% (v/v) FCS.
ELISA washing buffer	0.1% PBST.

2.1.3b Antibodies for ELISA

Antibody	Catalogue no.	Supplier
Anti-human IL-1β capture	14-7018	eBioscience
Anti-human IL-1β detection	13-7016	
Anti-human IL-4 capture	554515	BD Pharmingen
Anti-human IL-4 detection	554483	
Anti-human IL-6 capture	554543	BD Pharmingen

Anti-human IL-6 detection	554546	
Anti-human IL-12p40 capture	551227	BD Pharmingen
Anti-human IL-12p70 capture	555065	
Anti-human IL-12p40/p70 detection	554660	
Anti-human IL-13 capture	14-7139	eBioscience
Anti-human IL-13 detection	13-7138	
Anti-human IL-10 capture	554705	BD Pharmingen
Anti-human IL-10 detection	554499	
Anti-human GM-CSF capture	88-7339	eBioscience
Anti-human GM-CSF detection		
Anti-human IFN-γ capture	554699	BD Pharmingen
Anti-human IFN-γ detection	554550	
Anti-human TNF-α capture	551220	BD Pharmingen
Anti-human TNF-α detection	554511	
Anti-human TGF-β capture	88-8350	eBioscience
Anti-human TGF-β detection		
Anti-human sCD40L matched Ab pair	BMS239MST	eBioscience
Anti-mouse IL-1β capture	14-7012	eBioscience
Anti-mouse IL-1β detection	13-7112	
Anti-mouse IL-6 capture	14-7061	eBioscience
Anti-mouse IL-6 detection	13-7062	
Anti-mouse IL-10 capture	14-7101	eBioscience
Anti-mouse IL-10 detection	13-7102	

2.1.4 Flow Cytometry, FACS sorting and MACS sorting reagents

2.1.4a Materials for FACS and MACS

Material	Description
FACS buffer	PBS containing 10% (v/v) FCS.
MACS buffer	PBS containing 2 mM EDTA and 10% (v/v) FCS.

MACS LS columns	From Miltenyi Biotec, for positive selection
MACS LD columns	From Miltenyi Biotec, for negative depletion

2.1.4b Antibodies for FACS and MACS

FACS Antibody	Clone	Isotype	Conjugate	Supplier
Anti- mouse CD11b	M1/70	Rat IgG2b	PE, APC	eBioscience
Anti-mouse CD11c	N418	Hamster IgG	APC, PE-Cy7	eBioscience
Anti-mouse F4/80	BM8	Rat IgG2a	APC, PE-Cy7	eBioscience
Anti-mouse Ly6G	1A8	Rat IgG2b	FITC	eBioscience
Anti-human/mouse TLR2	T2.5	Mouse IgG1	PE	eBioscience
Anti-human/mouse FPR2	GM-1D6	Mouse IgG1	Purified	Genovac
Anti-human CD80	L307.4	Mouse IgG1	PE	BD Pharmingen
Anti-human CD83	HB15e	Mouse IgG1	FITC	BD Pharmingen
Anti-human CD86	2331 (FUN-1)	Mouse IgG1	APC	BD Pharmingen
Anti-human CD2	RPA0-2.10	Mouse IgG1	PE	eBioscience
Anti-human CD40L	TRAP1	Mouse IgG1	FITC	BD Pharmingen
α-GalCer/CD1d tetramer	-	-	APC	
Propidium iodide	-	-	-	Sigma

MACS Beads	Supplier
CD14 MACS Beads (Human)	Miltenyi Biotec
CD11c MACS Beads (Mouse)	Miltenyi Biotec

2.1.5 Antibodies for functional assays

Antibody	Clone	Isotype	Supplier
Anti-human TLR2	T2.5	Mouse IgG1	eBioscience
Anti-human FPR2	GM-1D6	Mouse IgG1	Genovac
Anti-human CD40L	MK13A4	Mouse IgG1	eBioscience

Anti-human CD1d	CD1d42	Mouse IgG1	BD Pharmingen
Anti-human IL-13	JES10-5A2	Rat IgG1	BD Pharmingen

2.1.6 Materials for real time PCR

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
Taqman Gene Expression assays, human IL-1β (Hs01555410_m1)	Applied Biosystems
Taqman Gene Expression assays, mouse IL-1β (Mm00434228_m1)	Applied Biosystems

2.1.7 Materials for EMSA

Buffer	Composition
Buffer A (Hypotonic)	10 mM HEPES (pH 7.9), 10 mM KCl, 1.0 mM DTT, and 0.5 mM PMSF
10% Igepal	From Sigma
Buffer C (Hypertonic)	20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF
Binding Buffer	100 mM HEPES (pH 7.9), 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM DTT, 5 mM EDTA, and 250 mM NaCl), 2 µg of poly(dI-dC) and 10 µg of nuclease-free BSA
Native Gel	35.1ml ddH_2O, 6.25ml acrylamide, 5ml 5xTBE, 500µl APS, 120µl TEMED
Running Buffer	1xTBE
NF-κB probe	5'-AGTTGAGGGGACTTTCCCAGGC-3'
[γ-^{32}P] dATP	From Perkin Elmer
T4 polynucleotide kinase	From New England Biolabs

2.1.8 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. 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.

The C57BL/6 (WT), *Il1r1*$^{-/-}$ and *Jα18*$^{-/-}$ were bred in the BMS, University of Oxford. *Tlr2*$^{-/-}$, *Asc*$^{-/-}$, *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ animals were kindly provided by Dr. Kevin Maloy, *Casp1*$^{-/-}$ mice by Dr. Alan Sher and *Fpr2*$^{-/-}$ mice by Professor Rod Flower.

2.2 Methods

2.2.1 Cell culture

2.2.1a Generation of human monocyte-derived dendritic cells

Leukocyte (PBMC) isolation from buffy coat using Lymphoprep

Blood from healthy donors (~50ml) was diluted in 130ml of RPMI supplemented with HEPES. 30ml of this mixture was gently layered over 15 ml of Lymphoprep in a 50ml falcon tube and spun at 2000rpm for 30min with no centrifuge brakes. The opaque ring between plasma and Lymphoprep was collected, transferred to a new 50ml falcon tube and washed with fresh RPMI. Cells were pelleted by spinning for 15min at 1500rpm. The pellet was collected, resuspended in fresh RPMI and spun for 10 minutes at 1000 rpm to remove platelets. The pellet was collected and cells washed once more in R10 (by spinning at 1500rpm for 5min).

Purification of monocytes

Monocytes were positively selected from buffy coat leukocytes using MACS sorting (see below), based on CD14 expression.

Differentiation of monocyte-derived dendritic cells

$CD14^+$ cells were resuspended in R10 supplemented with 3% (v/v) IL-4 and 50ng/ml recombinant GM-CSF. Cells were plated in 1.5 ml per well in 6 well plates at a density of $5x10^5$ cells/ml and differentiated for 4-5 days at 37°C, 5% CO_2.

2.2.1b Generation of human monocyte-derived macrophages

Monocytes were generated as above, and $CD14^+$ cells were differentiated for 7-9 days in 6 well plates at a density of $1x10^6$ cells per well in 3ml of R10 supplemented with 50ng/ml recombinant M-CSF.

2.2.1c Generation and maintenance of human iNKT cells

iNKT cells were generated from healthy blood donors. Total PBMCs were isolated via Lymphoprep separation (as above). $2x10^6$ PBMCs were cultured in iNKT cell medium in the presence of 100ng/ml α-GalCer. From day 5 to day 15, cells were fed every other day with fresh iNKT cell medium

supplemented with IL-2, and at day 15 stained with α-GalCer/CD1d tetramers and flow cytometry performed to measure the purity of iNKT cells. Cells were further expanded until day 21, harvested and stained with α-GalCer/CD1d tetramer, and sorted by FACS.

In order to expand and maintain a polyclonal iNKT cell line in culture, α-GalCer/CD1d tetramer sorted cells were restimulated by mixing with irradiated PBMCs in iNKT cell medium supplemented with IL-2 and 1 μg/ml PHA. Fresh iNKT cell medium with IL-2 was added every 2 days. At day 15 cells were stained with α-GalCer/CD1d tetramer to check for purity. iNKT cells in all experiments were at least 98% α-GalCer/CD1d tetramer positive and used at least 20 days after restimulation. Restimulation with irradiated PBMCs and PHA was performed every 25 days.

2.2.1d Isolation of human naïve T cells

PBMCs were generated as above before MACS separation was used to negatively select for naïve T cells, following the manufacturer's protocol (Miltenyi Biotec). Briefly, PBMCs were incubated with a cocktail of biotinylated antibodies (CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCRγ/δ, anti-HLA-DR, and CD235a) before anti-biotin magnetic beads were added for negative selection.

2.2.1e Generation of mouse bone marrow-derived dendritic cells

Bone marrow from various strains were plated in $10cm^2$ dishes at a density of $5x10^6$ cells per plate, in R10 supplemented with 20ng/ml GM-CSF for 4-6 days, with fresh medium added every 3 days. After harvesting, cells were enriched for DCs by positive selection for $CD11c^+$ cells by MACS separation.

2.2.1f Generation of mouse bone marrow-derived macrophages

Bone marrow was plated in $10cm^2$ dishes at a density of $5x10^6$ cells per plate, in R10 supplemented with 30% (v/v) conditioned medium from L929 cells, which contain M-CSF. 7-9 days later, adherent cells were harvested and checked for purity by staining for F4/80 expression by flow cytometry.

2.2.1g Isolation of mouse splenic dendritic cells

Splenocytes from WT C57BL/6 mice were obtained by homogenisation using a syringe plunger through a cell strainer. The resulting cell suspension was positively selected for $CD11c^+$ cells using MACS separation

2.2.1h THP-1 differentiation

THP-1 cells were maintained in culture in R10 and passaged following confluence every 4-7 days. Prior to use in experiments, THP-1 cells were differentiated into macrophage-like cells. For this, THP-1 cells were plated in $10cm^2$ dishes at a density of $5x10^6$ cells per plate, in R10 containing 50ng/ml PMA. Cells were left for 24-48 hours, before harvesting and washing in R10 alone. Cells were then replated and rested in R10 for at least 24 hours before use in experiments.

2.2.1i MACS microbead sorting

Cells to be sorted were resuspended in 2ml of cold MACS buffer and incubated for 20min at 4°C with magnetic beads conjugated to antibodies against the applicable cell surface molecules. Cells were then washed with 50 ml of cold MACS buffer and pelleted. The resulting pellet was resuspended in 1ml of cold MACS buffer, filtered through a cell strainer to remove cell clumps and passed through a MACS LS column for positive selection, or a MACS LD column for negative selection, following the manufacturer's protocol (Miltenyi Biotec). Column-retained cells or flow-through, for positive or negative selection, respectively, were harvested and washed in complete medium twice before use.

2.2.1j Fluorescence-activated cell sorting (FACS)

Cells to be sorted were stained with fluorochrome-conjugated antibodies as for flow cytometry (see below). 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 using a MoFlo sorter (Beckman Coulter) or FACS Aria II sorter (BD Biosciences) with the expert help of Craig Waugh and Paul Sopp.

2.2.2 Cellular assays

2.2.2a Stimulation of IL-1β production

For stimulation of IL-1β production, cells were plated in 150µl R10 per well in 96-well culture plates, at a density of $5x10^4$ cells per well for human cells, and $1x10^5$ cells per well for mouse cells. Cells were then allowed to settle and rest for 2 hours at 37°C before stimulation.

SAA – at 1µg/ml (or stated concentration) for 24 hours.

LPS/ATP – LPS at 10-100ng/ml for 16-24 hours, followed by ATP at 5mM for 30min to 5 hours.

Pam_3Csk_4/ATP – at 1µg/ml for 16-24 hours, followed by ATP at 5mM for 30min to 5 hours.

UV-killed bacteria – at multiplicity of infection (m.o.i.) of 50 for 24 hours. Bacteria were prepared by growing overnight in LB solution at 37°C, with agitation. Bacterial suspensions were then washed 4 times in PBS before being resuspended in PBS and transferred to a 6-well plate. Bacteria were then killed by exposure to UV-light for 7.5 minutes, before determination of cell number by measurement of optical density at 600nm.

For ELISA analysis, cells were retained in R10 for the whole duration of the incubation. For analysis of supernatants by immunoblotting, R10 was replaced by OptiMEM for the final incubation step.

2.2.2b Neutralisation assays

For neutralisation assays, cells were pre-incubated with the stated concentration of blocking antibodies for 1-2 hours before stimulation. The neutralising antibodies were retained in the culture during stimulation.

2.2.2c iNKT cell-MdDC crosstalk assays

Pre-conditioning using basal autoreactivity

MdDCs were harvested and washed 3 times with fresh R10 to remove IL-4 and GM-CSF from the differentiation medium. iNKT cells were similarly harvested and washed. For pre-conditioning, $5x10^4$ MdDCs were either left untreated or co-cultured with iNKT cells (at 3:1 or stated ratio) in 150µl R10 per well in 96-well plates, for 12-16 hours. After this pre-conditioning step, IL-1β production was stimulated in MdDCs without the removal of iNKT cells.

iNKT cell stimulation using α-GalCer

The iNKT cell agonist α-GalCer was sonicated for 10min to disrupt liposomes which may have formed during storage. MdDCs were combined with iNKT cells as above in the presence of 2ng/ml α-GalCer.

2.2.2d Pre-conditioning with recombinant sCD40L or cytokines

MdDCs were harvested and plated as above, in the presence of recombinant sCD40L, various recombinant cytokines or supernatants at the stated concentrations, for 14-16 hours. Recombinant molecules and supernatants were not washed out prior to stimulation of IL-1β production.

Recombinant sCD40L exists as a FLAG-tagged monomeric form; to generate the multimer, monomeric sCD40L was incubated with enhancer for ligands (an anti-FLAG antibody) at a ratio of 9:1 (v/v) for 30min at 4°C.

2.2.2e Generation of basal autoreactivity supernatant

Basal autoreactivity supernatant was generated by co-incubating MdDCs with iNKT cells as above, in the absence of α-GalCer, for 12-16 hours in R10. Subsequently, the culture supernatant was harvested, pooled, and centrifuged to remove contaminating cells. The supernatant was then passed through a filter to ensure the removal of any cells, and stored in aliquots at -80°C for later use. DC supernatants were generated exactly as above, but without the addition of iNKT cells during the 12-16 hour incubation.

2.2.2f CFSE-labelling of bacteria

Bacteria were grown overnight in 5ml LB broth at 37°C, with agitation. After washing 3 times in PBS, bacteria were resuspended in 500µl PBS at an optical density of 0.8 and CFSE added (final concentration 20µM). The bacteria were incubated for 30min at 30°C, with agitation, before CFSE was quenched using 10ml of warmed FCS. The labelled cells were then pelleted by spinning at 3500rpm for 10min, before being washed 4 times in FACS buffer. The resulting cells were then killed by UV and used as above.

2.2.2g *In vitro* influenza virus infection

MdDCs were harvested as above and plated at a density of $4x10^5$ cells per well in a 24-well plate, in 200µl OptiMEM. The cells were then rested at 37°C for 1 hour. Influenza A virus was then added, at the stated m.o.i., and the cells incubated at room temperature for 1 hour. Subsequently, the MdDCs were washed with PBS and incubated in 300µl R10 for 24 hours before analysis of cell lysates and supernatants.

In pre-incubation assays with iNKT cells, MdDCs were plated in 24-well plates with iNKT cells at a ratio of 3:1 in R10 for 14-16 hours. After this pre-incubation step, the cells were washed once with OptiMEM before infection was carried out as above.

2.2.2h Splenocyte restimulation

Splenocytes were obtained from influenza-infected mice of different strains and single cell suspensions prepared by homogenisation through a cell strainer. Splenocytes were then plated in 0.5ml R10 in a 24-well plate at a density of $1x10^6$ cells per well. Various restimulation stimuli were added, and cells cultured for 72 hours before analysis of cytokine release.

PR8 virus was added at 10^6 pfu; NP peptide (ASNENMETM) and OVA peptide (SIINFEKL) at 20μM; PMA/Ionomycin at 1μg/ml each.

2.2.3 Analysis of cellular and cytokine content

2.2.3a Immunoblotting

Protein precipitation from cell supernatant

In order to detect cleaved IL-1β and caspase-1 in the cell supernatant, experiments were performed in OptiMEM to avoid non-specific binding of anti-IL-1β antibody to serum proteins. Supernatants were transferred to 1.5ml eppendorf tubes and mixed with 1 volume of methanol and 0.25 volume of chloroform in order to precipitate the proteins. The vials were then spun at 13k rpm for 10min at 4°C. The supernatant above the layer of precipitated protein was gently removed and another volume of methanol was added. Vials were spun for an additional 10min at 4°C to pellet the proteins, before supernatants were removed and vials were left to dry upside down to remove traces of methanol. The precipitated proteins were then dissolved in 1x reducing loading buffer for loading onto SDS-PAGE gels.

Preparation of cell lysates

Cells were harvested from experiments, with adherent cells dislodged using PBS+2mM EDTA, and transferred into 1.5 ml eppendorf tubes. Cells were spun down at 6000rpm for 5min and washed two times with sterile PBS. The pellet was lysed with 10-15μl of cell lysis buffer. Protein concentration was

then determined by Bradford assay. Briefly, 1µl of cell lysate was mixed with 200µl Bradford solution (prepared according to manufacturer's instructions, BioRad) and after 5min, absorbance at 595nm measured and absolute concentration calculated relative to a standard curve obtained using known concentrations of bovine serum albumin (BSA). Samples were then normalised to the same protein content by dilution with cell lysis buffer, and reducing loading buffer added.

Immunoblotting

Samples were heated for 10 minutes at 95°C and loaded onto polyacrylamide gels for SDS-gel electrophoresis. 3µl of protein ladder (250-10 kDa) were also loaded to identify the molecular weight of target proteins. Proteins were migrated through the gel at 140V, constant voltage, until the loading dye front reached the bottom of the gel.

Subsequently, proteins were transferred to Hybond-P PVDF membrane, which was hydrated in methanol for 5 seconds and then left in water for 5min. Both the membrane and the gel were equilibrated in the transfer buffer for 5 minutes and then the transfer pack assembled for the transfer as per manufacturer's instructions (BioRad). The transfer tank was filled with transfer buffer, with an ice pack included to maintain the transfer at 4°C. The transfer was performed for 1hr at 50V for supernatant analysis or at 80V for the lysates.

The membrane was then removed from the pack and fixed at room temperature for 20min with 0.5% PFA in PBS. It was then washed with 0.1% PBST (wash buffer) and blocked with blocking buffer for 45min. After blocking, the membrane was rinsed with wash buffer and the primary antibody was added (diluted in antibody dilution buffer) and incubated overnight at 4°C. Subsequently, the membrane was washed 3 times for 5min and incubated with the secondary antibody for 1-2hrs at room temperature. Excess secondary antibody was washed off with wash buffer (3x5min).

The membrane was then developed by the addition of 1.5 ml ECL solution onto the membrane for 5min. Chemiluminescence was detected by exposure to X-ray film.

2.2.3b ELISA

ELISA plates (96-well, Corning) were incubated overnight with 25µl of capture antibody (diluted in coating buffer) at 4°C. Plates were then washed 5 times with wash buffer and blocked with 100µl

blocking buffer at 37°C for 2 hours. Blocking buffer was removed and 20µl cell supernatants to be tested added into the wells and incubated overnight at 4°C. Dilutions of samples were done in blocking buffer. Plates were then washed 5 times in wash buffer and incubated at room temperature for 1hr with 25µl of the specific detection/biotinylated antibody (diluted in blocking buffer). Plates were then washed 5 times and 25µl of streptavidin-HRP was added to the well for 30min at room temperature. Plates were washed 9 times, with the final wash performed with PBS. To develop the plate, 25µl of TMB substrate was added to the wells and developed for 3-10 minutes on average. When a suitable colour intensity was achieved, the reaction was stopped with 25µl of stop solution. The absorbance was then measured at 450nm using a microplate reader and the concentration of the cytokines calculated on the basis of the standard curve.

2.2.3c Flow cytometry

For analysis by flow cytometry, cells were harvested and transferred into 96-well plates (U bottom), washed with 200µl FACS buffer and spun for 5min at 1500rpm. Cells were then stained with the relevant fluorochrome-conjugated antibodies for 20min at 4°C, or purified antibodies followed by the relevant secondary antibody. α-GalCer/CD1d tetramer staining was performed before the addition of other antibodies and done at 37°C for 15min. Cells were then washed twice with FACS buffer before being resuspended in FACS buffer for analysis on FACSCalibur (BD Biosciences) or Cyan (Dako). Propidium iodide (at 5µg/ml) was added shortly before analysis to stain for dead cells. Flow cytometry data was analysed using FlowJo software.

2.2.3d Real time PCR

For PCR analysis, cells were lysed in 350µl RLT buffer, and frozen at -80°C until required. RNA was extracted using a Qiagen RNeasy mini kit according to the manufacturer's instructions. Samples were treated with Turbo DNase to remove genomic DNA as per kit instructions.

cDNA was obtained by denaturation of RNA followed by reverse trancription using the Superscript III Reverse transcriptase kit. Briefly, random primers were annealed to the RNA and then reverse transcription was performed by incubation in the presence of the Superscript III reverse transcriptase at 50°C for 60 minutes. The reaction was subsequently inactivated by heating to 70°C for 15 minutes. The cDNA obtained at this point was then used as a template for amplification in PCR.

Real-time quantitative PCR was performed with the TaqMan Gene Expression Master Mix, according to the manufacturer's protocol. qPCR reactions were set up by mixing 5µl of qPCR master mix, 4.5µl of sample cDNA (10-100ng) and 0.5µl of the relevant 20x TaqMan probe. Reactions were run in a 7500 Fast Real-time PCR system. Fold induction was calculated as $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T = (C_T - C_{T\ ref\ gene}) - (C_T - C_{T\ ref\ gene})_{ref\ sample}$

2.2.3e EMSA

For EMSA, nuclear extracts were prepared by resuspending cells in 400µl cold buffer A (hypotonic). After 15min on ice, 25µl of 10% igepal (v/v) were added, and the lysate was vortexed for 10s and centrifuged for 30s at 12,000g. The supernatant was discarded and the cell pellet was resuspended in 100µl cold buffer B (hypertonic). Cells were then rocked vigorously at 4°C for 15min. Cellular debris was removed by centrifugation at 12,000g for 5min at 4°C, and the supernatant containing the nuclear fraction removed.

^{32}P was incorporated into the oligonucleotide probe by incubating 20ng oligonucleotide with 10µl dH_2O, 2µl PNK Buffer A and 1µl T4 polynucleotide kinase at 37°C for 30min

Nuclear extracts were incubated with binding buffer containing 1ng of [γ-^{32}P] dATP-radiolabelled double-stranded DNA oligonucleotide for 20min at room temperature. The DNA binding consensus sequence used for NF-κB was (5'-AGTTGAGGGGACTTTCCCAGGC-3'). DNA-protein complexes were loaded and resolved by electrophoresis in native 4% (w/v) polyacrylamide gels. The gels were then dried and autoradiographed. EMSA protocols were performed with the expert assistance of Dr. Issa Abu-Dayyeh.

2.2.4 Preparation of samples from *in vivo* experiments

2.2.4a Blood

For blood analysis, mice were bled from the tail vein, with blood collected into an eppendorf tube containing 40µl heparin. Erythrocytes were then removed by osmotic lysis by resuspending pellets in 1ml of red blood cell (RBC) lysis buffer for 5mins. Cells were then pelleted, washed in FACS buffer, and stained for flow cytometry.

When serum was required, blood was collected from the tail vein in non-heparinised tubes. Samples were allowed to stand for 90-120mins at room temperature to enable coagulation and sedimentation. Samples were then centrifuged at 13k rpm for 15min and the serum settling above the cells was then collected for ELISA analysis.

2.2.4b Broncho-alveolar lavage (BAL)

Immediately after sacrifice of animals, tubing was inserted into the trachea and 1ml of PBS introduced via a syringe to inflate the lungs. The BAL fluid was withdrawn and re-introduced a total of three times before being collected. BALF was then centrifuged at 6000rpm for 5min and BALF supernatant collected for ELISA, whilst pelleted cells were counted by haemocytometer (with Trypan Blue exclusion) and analysed by FACS after RBC lysis.

2.2.4c Isolation of lung leukocytes

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 3ml of type II collagenase (3mg/ml) in R0 for 30 minutes at 37°C, with agitation. Following incubation, cells were filtered through a 70µm cell strainer and immediately washed in FACS buffer. Cell suspensions were pelleted (1500 rpm, 5 minutes at 4°C), and erythrocytes removed using RBC lysis buffer for 5mins. Cells were then washed in FACS buffer, filtered through a 70µm cell strainer for a second time and resuspended in FACS buffer for staining.

2.2.4d Bone marrow

Hind limbs were harvested from mice of various strains. Intact bones were washed with 70% ethanol and RPMI, and then cut at both ends. Bone marrow was then flushed out with FACS buffer using a syringe and needle. Bone marrow cells were then passed through a cell strainer to remove debris and washed twice in R10 before differentiation as described above.

CHAPTER 3

Induction of IL-1β Synthesis and Maturation by Serum Amyloid A

Chapter 3: Induction of IL-1β Synthesis and Maturation by Serum Amyloid A

SAA is an acute phase protein which can increase up to 1000-fold from basal levels during infection. As such, it is believed to be a key modulator of the immune response. Synthesised mainly by hepatocytes in response to IL-1 and IL-6, SAA can drive the synthesis of a variety of pro- and anti-inflammatory cytokines. In addition, it has been reported to induce the migration of leukocytes towards the site of infection.

The pleiotropic activities of SAA have been attributed to its ability to engage several different cell surface receptors and induce different signalling pathways. In particular, previous reports point to TLR2 and FPR2 as key receptors in mediating the effects of SAA on immune cells.

In this chapter, we will examine the ability of SAA to induce production of the pro-inflammatory cytokine IL-1β. The interplay between SAA and IL-1β can potentially establish a positive feedback mechanism to drive inflammatory responses. We will investigate the receptors involved in sensing SAA, as well as the molecular mechanisms downstream of receptor engagement. In addition, the physiological role of SAA in inducing IL-1β will be examined. Our data will be important in adding to our understanding of the role of SAA in the inflammatory response.

3.1 SAA induces the expression of pro-IL-1β

In order to investigate the induction of bioactive IL-1β by SAA, we first confirmed the ability of SAA to induce the synthesis of the inactive precursor, pro-IL-1β. SAA has been reported to be able to induce the secretion of IL-1β in human monocytes [256]. However, as human monocytes possess a constitutively active inflammasome owing to continued secretion of ATP into the culture medium [257], this report therefore does not investigate the ability of SAA to induce pro-IL-1β cleavage.

In *in vitro* assays, human monocyte-derived DCs (MdDCs) were stimulated with human SAA for 24 hours, and LPS, with or without the inflammasome activator ATP, which was used as a positive control for pro-IL-1β induction. Following stimulation, cells lysates were analysed by immunoblotting **(Fig. 3.1a)**, which can distinguish the pro- and cleaved forms of IL-1β due to their different sizes. Pro-caspase-1 was used as a loading control. Confirming the previous report using human monocytes, we observed the induction of the 31kDa pro-IL-1β at all concentrations of SAA tested in MdDCs.

Next, we investigated the induction of pro-IL-1β by SAA at the transcriptional level. As activation of the transcription factor NF-κB and its translocation into the nucleus is a pre-requisite for driving pro-IL-1β transcription [73, 74, 258], the ability of SAA to activate the NF-κB pathway was analysed by electrophoretic mobility shift assay (EMSA). A radiolabelled DNA probe with the NF-κB binding motif was incubated with total nuclear extract from stimulated cells, before being migrated through a native agarose gel. Any active NF-κB in the nuclear extract would bind to the radiolabelled probe and therefore slow its movement through the gel. This results in a shift of the band corresponding to the probe, which can be visualised using radiosensitive film. We found that treatment of human MdDCs with SAA induces accumulation of active NF-κB in the nucleus, with the signal increasing with time **(Fig. 3.1b)**. We also showed that activation of NF-κB was concurrent with the induction of *Il1b* mRNA, as detected by quantitative real-time PCR **(Fig. 3.1c)**. By this method, we found that induction of the *Il1b* transcript by SAA is rapid, already reaching 50-fold after 30 minutes and peaking at around 1000-fold after 2 hours.

We then performed experiments to exclude the possibility that the induction of pro-IL-1β observed was a result of LPS contamination in our recombinant SAA stocks, which were generated in *E. coli*. Heat-mediated denaturing of SAA at 95°C significantly diminished its ability to induce pro-IL-1β in the cell lysate, whereas a more modest decrease in signal was detected when using LPS subjected to a

similar pre-treatment **(Fig. 3.1d)**. In addition, when polymyxin B (an antibiotic which neutralises the action of endotoxin) was added, pro-IL-1β induction by SAA was still detected, whereas LPS-induced pro-IL-1β was completely abolished **(Fig. 3.1e)**. These experiments show that SAA itself, and not contaminating LPS, is responsible for stimulating the production of pro-IL-1β.

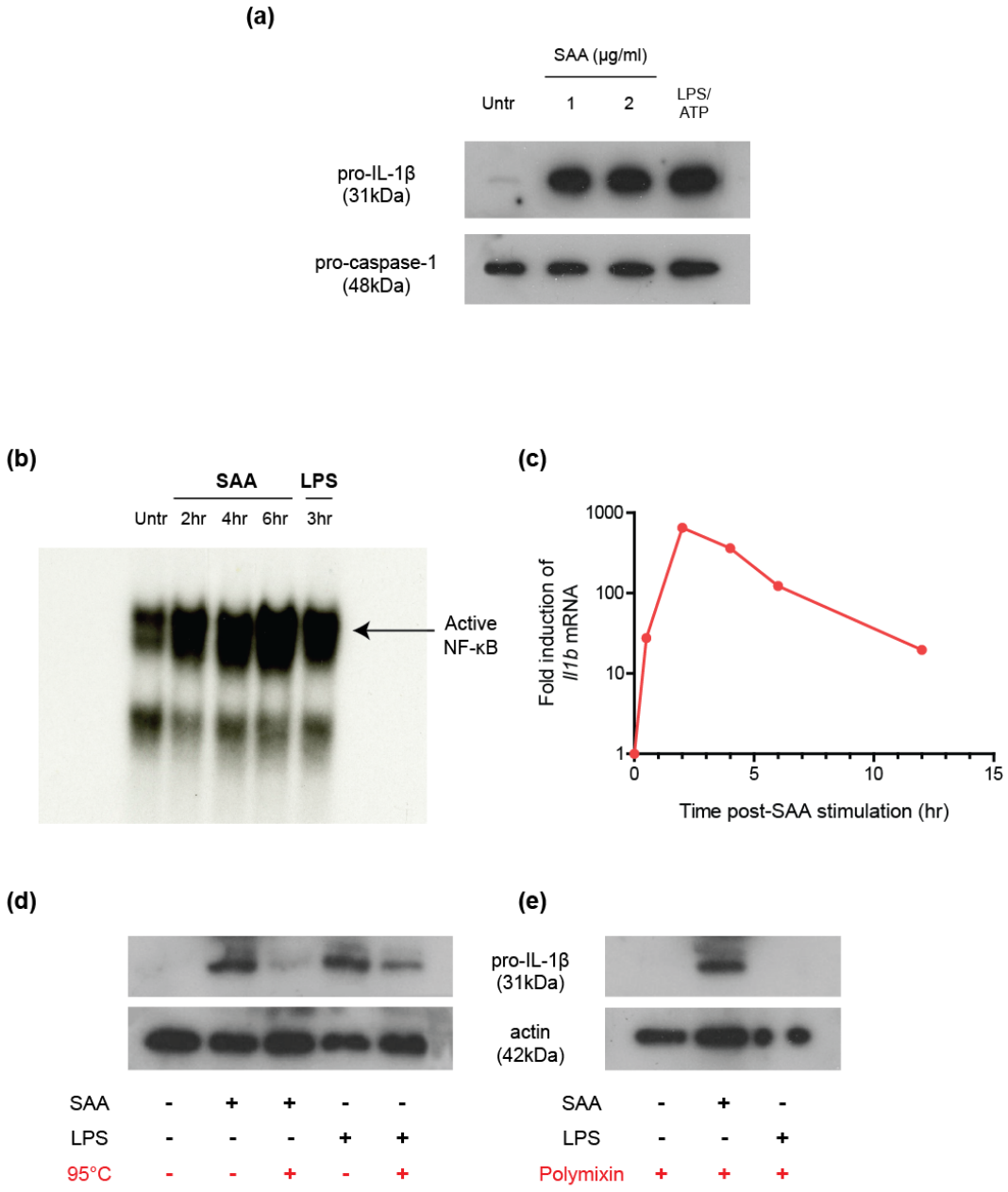

Fig. 3.1 SAA induces the expression of pro-IL-1β in human MdDCs. (a) The induction of pro-IL-1β by SAA (1μg/ml) in human MdDCs after 24hrs stimulation was analysed in cell lysates by immunoblotting. LPS (100ng/ml for 14hrs) followed by ATP (5mM) for 30min was added as a positive control. Pro-caspase-1 was used as a loading control. **(b)** Analysis of NF-κB activation by EMSA following stimulation of human MdDCs with SAA and LPS. **(c)** Induction of pro-IL-1β mRNA in human MdDCs was analysed by real time PCR after stimulation with SAA (1μg/ml). **(d)** Induction of pro-IL-1β by SAA was not due to LPS contamination. SAA (1μg/ml) or LPS (100ng/ml) were heated to 95°C for 2hrs prior to addition to MdDCs, or **(e)** added together with polymyxin B (25μg/ml), for 24hrs. All data are representative of at least 2 independent experiments.

3.2 SAA induces maturation and secretion of IL-1β with concurrent cleavage of caspase-1

Having confirmed the ability of SAA to drive pro-IL-1β expression, we proceeded to determine whether signal 2, the cleavage of pro-IL-1β to yield the bioactive cytokine, could also be induced by SAA. The secretion of cleaved IL-1β (a 17kDa protein), as detected by immunoblotting of protein precipitated from the cell supernatant, was used as a readout of IL-1β maturation. In addition, activation of the inflammasome and IL-1β cleavage has been observed to be accompanied by the secretion of active, cleaved caspase-1 itself [99], potentially as a negative feedback mechanism to shut down IL-1β production. We found that the human monocyte cell line THP-1 (differentiated with PMA to induce a macrophage-like phenotype) secreted the cleaved form of IL-1β upon SAA treatment, in contrast to LPS in the absence of ATP, which was unable to mature the cytokine **(Fig 3.2.1a)**. In addition, we detected the cleaved form of caspase-1 (20kDa) in the cell supernatant, correlating with the release of bioactive IL-1β.

As well as detecting IL-1β by immunoblotting, we used ELISA to give a more quantitative approach to assay the release of IL-1β upon stimulation. Despite the antibodies used for ELISA being unable to unequivocally distinguish the pro- or cleaved forms of IL-1β, studies have shown that they show a preference for binding to the cleaved form [96, 259]. In addition, cleavage of IL-1β has been proposed to be a pre-requisite for its secretion [97]. We found that, as in our immunoblotting assays, SAA stimulated the release of IL-1β from human myeloid cells into the supernatant, which was detectable by ELISA **(Fig 3.2.1b, c)**. In addition, we observed a dose response, with increasing levels of IL-1β at higher concentrations of SAA used.

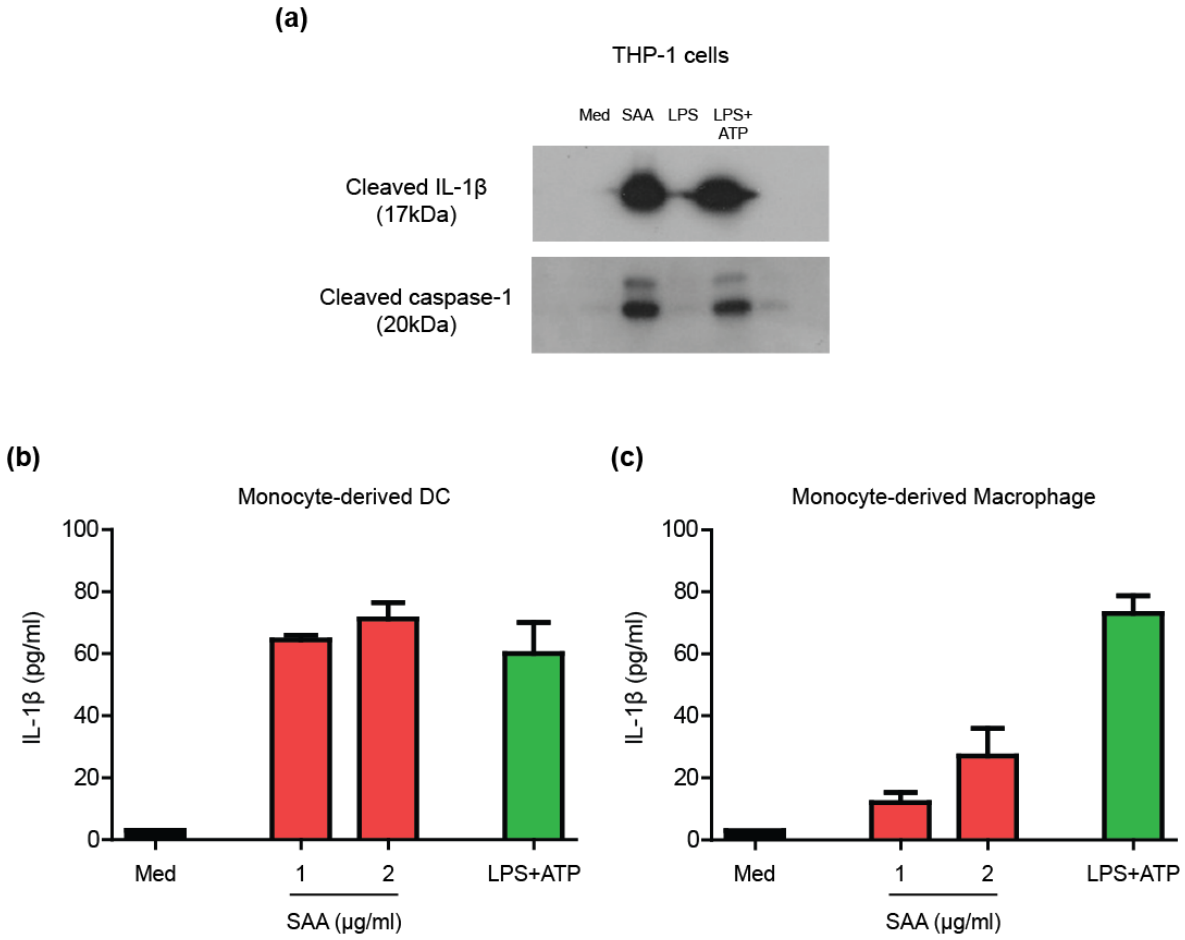

Fig. 3.2.1 SAA induces activation of caspase-1 and maturation of IL-1β in human myeloid cells. (a) Total protein was precipitated from THP-1 cell supernatant after stimulation with SAA (1μg/ml) or LPS (100ng/ml) for 24hrs, with or without ATP (5mM for 2hrs) and analysed by immunoblotting. **(b)**, **(c)** Secreted IL-1β was quantified by ELISA in monocyte-derived DCs or macrophages. Data are representative of 3 independent experiments. Data bars show the mean of duplicates +/- SEM.

As well as human myeloid cells, we also tested the induction of bioactive IL-1β using murine bone marrow-derived DCs and macrophages. We demonstrated similar results when mouse myeloid cells were stimulated with SAA, observing the secretion of cleaved IL-1β and cleaved caspase-1, by both immunoblotting **(Fig. 3.2.2a)** and ELISA **(Fig. 3.2.2b, c)**. These data suggest that SAA induction of pro-IL-1β and IL-1β maturation is conserved between multiple species, emphasising the importance of SAA in generating an inflammatory response.

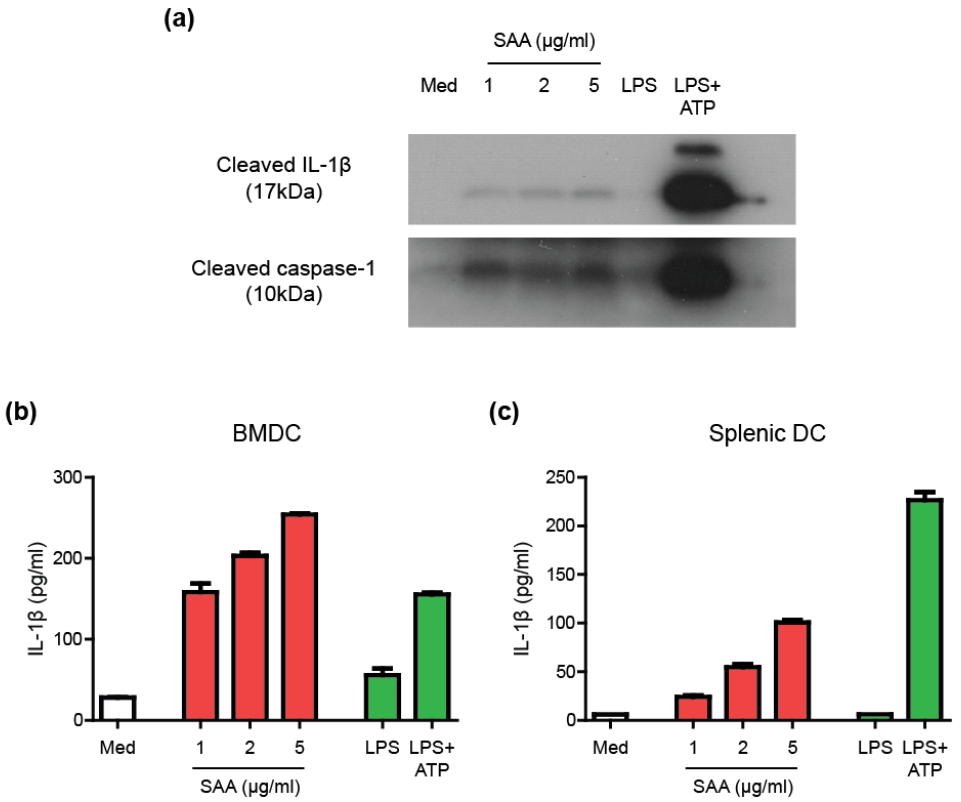

Fig. 3.2.2 SAA induces activation of caspase-1 and maturation of IL-1β in mouse myeloid cells. (a) Total protein was precipitated from bone marrow-derived DC supernatant after stimulation with SAA or LPS (10ng/ml) for 24hrs, with or without ATP (5mM for 2hrs) and analysed by immunoblotting. **(b)**, **(c)** Secreted IL-1β was quantified by ELISA in bone marrow-derived DCs or splenic DCs. Data are representative of at least 3 independent experiments. Data bars show the mean of duplicates +/- SEM.

3.3 Induction of a panel of pro- and anti-inflammatory cytokines by SAA

In order to better understand the cytokine milieu in which cells stimulated with SAA are exposed, we tested, by ELISA, the levels of various pro- and anti-inflammatory cytokines in the supernatant of human MdDCs stimulated with SAA **(Fig 3.3)**. In addition to IL-1β, we found that SAA stimulated the release of appreciable levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-12p40, as well as the anti-inflammatory cytokines IL-10 and TGF-β. This is in agreement with reports that SAA induces a variety of different cytokines from both human and mouse cells [63, 260]. We also detected the release of low levels of IL-12p70 (around 200pg/ml), but this was not consistently observed across all donors (data not shown). With TNF-α and IL-6 being important innate pro-inflammatory cytokines, IL-12 being a key player in Th1 responses and IL-10 and TGF-β playing a central role in immune regulation, this finding highlights the ability of SAA to modulate the plasticity of different cell types, with the potential to polarise immune responses.

Furthermore, our finding that SAA can induce the production of anti-inflammatory cytokines, such as IL-10 and TGF-β, could explain the observation that when plasma SAA levels *in vivo* increase up to 1000-fold during inflammation [261], systemic IL-1β levels, whilst elevated, remain controlled. The consequences of uncontrolled IL-1β production are well-described in disorders affecting the inflammasome, such as Muckle-Wells syndrome, as well as animal models exhibiting dysregulation of the NF-κB pathway [42, 143, 262]. We can speculate that SAA-induced IL-10 and TGF-β may play an important role in preventing overzealous systemic inflammation, thereby protecting the host from systemic shock and severe immunopathology.

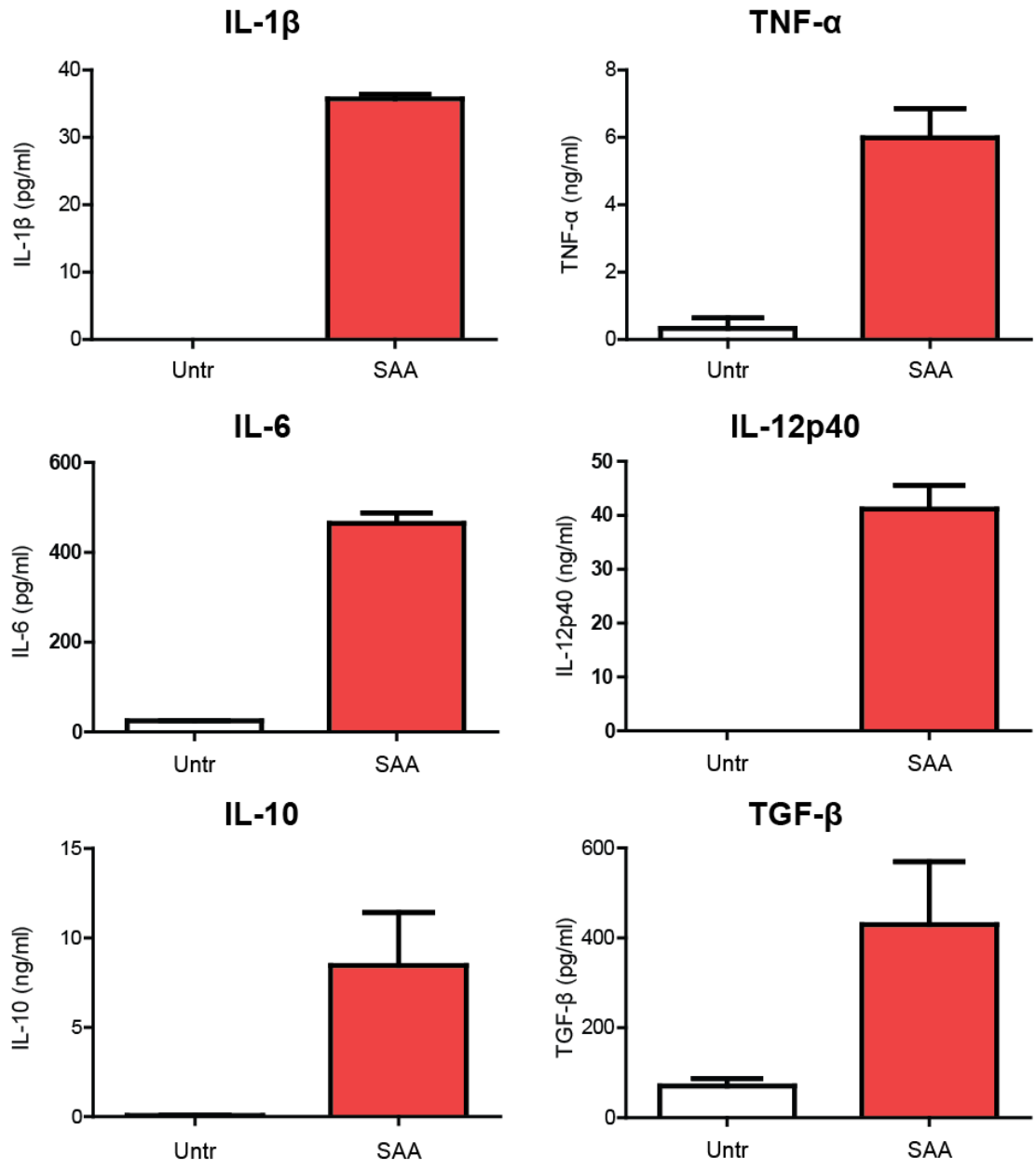

Fig. 3.3 SAA induces the production of various pro- and anti-inflammatory cytokines from human MdDCs. As well as IL-1β, release of TNF-α, IL-6, IL-12p40, IL-10 and TGF-β were detected in the supernatant of human monocyte-derived DCs stimulated with 1μg/ml SAA for 24hrs. Data are representative of 3 independent experiments, each performed with cells from a different donor. Bars show the mean of triplicates +/- SEM.

3.4 TLR2, and not FPR2, is required for the induction of pro-IL-1β by SAA

The ability of SAA to induce a variety of cytokines could be due to its capacity to engage various different receptors on the cell surface and therefore trigger diverging intracellular signalling pathways. In the context of pro-IL-1β induction, we have shown that SAA is able to activate NF-κB and, in so doing, drive *Il1b* transcription. Of note, SAA has been shown to activate NF-κB in intestinal epithelial cells [263], with TLR2 identified as the functional receptor upstream in another report [264]. Another cell surface receptor, FPR2, is a G-protein coupled receptor shown to regulate inflammatory processes, including SAA-mediated neutrophil recruitment *in vivo* [265]. In addition, blockade of FPR2, but not TLR2, was demonstrated to block the IL-10-inducing capacity of SAA on human neutrophils [62]. Consequently, we decided to investigate whether TLR2 and FPR2 were required for the induction of pro-IL-1β by SAA, using BMDMs originating from mice deficient in either of these receptors.

Firstly, we verified that BMDMs from wild-type (WT) C57BL/6 mice express TLR2 and FPR2, using flow cytometry. After 7 days of differentiation in medium containing M-CSF, 98% of cells expressed both the myeloid marker CD11b and the macrophage marker F4/80 **(Fig. 3.4.1a and 3.4.2a, left panels)**. Using anti-TLR2 and anti-FPR2 antibodies, we showed that BMDMs differentiated in this way express both receptors **(Fig. 3.4.1a and 3.4.2a, right panels)**.

Using macrophages derived from *Tlr2*$^{-/-}$ bone marrow, we tested whether stimulation of pro-IL-1β by SAA was dependent on TLR2. By immunoblotting of cell lysates, we were able to show that SAA induces pro-IL-1β in WT BMDMs only, and not in TLR2-deficient cells **(Fig. 3.4.1b)**. However, pro-IL-1β was upregulated in cells of both genotype upon LPS and ATP treatment. As expected, Pam_3Csk_4, a synthetic lipoprotein which is a TLR2 agonist, was unable to induce pro-IL-1β expression in TLR2-deficient cells. From these data we conclude that SAA-mediated pro-IL-1β expression is TLR2-dependent.

We confirmed this observation by testing the supernatants of WT or *Tlr2*$^{-/-}$ BMDMs by ELISA after stimulation **(Fig. 3.4.1c)**. In agreement with our immunoblotting results, TLR2-deficient cells did not release detectable levels of IL-1β upon exposure to SAA or Pam_3Csk_4 with ATP, however, their capacity to secrete IL-1β in response to the TLR4 agonist LPS (in conjunction with ATP) remained unaffected.

Similarly, *Tlr2*$^{-/-}$ BMDMs showed no defect in their ability to secrete IL-10 and IL-6 in response to SAA, although their response to Pam_3Csk_4 was significantly diminished **(Fig. 3.4.1d)**. This confirms the previous report from our laboratory that SAA elicits IL-10 secretion in a TLR2-independent manner [62].

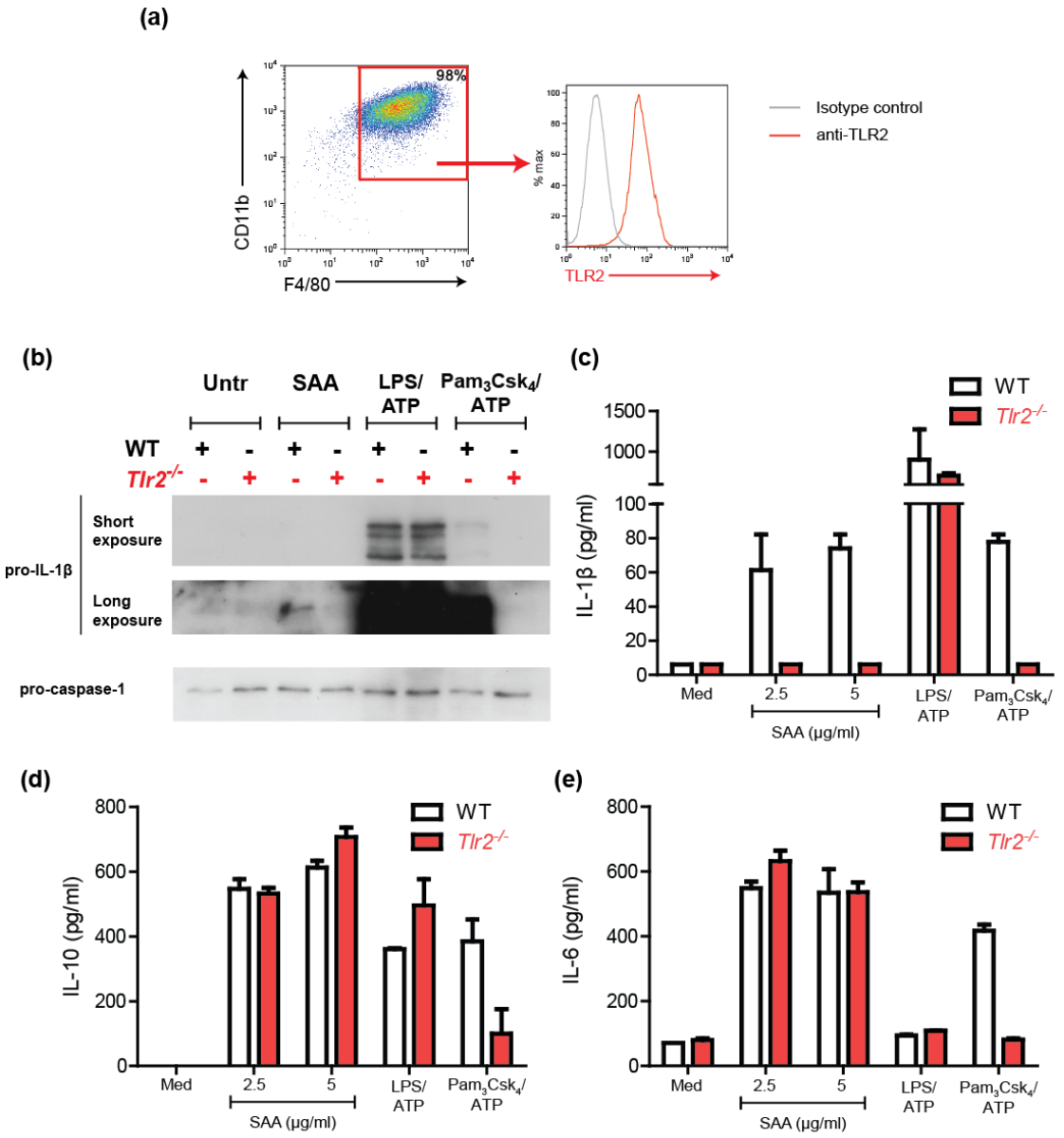

Fig. 3.4.1 SAA signals through TLR2 to induce pro-IL-1β. **(a)** Mouse bone marrow-derived macrophages, defined as $CD11b^+F4/80^+$, express TLR2, as detected by flow cytometry. **(b)** Immunoblot of mouse BMDM lysates after stimulation with SAA (1μg/ml) for 24hrs, or LPS (10ng/ml) or Pam3Csk4 (1μg/ml) for 14hrs with ATP (5mM) for 30min. **(c-e)** Levels of secreted cytokines from mouse BMDMs derived from WT or *Tlr2*$^{-/-}$ mice after stimulation as above, as detected by ELISA. Data are representative of 3 independent experiments, with bars showing the mean of triplicates +/- SEM.

Next, we performed similar experiments comparing BMDMs from the 129 (WT) and *Fpr2*$^{-/-}$ strains. In contrast to TLR2-deficient cells, FPR2 deficiency had no effect on the induction of pro-IL-1β by SAA **(Fig. 3.4.2b)**. By ELISA, we also observed no differences in the secretion of IL-1β between WT and *Fpr2*$^{-/-}$ BMDMs, although the production of IL-10 was significantly impaired **(Fig. 3.4.2c, d)**.

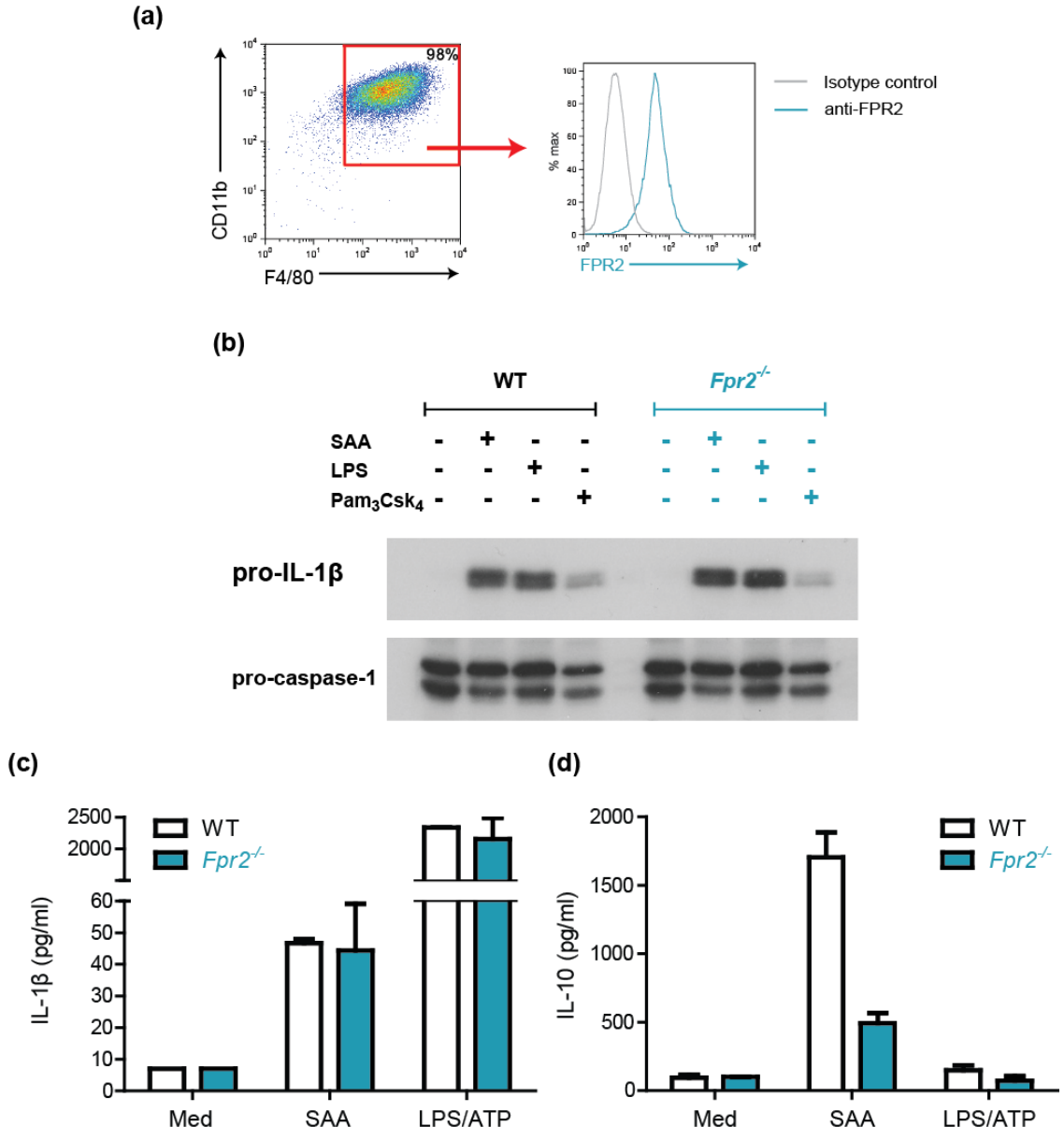

Fig. 3.4.2 FPR2 is not required for pro-IL-1β induction by SAA. (a) Expression of FPR2 on BMDMs was analysed by flow cytometry. **(b)** Immunoblot of mouse BMDM lysates after stimulation with SAA (1μg/ml), LPS (10ng/ml) or Pam3Csk4 (1μg/ml) for 24hrs. **(c-d)** Levels of secreted cytokines from mouse BMDMs derived from WT or *Fpr2*$^{-/-}$ mice after stimulation with SAA (1μg/ml, 24hrs) or LPS (10ng/ml, 24hrs) with ATP (5mM, 30min), as detected by ELISA. Data are representative of 3 independent experiments, with bars showing the mean of triplicates +/- SEM.

Following these experiments using different knockout mice, we extended our results to human cells. THP-1 cells were pre-incubated with an isotype control antibody, an anti-TLR2 or an anti-FPR2 blocking antibody to neutralise signalling through these receptors, prior to incubation with SAA. We demonstrated a similar pattern to that obtained from mouse BMDMs, observing that blockade of TLR2, but not FPR2, decreased IL-1β secretion in response to SAA **(Fig. 3.4.3)**.

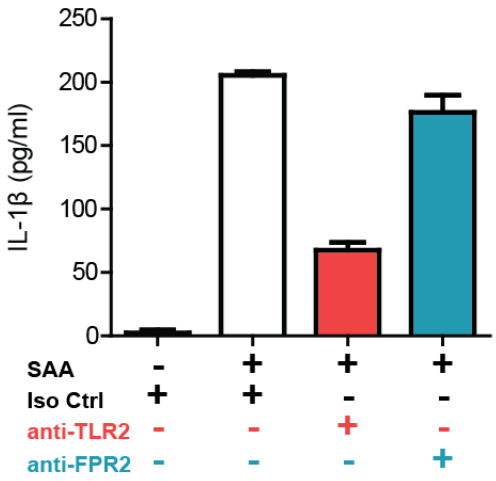

Fig. 3.4.3 Blockade of TLR2, but not FPR2, inhibits IL-1β production by human THP-1 cells. THP-1 cells were pre-incubated with 1µg/ml of anti-TLR2 or anti-FPR2 antibody for 2 hours before addition of SAA (1µg/ml) for 24 hours. Mouse IgG was used as an isotype control. Cell supernatants were analysed by ELISA. Data are representative of 2 independent experiments, with data bars showing the mean of duplicates +/- SEM.

These data corroborate reports of the ability of SAA to bind to various different cell surface receptors [56]. Importantly, we show that SAA-induced signalling via different receptors give rise to the induction of different cytokines **(Fig. 3.4.1, 3.4.2)**. Analysis of differential expression levels of these receptors in different cell types could contribute to our understanding of the differences in the quality and variety of cytokine responses elicited by SAA.

3.5 Maturation of IL-1β by SAA is dependent on the NLRP3 inflammasome

Having identified TLR2 as the receptor responsible for SAA-mediated pro-IL-1β induction, we next investigated the molecular mechanism responsible for induction of IL-1β maturation.

We firstly tested whether ROS production was required for SAA-mediated IL-1β cleavage. To do this in human cells, we used the THP-1 cell line, which were stimulated with SAA in the presence of 200μM butylated hydroxyanisole (BHA), a free radical scavenger. As BHA was dissolved in 100% EtOH, an equivalent volume of 100% EtOH only was added to control samples. Analysis of the cell supernatant by ELISA and cell lysates by immunoblotting revealed that BHA inhibited the secretion of mature IL-1β **(Fig. 3.5.1a, b)**. Importantly, secretion of cleaved caspase-1 was also undetectable in the presence of BHA, suggesting sequestration of ROS abolished activation of caspase-1 and subsequent IL-1β cleavage. In addition, we checked the toxicity of BHA on THP-1 cells by flow cytometry, using propidium iodide (PI), a DNA intercalating agent, to stain dead cells **(Fig. 3.5.1c)**. We found that addition of BHA had only a small toxicity effect as compared to addition of the solvent alone.

Having established the role of ROS in SAA-mediated IL-1β cleavage, next we tested whether K^+ efflux was required. To do this, we performed stimulations in cell medium containing a high concentration (150mM) of KCl, thereby abolishing the K^+ gradient required for efflux out of the cell. As a control, we used the same concentration of NaCl, containing another ion with a single positive charge. We observed the abolishment of IL-1β maturation when K^+ efflux was inhibited **(Fig. 3.5.1d)**; however in the presence of Na^+ this remained unaffected.

The requirement of both ROS and K^+ efflux for SAA-mediated IL-1β cleavage led us to investigate the involvement of the NLRP3 inflammasome. Whilst the exact mechanism of activation of this inflammasome remains unclear, ROS generation [116, 266, 267] and K^+ efflux [113, 117] are two of various pathways which have been postulated, others being disruption of lysosomes, and direct binding of an unidentified ligand to the LRR of NLRP3 [109]. Given the ability of both ROS and K^+ efflux to control inflammasome activation by NLRP3 activators, it is unlikely that these mechanisms are mutually exclusive.

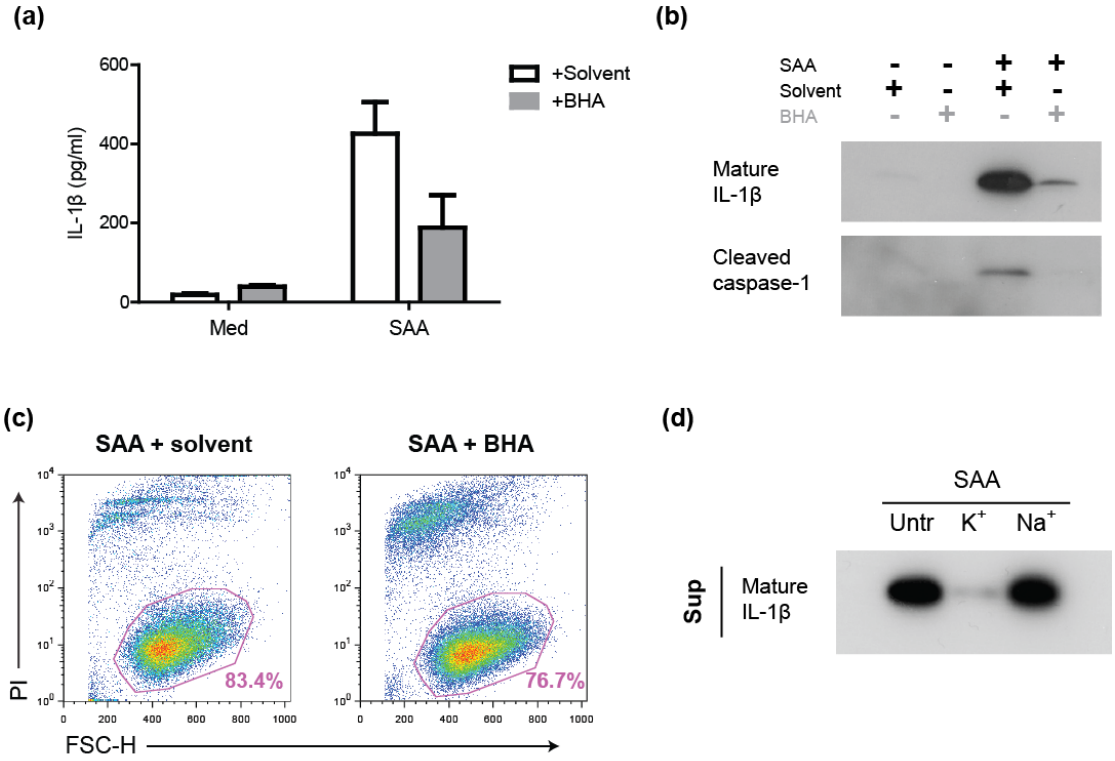

Fig. 3.5.1 Inhibition of ROS or K^+ efflux abolishes SAA-induced IL-1β cleavage. Human THP-1 cells were stimulated with 1µg/ml SAA for 24 hours in the presence of 200µM BHA (a ROS scavenger) dissolved in EtOH or an equivalent volume of EtOH alone (solvent). Supernatants were then analysed by ELISA **(a)** or immunoblotting **(b)** and toxicity checked by propidium iodide staining and flow cytometry **(c)**. **(d)** THP-1 cells were stimulated with SAA in the presence of 150mM KCl to block K^+ efflux, or NaCl as a control. Proteins in the cell supernatant were precipitated before analysis by immunoblottting. Data are representative of 3 independent experiments, with data bars showing the mean of duplicates +/- SEM.

We started by confirming the requirement of SAA for caspase-1 in IL-1β cleavage. Using BMDMs, we were able to show that cells derived from *Casp1*$^{-/-}$ animals were unable to cleave IL-1β into the mature cytokine, despite priming of pro-IL-1β being unaffected **(Fig. 3.5.2a, b)**. This was observed both with SAA and the caspase-1 dependent stimulus ATP.

Next we used BMDMs from mice deficient in NLRP3, ASC or NLRC4 to test the requirement of SAA for components of the NLRP3 inflammasome. Analysis of the cell supernatant by ELISA revealed that whilst *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ cells failed to secrete mature IL-1β in response to SAA, *Nlrc4*$^{-/-}$ cells were still able to process and secrete IL-1β **(Fig. 3.5.2c)**. As expected, a similar pattern was observed when LPS/ATP were used as the stimulus, with NLRP3 and ASC, but not NLRC4, being required. Immunoblotting of cell lysates from the same experiment revealed that priming of pro-IL-1β remained intact in all the cells, regardless of genotype **(Fig. 3.5.2e)**. This suggests that the defect in IL-1β

production from *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ BMDMs occurs downstream of pro-IL-1β induction, namely at the level of cleavage. We also verified that SAA was able to induce IL-10, an inflammasome-independent cytokine, from cells of all genotype **(Fig. 3.5.2d)**. Taken together, these data suggest that SAA utilises the NLRP3 inflammasome to provide signal 2, the cleavage of pro-IL-1β into the bioactive cytokine.

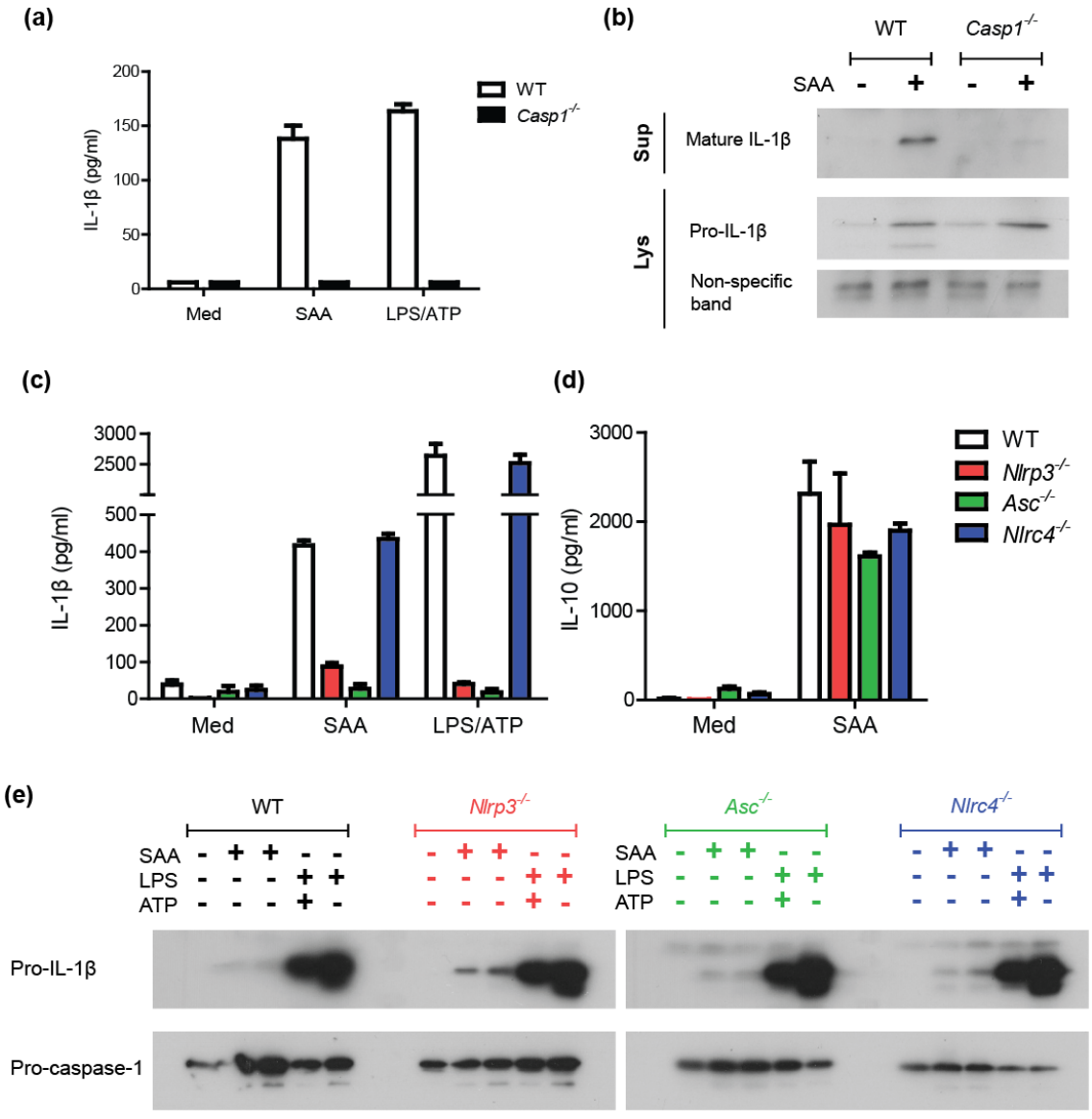

Fig. 3.5.2 Induction of bioactive IL-1β release is dependent on the NLRP3 inflammasome. BMDMs from C57BL/6 (WT) or mice deficient in components of the NLRP3 inflammasome, or NLRC4, were stimulated with SAA (1μg/ml) for 24hrs or LPS (10ng/ml) for 16hrs followed by ATP (5mM) for 30min. Cell supernatant and lysates were analysed by ELISA **(a, c, d)** or immunoblotting **(b, e).** Data are representative of 2 independent experiments, and bars show the mean of triplicates +/- SEM.

3.6 IL-1β induction by SAA *in vivo*

Our finding that SAA is able to provide both signals for IL-1β production *in vitro* prompted us to investigate whether SAA could be used to induce IL-1β production and its associated inflammatory effects *in vivo*.

A previous report has demonstrated that subcutaneous injection of SAA every 24 hours leads to systemic neutrophilia in mice, which is abolished in TLR2-deficient animals [268]. The authors linked SAA administration to TLR2-dependent G-CSF production, which led to an increase in the number of neutrophils in the peripheral blood. In addition, signalling through IL-1R1 is essential in reactive neutrophilia in response to alum injection, with G-CSF production in this model found to be dependent on IL-1R1 signalling [161, 269]. With our observation that SAA can induce pro-IL-1β expression via TLR2, we wanted to investigate whether the neutrophilia seen upon SAA administration is dependent on IL-1R1 signalling.

WT C57BL/6 or *Il1r1*$^{-/-}$ mice were injected subcutaneously with 2.4µg SAA in the right flank every 24 hours. Blood was drawn from the tail vein at days 0 (shortly before the 1st injection), 8 and 10 after the 1st injection. After lysis of erythrocytes, peripheral blood cells were analysed by flow cytometry to determine the percentage of neutrophils (CD11b^{+}Ly6G^{+} cells) in the blood **(Fig. 3.6.1a)**. We observed a significant expansion of neutrophils in WT mice 8 days after the 1st injection, with neutrophil numbers decreasing at day 10 **(Fig. 3.6.1b)**. However, in IL-1R1-deficient animals there was no significant increase in the percentage of CD11b^{+}Ly6G^{+} cells at day 8 as compared with day 0, suggesting SAA-induced neutrophilia is dependent on IL-1R1 signalling.

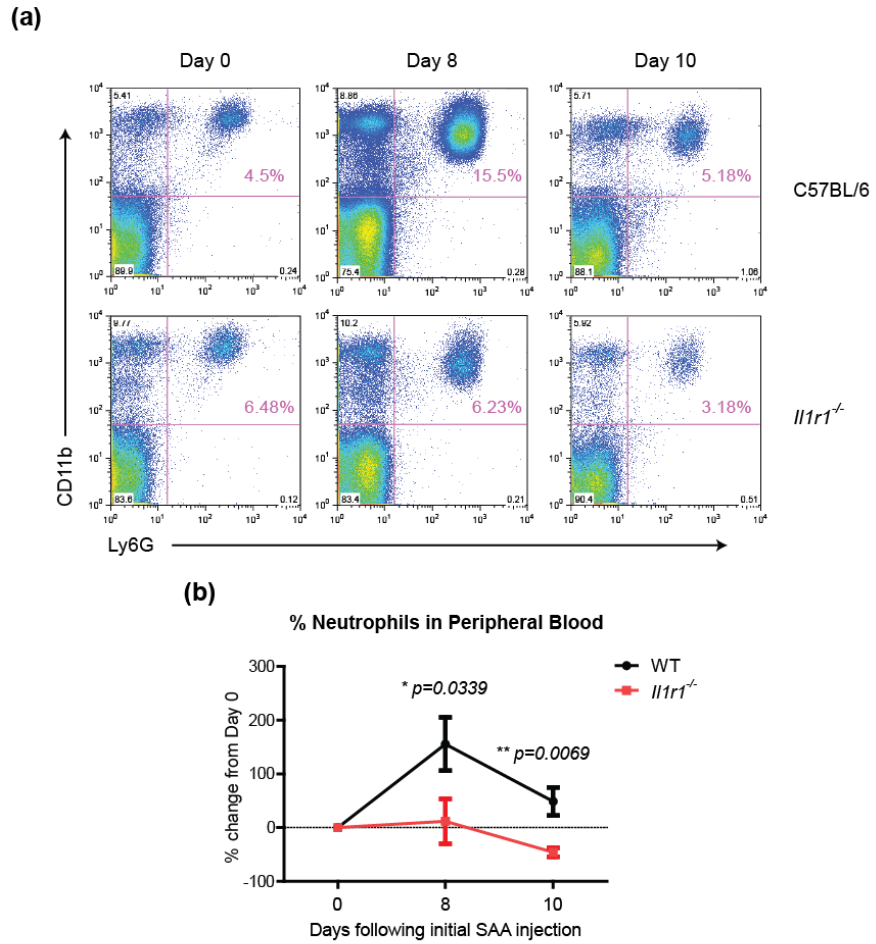

Fig. 3.6.1 Subcutaneous administration of SAA leads to blood neutrophilia in an IL-1R-dependent manner. C57BL/6 or IL-1R1-deficient mice were injected daily with 2.4µg SAA subcutaneously. At days 0, 8 and 10 blood was analysed by flow cytometry. **(a)** Representative flow cytometry plot of peripheral blood cells, stained for CD11b and Ly6G to determine the percentage of neutrophils. **(b)** The percentage change in total neutrophil counts ($CD11b^{+}Ly6G^{+}$) compared to day 0 were calculated. Data represent the mean of 4 animals per group +/- SEM. Statistical analysis was performed using Student's t test.

Following from these observations from prolonged administration of SAA, we decided to extend our results to a model of acute inflammation. Unlike the model of subcutaneous injection, we administered one single, high dose of SAA to a physiological site where it would remain concentrated for a longer period. With these considerations in mind, we instilled SAA into the airways of WT mice. SAA was administered intranasally (10µg in 20µl PBS), with control animals receiving the same volume of PBS alone. Four hours later, broncho-alveolar lavage (BAL) was performed to wash the airways with 1ml PBS, before the BAL fluid (BALF) was analysed for cytokine and cellular content. We found a modest but consistent increase in the level of IL-1β in the BALF after SAA instillation **(Fig. 3.6.2a)**, suggesting

that SAA was able to induce IL-1β secretion *in vivo*. In addition, we counted the number of cells in the BALF by haemocytometer, and found that SAA significantly increased in the number of cells infiltrating the airways **(Fig. 3.6.2b)**. Further analysis of the cellular content via flow cytometry revealed an increase in both the percentage and absolute number of neutrophils ($CD11b^+Ly6G^+$ cells) in the BALF **(Fig. 3.6.2c, d)**, indicating that, like subcutaneous injection, SAA is able to induce neutrophilia upon *in vivo* administration.

These experiments demonstrate the ability of SAA to induce the secretion of IL-1β *in vivo*. This suggests that increases in the levels of this protein during the acute phase response could contribute to the production of IL-1β and drive a self-amplifying loop to establish and prolong the inflammatory state.

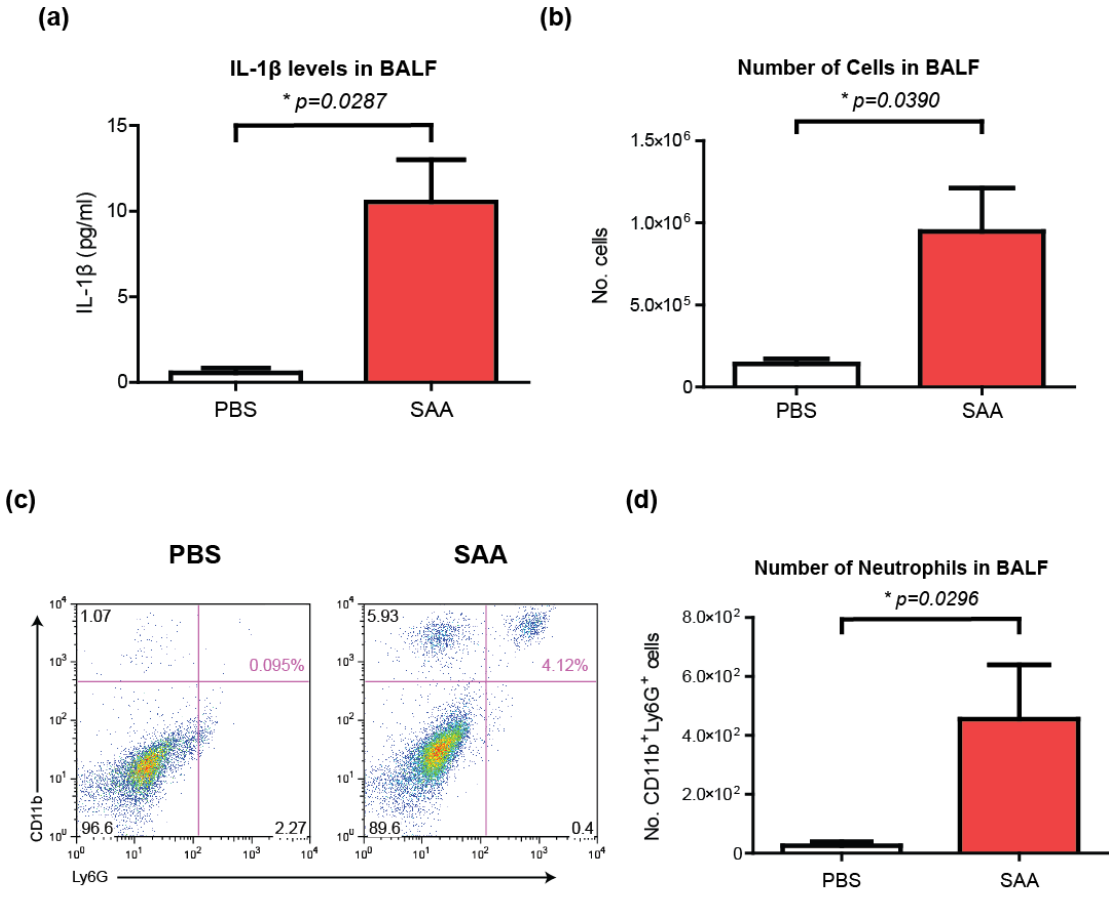

Fig. 3.6.2 Intranasal administration of SAA results in local IL-1β release and neutrophil recruitment. C57BL/6 mice were given 10μg SAA (or an equivalent volume of PBS) per animal intranasally. 4hrs later, the animals were sacrificed and BALF collected. **(a)** IL-1β levels were determined by ELISA. **(b-d)** Total cell number in the BALF was counted before analysis by flow cytometry and % neutrophils ($CD11b^+Ly6G^+$) calculated. Data are representative of 3 independent experiments, each with n=4. Data bars show the mean +/- SEM. Statistical analysis was performed using Student's t test.

3.7 Discussion and future perspectives

3.7.1 Receptor usage by SAA

Our finding that SAA can directly induce IL-1β synthesis and inflammasome activation adds to the complexity of SAA biology. Our observation of the requirement for TLR2 in NF-κB-mediated pro-IL-1β induction is consistent with its ability to act as a sensor for lipoproteins. Given its ability to form heterodimers with TLR1 or TLR6, which show different preferences for TLR2 ligands [12], it would be interesting to investigate whether TLR1 or TLR6 are also involved in SAA-mediated induction of pro-IL-1β. Indeed, a report identifying TLR2 as a functional receptor for SAA observed that co-expression of TLR1 and TLR2, but not TLR6, in HeLa cells could enhance NF-κB activation by SAA as compared to expression of TLR2 alone, suggesting that a TLR1-TLR2 heterodimer could be a functional receptor for SAA [264]. In fact, our results have been confirmed in a study where similar analyses were performed using blocking antibodies on human macrophages to elucidate the requirements of SAA-induced IL-1β secretion [270]. In this study, the authors reported that a combination of TLR2 and TLR4 signalling were required for the induction of pro-IL-1β transcription by SAA.

In our *in vitro* experiments, the finding that FPR2 was not required was perhaps more surprising, given the ability of FPR2 to also activate the NF-κB pathway [56]. This observation must be interpreted in the context of FPR2 being a pleiotropic receptor capable of inducing the production of both pro- and anti-inflammatory cytokines [63]. Despite being able to bind FPR2, different agonists appear to be able to activate different signalling pathways. For example, whereas both the lipoxin LXA_4 and SAA are able to bind FPR2, only LXA_4 is able to induce phosphorylation of STAT3 in macrophages [271]. This and other observed differences could be due to LXA_4 and SAA interacting with different domains of FPR2, with SAA (and not LXA_4) requiring the glycosylated extracellular loops of the receptor [56]. Nevertheless, SAA activation of FPR2 has been shown to activate the NF-κB pathway and the secretion of IL-8 and MMP-3 [272]. The exact mechanism of NF-κB activation downstream of FPR2 remains unclear, although ROS production and MAPK pathways have been implicated, pointing to a non-classical pathway [271]. Activation of the NF-κB pathway via extracellular TLRs and TRAF6 (the classical pathway), and receptors of the TNFR superfamily and NIK (the alternative pathway) have been shown to give rise to different active NF-κB dimers, with the classical pathway commonly yielding the p50/p65 heterodimer, and the alternative pathway producing p52/RelB heterodimers [273]. In this

respect, the different intracellular pathways downstream of TLR2 and FPR2 could conceivably induce different NF-κB proteins, with TLR2 favouring the p50/p65 heterodimer which drives pro-IL-1β transcription [74]. In addition, SAA activation of FPR2 induces PI(3)K, ERK and p38 phosphorylation, which are essential for the induction of IL-10 by SAA [62]. In the context of our data, the decrease in IL-10 (a suppressor of pro-IL-1β induction) in the supernatant of *Fpr2*$^{-/-}$ cells could also mask potential defects in IL-1β induction by relieving suppression.

Our observation that the NLRP3 inflammasome is required for SAA-mediated IL-1β maturation was also confirmed in another study [270]. With the exact mechanism of NLRP3 activation still unclear, the authors also identified the purinergic P2X7 receptor and cathepsin B activity as important in SAA-signalled inflammasome activation. Despite P2X7R being key in the activation of NLRP3 by ATP, the involvement of ATP in the action of SAA was excluded, with a direct action of SAA on P2X7R postulated. However, as yet no studies have shown direct binding of SAA to P2X7R; in fact studies demonstrating the anti-apoptotic effect of SAA in neutrophils, although discovered to be dependent on a P2X7R-sensitive pathway, found no ligand selectivity in the action of P2X7R [274]. It was therefore concluded that P2X7R was not a *bona fide* receptor for SAA. The discovery that cathepsin B was involved in bridging SAA signalling and NLRP3 activation is reminiscent of the mechanism shown for amyloid-β, with phagocytosis of the fibrillar deposits causing lysosomal damage and cathepsin B release, leading to inflammasome activation [128]. However, the formation of SAA fibril formation was also excluded as cathepsin B was shown to remain inside the lysosomes upon SAA stimulation, as well as the inhibition of phagocytosis with cytochalasin D being dispensable. These results suggest that SAA remains in the soluble phase when activating the NLRP3 inflammasome, and further study is required to determine the exact molecular mechanism linking SAA and IL-1β cleavage.

3.7.2 SAA induction of IL-1R1-dependent neutrophilia

One physiological application of our findings is the IL-1R1-mediated recruitment of neutrophils in response to SAA administration. Our data in the lung was confirmed in models of allergic asthma and acute lung injury in mice, induced by intranasal SAA delivery [275, 276]. Similar to our findings, TLR2- and NLRP3 inflammasome-dependent IL-1β was seen in the BALF accompanying neutrophilia. Despite the ability of SAA to directly induce chemotaxis [265], possibly via the production of chemokines and G-CSF [263, 268], the dependence on IL-1R1 signalling suggests an indirect

mechanism. IL-1R1 signalling is important in driving the expression of various chemokines [277] as well as the expansion of the haematopoietic stem cell compartment, especially granulocyte/macrophage progenitors, thus supporting emergency granulopoiesis [161].

3.7.3 Chronic inflammation, SAA and IL-1β

To date, few endogenous, non-microbial stimulators of pro-IL-1β induction have been described, especially *in vivo*. This was especially noted in the case of alum-adjuvanted vaccines, which could induce bioactive IL-1β *in vivo*, despite its inability to stimulate pro-IL-1β production *in vitro*. The significant increase in plasma SAA levels during inflammation [278] gives SAA the potential to be a key player in inducing pro-IL-1β *in vivo*. In addition, elevated SAA levels during chronic inflammation or autoimmunity may also contribute to immunopathology. In fact, stimulation of human synovial fibroblasts with SAA as signal 1, followed by MSU as signal 2, thus mimicking the synovial environment in arthritic joints, successfully induces the secretion of bioactive IL-1β [279]. The association of SAA with atherosclerosis and its detection within atherosclerotic lesions [280] also coincides with increasing evidence of IL-1 signalling and inflammasome activation driving plaque formation [129].

The chronic upregulation of SAA in cancer, together with its secretion by tumour cells themselves [56], may also be significant. A role for inflammation and IL-1β is well described in tumorigenesis [162, 165]. SAA and IL-1β could reciprocally induce each other's expression, establishing a positive feedback loop which, in a chronic setting, could play an important role in maintaining the inflammatory environment required for tumour development. In addition, SAA and IL-1β could potentially attract MDSCs to the tumour site via their chemotactic properties and/or polarise inflammatory myeloid cells towards a suppressive (eg. IL-10-producing) phenotype, thus dampening the anti-tumour response [62, 168].

3.7.4 Future perspectives and therapeutic potential

Therapeutically, our findings, together with similar investigations in the literature, point to SAA as an avenue to enhance IL-1β-dependent immune responses. In particular, an SAA-induced model of allergic asthma was shown to induce IL-1-dependent IL-17 in the lungs, with SAA able to polarise Th17 cells *in vitro* [275, 281]. The scarcity of vaccine adjuvants licensed for use in the clinic, due to

concerns over safety, means that vaccination efficacy is low in the case of certain pathogens, including *M. tuberculosis* [282]. Given its Th17-inducing capacity, SAA could potentially be used to develop a novel adjuvant to enhance IL-17-mediated responses, with one significant advantage being the use of an endogenous protein and hence a decrease in the risk of inducing exacerbated immune activation and tissue damage. However, careful investigation will be required to address concerns over the potential of high local concentrations of SAA to form amyloid fibrils and initiate an amyloid A amyloidosis-like disease, as well as the induction of various anti-inflammatory cytokines, especially high levels of IL-10, which could polarise the ensuing immune response towards a tolerogenic phenotype.

In summary, these data add to our understanding of the pleiotropic effects mediated by SAA. Further study of its role in initiating and potentiating pro-inflammatory responses may lead to potential developments in its use in a therapeutic setting. In addition, advances in our understanding of the various receptors utilised by SAA and their intracellular signalling pathways may enable us to target the secretion of specific cytokines detrimental to the host in certain circumstances, whilst preserving the induction of beneficial responses.

CHAPTER 4

Modulation of IL-1β Production in Human Dendritic Cells by Invariant Natural Killer T Cells

Chapter 4: Modulation of IL-1β Production in Human Dendritic Cells by Invariant Natural Killer T Cells

With their ability to play both pro- and anti-inflammatory roles, iNKT cells are important modulators of the immune response. Their ability to recognise self ligands presented onto the CD1d molecule gives them the unique ability to react at a basal level to resting APCs.

IL-1β is a key inflammatory cytokine which drives the innate response upon recognition of PAMPs by myeloid cells. In particular, IL-1β is produced in high levels by APCs such as monocytes/macrophages and DCs. Its importance is reflected in its role in controlling infection. However, its association with autoinflammatory diseases also points to a detrimental effect on the host if its production becomes dysregulated.

In this chapter, we will investigate whether the interaction between iNKT cells and DCs can result in modulation of IL-1β production. In particular, we will use *in vitro* assays to examine the crosstalk between human iNKT cells and MdDCs, and the effect this has on IL-1β synthesis. We will also mimic the activity of the iNKT cells by using recombinant molecules expressed by iNKT cells, and, in doing so, study the mechanism by which iNKT cells can influence IL-1β release in DCs. Our results will be significant in identifying a novel role of iNKT cells in controlling the inflammatory process.

4.1 Modulation of IL-10 production by iNKT cells

In Chapter 3, we have discussed the ability of SAA to induce bioactive IL-1β production from myeloid cells **(Fig. 3.2.1)**. In addition, we showed that other cytokines, such as IL-10, were also induced by SAA, via a different molecular pathway **(Fig. 3.3)**. This supports other published data from our laboratory, which also suggests an anti-inflammatory role for SAA, by inducing IL-10 secretion from neutrophils [62]. Interestingly, the addition of iNKT cells could reverse this anti-inflammatory phenotype by reducing IL-10 secretion. We therefore extended our observations using SAA and investigated whether iNKT cells could also modulate IL-10 production in human MdDCs. We found that, similar to neutrophils, SAA induced crosstalk between iNKT cells and MdDCs, leading to IFN-γ secretion **(Fig. 4.1a)**. In addition, co-incubation with iNKT cells led to a reduction in SAA-induced IL-10 release, implying that iNKT cells could modulate cytokine secretion in MdDCs **(Fig. 4.1b)**.

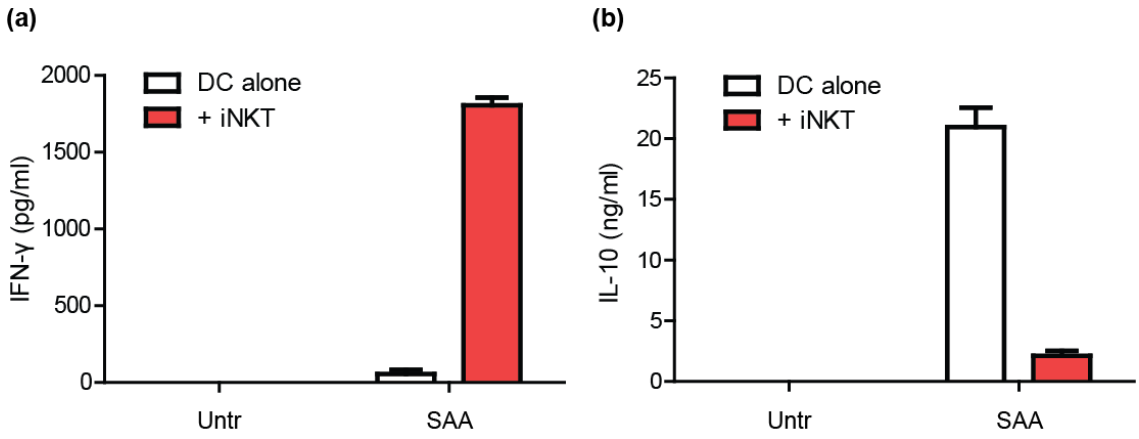

Fig. 4.1 iNKT cells decrease IL-10 secretion from DCs. Human MdDCs were pre-incubated with iNKT cells at a ratio of 3:1 for 14 hours prior to stimulation with 1μg/ml SAA for 24 hours. IFN-γ **(a)** and IL-10 **(b)** levels in the cell supernatant were quantified by ELISA. Data are representative of experiments performed with MdDCs from at least 2 donors. Data bars show the mean of duplicates +/- SEM.

4.2 Invariant natural killer T cells decrease IL-1β production by DCs

iNKT cells are known to have both pro- and anti-inflammatory properties, depending on the experimental system in question [217]. Their ability to crosstalk with antigen presenting cells [236, 243], as well as secrete high levels of both pro- and anti-inflammatory cytokines upon stimulation, endows them with the ability to coordinate and shape the immune response. In addition, modulation of IL-1β could have important consequences for both innate and adaptive immunity. Consequently, we investigated whether the synthesis of IL-1β could also be affected by interaction with iNKT cells.

Prior to induction of IL-1β synthesis, we co-incubated human MdDCs with human iNKT cells for 12-14 hours, in the absence of exogenous iNKT cell ligands. This allowed iNKT cells to crosstalk with MdDCs using their basal autoreactivity, the recognition of basal levels of self-lipids presented on CD1d. We performed this pre-conditioning step to mimic the physiological interaction of iNKT cells with immature DCs in the resting, uninfected state. As stimuli for IL-1β production, we used the non-microbial compound SAA or UV-killed whole bacteria, both of which are able to provide both signals 1 and 2 for bioactive IL-1β secretion. Significantly, after co-incubation with iNKT cells, we found that stimulation of MdDCs with SAA or bacteria (both the gram positive *Enterococcus fecalis* and the gram negative *Klebsiella atlantae*) led to decreased levels of IL-1β secretion **(Fig. 4.2.1)**. This implies that, during initial iNKT cell-DC interaction, the DCs are pre-conditioned to release lower levels of IL-1β

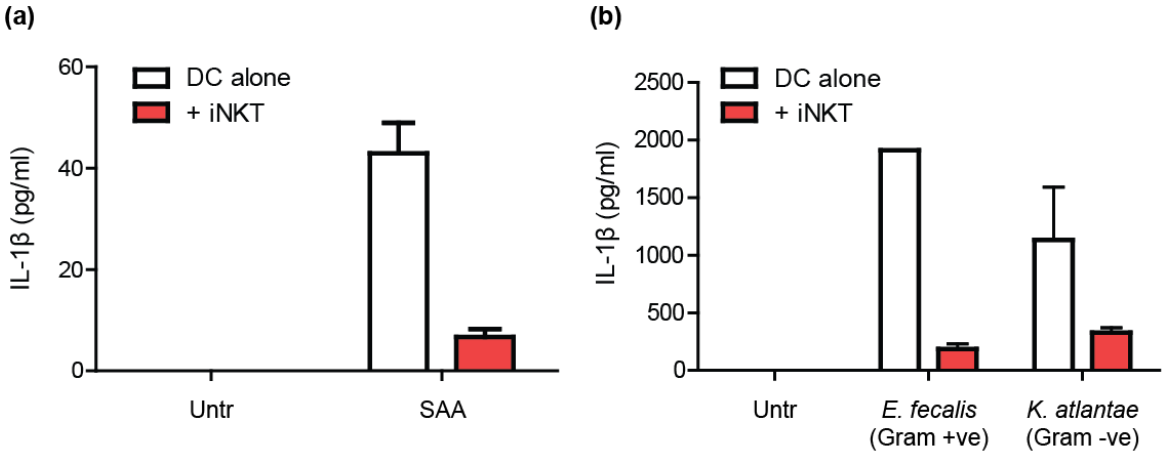

Fig. 4.2.1 Pre-conditioning by iNKT cells dampens the release of IL-1β from human MdDCs. Human MdDCs were incubated for 12-14 hours with human iNKT cells at a ratio of 3:1. SAA at 1μg/ml **(a)** or UV-killed bacteria at an m.o.i. of 50 **(b)** were then added to stimulate IL-1β synthesis and cleavage. 24 hours later, cell supernatants were harvested and analysed for IL-1β levels by ELISA. Data are representative of over 5 independent experiments, each performed with cells from a different donor. Data bars show the mean of triplicates +/- SEM.

upon further stimulation. This gives iNKT cells a novel anti-inflammatory property. Importantly, the production of IL-12 (both the p40 subunit and the active heterodimer p70) was not decreased by iNKT cells in the same experiments **(Fig. 4.2.2)**; in contrast the presence of iNKT cells further enhanced IL-12 levels, as has been previously described [283]. As a control, we also verified that iNKT cells were not able to produce IL-1β or IL-12 when stimulated with SAA or bacteria without MdDCs (data not shown). In addition, strong activation of iNKT cells can elicit IL-12 production from DCs in the absence of microbial stimulation, via the engagement of CD40/CD40L and the secretion of IFN-γ. We reproduced this by the addition of α-GalCer to potently activate iNKT cells and were able to show that this alone is sufficient to elicit IL-12 secretion from DCs.

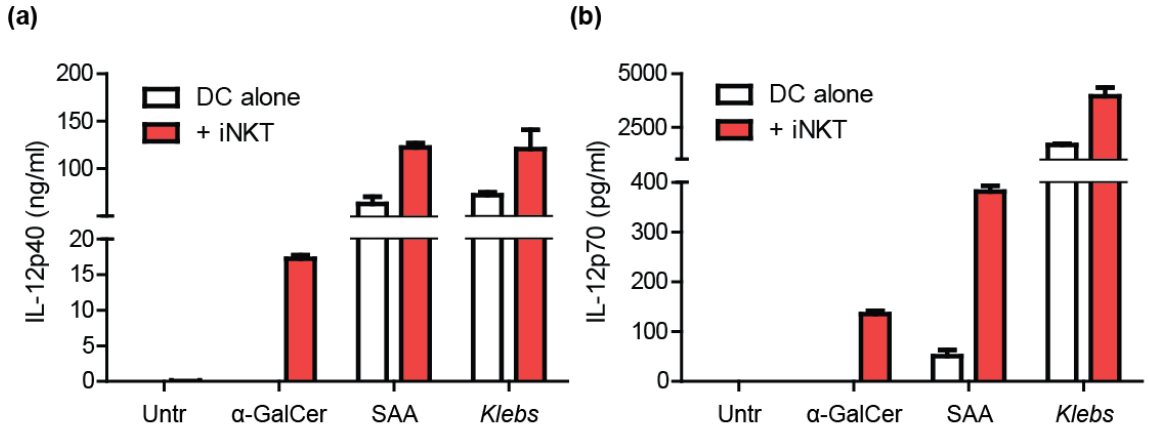

Fig. 4.2.2 Enhancement of IL-12 production by human MdDCs in the presence of iNKT cells. Human MdDCs were pre-conditioned with iNKT cells at a ratio of 3:1 for 14 hours, before stimulation with α-GalCer (2ng/ml), SAA (1μg/ml) or UV-killed *Klebsiella* (m.o.i. 50) for 24 hours. Cell supernatants were then harvested and analysed by ELISA for levels of IL-12p40 **(a)** and IL-12p70 **(b)**. Data are representative of at least 3 independent experiments, performed using cells from different donors. Bars show the mean of duplicates +/- SEM.

To exclude the possibility that IL-1β reduction was due to a decrease in the total capacity of DCs to respond to stimulation, we checked that DC maturation was unhindered, by looking at the upregulation of CD80, CD83 and CD86 by flow cytometry **(Fig. 4.2.3a)**. Mirroring IL-12 production, we found that iNKT cells enhanced the maturation of DCs in response to SAA and *Klebsiella atlantae*. This is in agreement with previous reports that iNKT cells can further increase the maturation of DCs in the presence of other stimuli [284], and shows that, despite inhibition of IL-1β, iNKT cells were not negatively affecting other aspects of the DC response. By enhancing DC maturation, iNKT cells have also been reported to decrease the phagocytic ability of DCs. Therefore, pre-conditioning of DCs with

iNKT cells could conceivably decrease their ability to uptake UV-killed bacteria, hence leading to a decrease in IL-1β secretion. Our observation that IL-1β elicited by SAA, a stimulus which does not require uptake, was also dampened implies that this is not applicable in our system. Nevertheless, this was verified by labelling *Klebsiella* using the fluorescent dye CFSE prior to UV-killing. These bacteria were then incubated with MdDCs (unconditioned or conditioned by iNKT cells) and internalisation of the bacteria assessed by flow cytometry. As a control, we also incubated labelled *Klebsiella* with MdDCs on ice. Under this condition, uptake by DCs is halted, showing the background level of CFSE fluorescence from labelled bacteria adhering to the cell surface. Crucially, we found no difference in the CFSE fluorescence intensity between unconditioned DCs and those which had interacted with iNKT cells, confirming that the inhibition of IL-1β by iNKT cells is not a result of decreased phagocytic ability **(Fig. 4.2.3b)**.

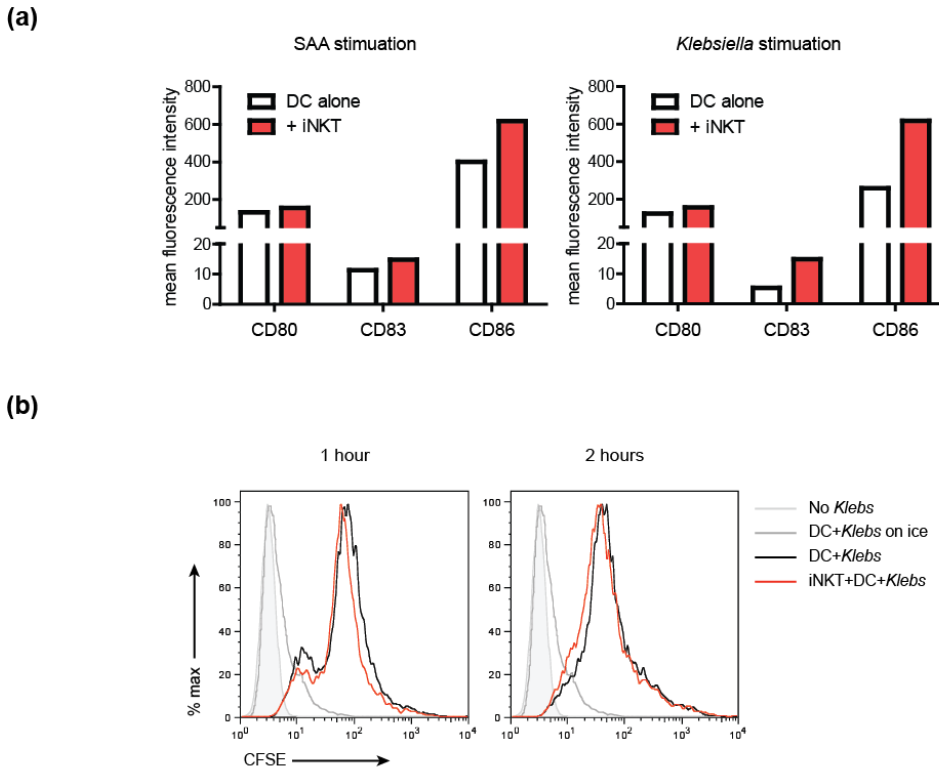

Fig. 4.2.3 DC maturation and phagocytic ability are not impaired by iNKT cells. Human MdDCs were conditioned for 12 hours with iNKT cells at a ratio of 3:1, before stimulation with SAA (1µg/ml) or UV-killed *Klebsiella atlantae* (m.o.i. 50) for 24 hours. Expression levels of the DC maturation markers CD80, CD83 and CD86 were assessed by flow cytometry. **(a)** Summary of flow cytometry data. **(b)** Unconditioned or iNKT cell-conditioned MdDCs were incubated with CFSE-labelled, UV-killed *Klebsiella* at 37˚C for 1 or 2 hours before analysis of phagocytic uptake by flow cytometry. As a control, MdDCs were fed UV-killed, labelled *Klebsiella* on ice. Data are representative of at least 2 independent experiments.

With bioactive IL-1β production separated into two steps, we then asked at which level iNKT cells were acting in order to dampen IL-1β release. A previous report in mouse macrophages has shown that activated and memory T cells are able to inhibit bioactive IL-1β synthesis by affecting NLRP1 and NLRP3 inflammasome activity [285]. With iNKT cells sharing various aspects of the activated, memory phenotype [217], we analysed IL-1β production by immunoblotting. Interestingly, we showed that iNKT cells decreased IL-1β production by reducing pro-IL-1β synthesis (signal 1) **(Fig. 4.2.4)**. Signal 1, as well as upregulating pro-IL-1β, is also important in priming signal 2 by inducing components of the NLRP3 inflammasome [98]. Therefore, our observation that iNKT cells inhibit signal 1 places the inhibitory signal at the apex of the IL-1β synthesis pathway, allowing iNKT cells to exert a powerful negative signal on IL-1β production.

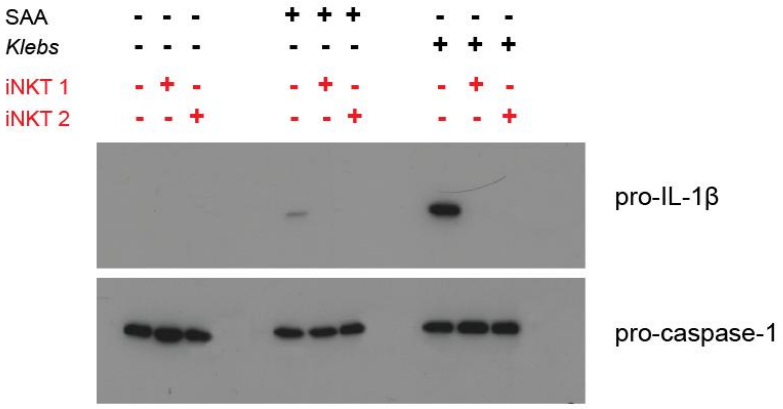

Fig. 4.2.4 iNKT cells dampen IL-1β production at the level of pro-IL-1β synthesis. Human MdDCs were incubated for 12-14 hours with 2 different lines of human iNKT cells at a ratio of 3:1. SAA at 1μg/ml or UV-killed *Klebsiella atlantae* at an m.o.i. of 50 were then added to stimulate IL-1β synthesis. 24 hours later, cells were harvested, lysed and analysed by immunoblotting. Pro-caspase-1 was used as a loading control. Data are representative of over 5 independent experiments, each performed with cells from a different donor.

Given the ability of conventional activated T cells to affect IL-1β, we verified that naïve T cells were unable to mediate a similar effect. Pre-conditioning of MdDCs with an equivalent number of naïve T cells from peripheral blood was not able to dampen subsequent IL-1β release **(Fig. 4.2.5)**. These data therefore highlight iNKT cells as a unique T cell subset which is capable of restraining IL-1β production in MdDCs, by inhibiting the accumulation of pro-IL-1β, the first step in IL-1β synthesis.

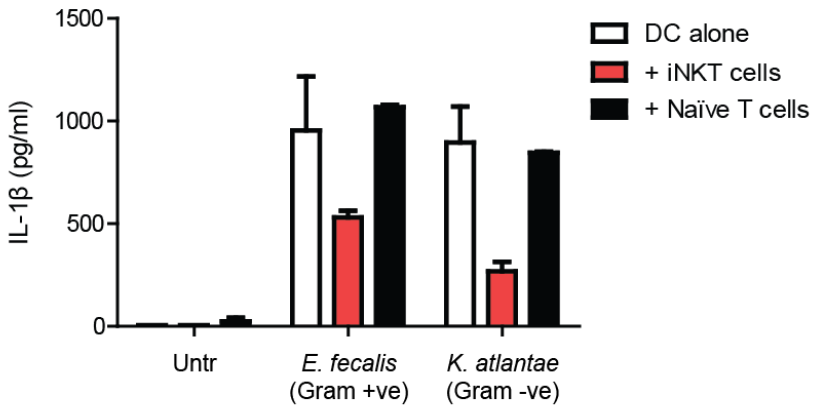

Fig. 4.2.5 Naïve T cells do not suppress IL-1β release by human MdDCs. Human MdDCs were pre-conditioned with iNKT cells or naïve T cells at a ratio of 1:3 for 14 hours. Cultures were then stimulated with UV-killed bacteria at an m.o.i. of 100 for 24 hours, before cell supernatants were analysed by ELISA. Data are representative of experiments performed using cells from 2 different donors. Data bars show the mean of duplicates +/- SEM.

Another unique property of iNKT cells is their self-reactivity – their ability to recognise self glycolipid in the context of CD1d. This endows them with a basal autoreactivity which allows them to recognise immature, unstimulated APCs displaying lipid-bound CD1d. After TLR stimulation, upregulation of CD1d-restricted self-ligands and cytokine secretion by APCs increase the level of signalling through the invariant TCR, thus increasing iNKT cell activation [236]. Similarly, addition of a high affinity iNKT cell ligand, such as α-GalCer, forces the interaction between iNKT cell and APC and results in powerful iNKT cell activation. Wang and colleagues have reported that basal autoreactivity leads to a specialised activation state [216]. With pre-conditioning of DCs in our system relying on basal autoreactivity, we investigated whether the presence of α-GalCer would affect IL-1β inhibition **(Fig. 4.2.6a)**. Interestingly, pre-conditioning of DCs in the presence of α-GalCer did not dampen subsequent IL-1β induction; on the contrary IL-1β release was marginally increased. This was also associated with the release of IFN-γ by iNKT cells upon α-GalCer recognition **(Fig. 4.2.6b)**. With IFN-γ being absent during basal recognition, this correlates with the reported ability of IFN-γ to boost IL-1β production [286].

These observations therefore identify iNKT cells as a novel inhibitor of IL-1β production. Crucially, their unique basal autoreactivity leads to a specialised activation state which pre-conditions MdDCs and reduces IL-1β secretion upon subsequent stimulation.

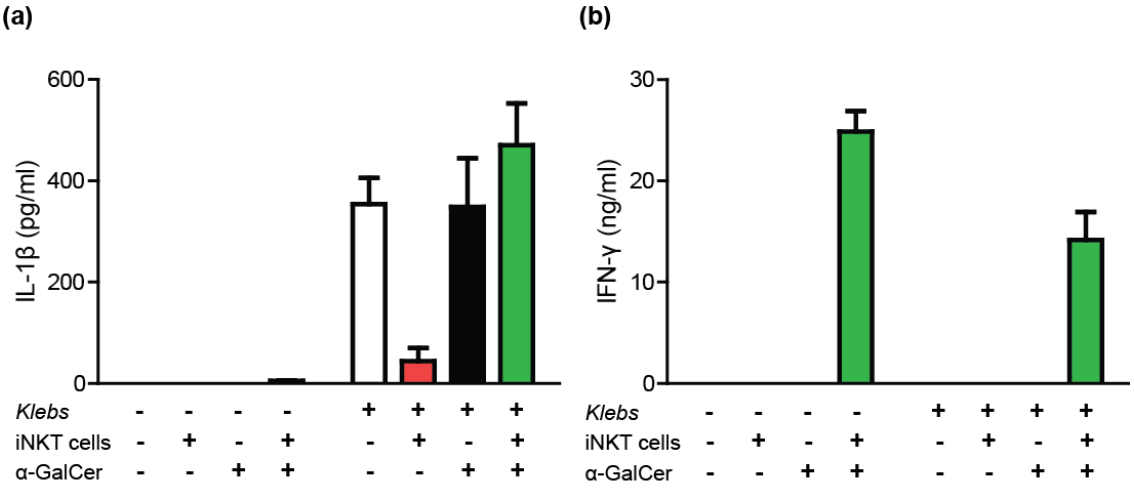

Fig. 4.2.6 Dampening of IL-1β requires iNKT cell-DC interaction via basal autoreactivity. Human MdDCs were pre-conditioned for 14 hours with iNKT cells at a ratio of 3:1 in the presence or absence of 2ng/ml α-GalCer. UV-killed *Klebsiella atlantae* was then added for 24 hours, at an m.o.i. of 50, before cell supernatants were collected and analysed by ELISA for IL-1β **(a)** and IFN-γ **(b)**. Data are representative of experiments performed using cells from 2 different donors. Bars show the mean of duplicates +/- SEM.

4.3 Inhibition of IL-1β production by CD40 ligand

Having established that iNKT cells are able to dampen IL-1β production in DCs, we proceeded to explore the mechanisms by which this is mediated. First, we considered molecules expressed on the iNKT cell surface which could interact with MdDCs. One of the best described ways by which iNKT cells can signal to DCs is via the CD40L-CD40 interaction [243]. CD40L is expressed on activated helper T cells and plays an important role in providing activatory signals by engaging CD40 on APCs.

Consistent with their activated, self-reactive phenotype, iNKT cells constitutively expressed CD40L as detected by flow cytometry **(Fig. 4.3.1a)**. This was rapidly upregulated upon activation using PMA and ionomycin, possibly via the mobilisation of preformed CD40L from the lysosomes to the plasma membrane [287, 288]. With CD40L also present physiologically as a soluble protein, we tested, by ELISA, whether CD40L could be shed by iNKT cells. We were unable to detect soluble CD40L (sCD40L) in the supernatant of iNKT cells co-cultured with MdDCs alone or stimulated by *Klebsiella* **(Fig. 4.3.1b)**, suggesting that iNKT cell-associated CD40L activity is exclusively membrane bound.

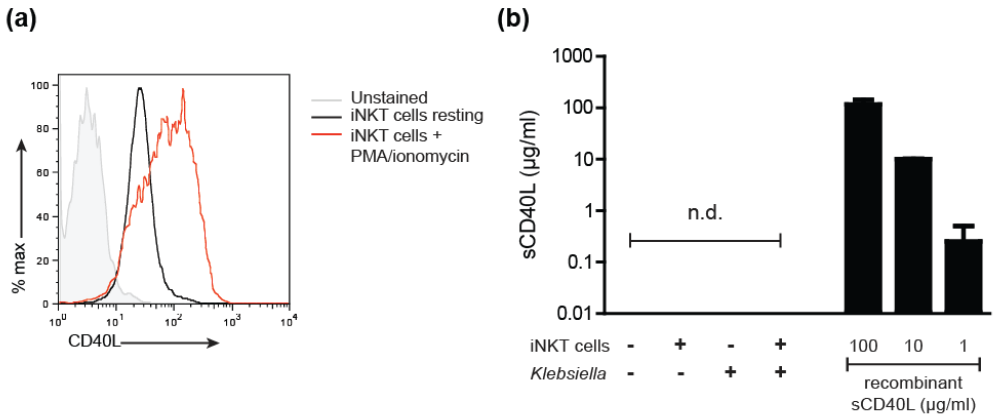

Fig. 4.3.1 Expression of CD40L on iNKT cells. (a) iNKT cells (resting and stimulated with PMA and ionomycin (1µg/ml) for 2 hours) were stained for CD40L expression and analysed by flow cytometry. Samples were gated on live cells by propidium iodide negativity. **(b)** Human MdDCs were co-incubated with iNKT cells at a ratio of 3:1 for 13 hours, before addition of *Klebsiella atlantae* at an m.o.i. of 50 for 24 hours. Cell supernatants were then tested for the presence of soluble CD40L by ELISA. Recombinant sCD40L was added at 3 different concentrations as a positive control (right 3 bars). Data are representative of 3 independent experiments with 2 different iNKT cell lines. Bars show the mean of duplicates +/- SEM.

To mimic the activity of iNKT cells, we then tested whether pre-conditioning MdDCs with sCD40L in place of iNKT cells could reproduce the inhibitory effect on IL-1β. Whereas recombinant sCD40L is produced as a monomer ($sCD40L_1$), physiological CD40L (membrane-bound and soluble) exists as a trimer, similar to other members of the TNF superfamily [191]. Significantly, higher-order multimers have been shown to trigger stronger CD40 signalling by enhancing receptor clustering [192]. We therefore compared the effects of $sCD40L_1$ or multimeric sCD40L ($sCD40L_m$) over a range of concentrations. $sCD40L_m$ was generated by the addition of an enhancer, an anti-FLAG antibody, to crosslink FLAG-tagged, monomeric sCD40L. We found that pre-conditioning of human MdDCs with higher concentrations of sCD40L decreased IL-1β secretion induced by subsequent SAA or *Klebsiella* stimulation **(Fig. 4.3.2a and b left panels)**, with the inhibition stronger when multimeric sCD40L was used. Furthermore, the effect titrated with the concentration of sCD40L. Mirroring the effect of iNKT cells, sCD40L enhanced IL-12p40 production induced by SAA and *Klebsiella*, with the stronger, multimeric sCD40L being more effective **(Fig. 4.3.2a and b right panels)**. Significantly, we also found that some concentrations of sCD40L alone (without further SAA or *Klebsiella* stimulation) were able to induce IL-12p40 expression in MdDCs **(Fig. 4.3.2c)**. In agreement with published data [193], high concentrations of $sCD40L_m$ were required. Our titrations revealed that 0.1 to 1μg/ml $sCD40L_1$ or 0.1μg/ml $sCD40L_m$ is sufficient to decrease IL-1β production by MdDCs, but insufficient to induce IL-12p40. In the context of iNKT cells, this suggests that levels of CD40L on the cell surface too low for inducing IL-12 (eg. in resting iNKT cells or iNKT cells activated by basal autoreactivity) may still be able to dampen IL-1β production.

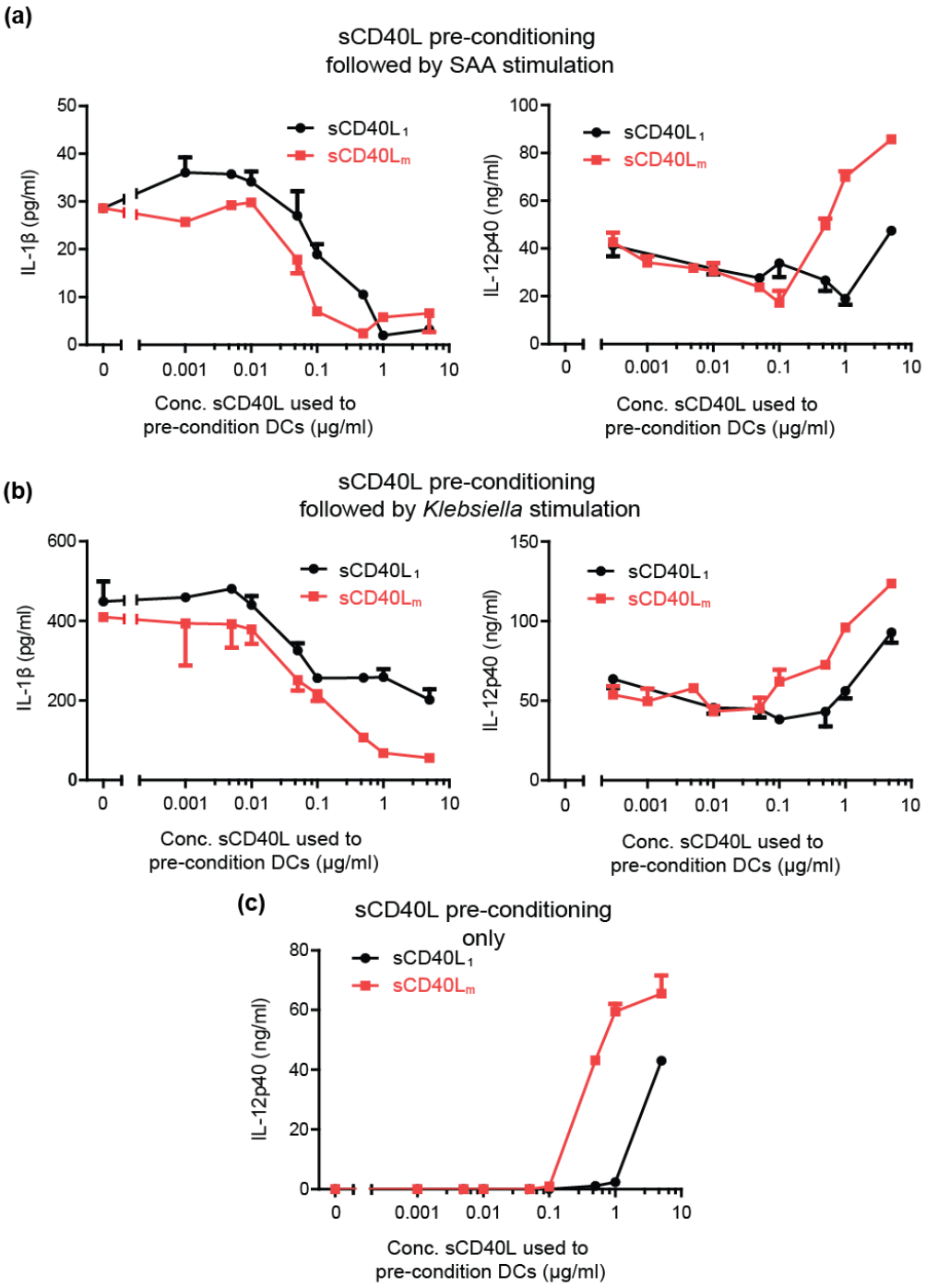

Fig. 4.3.2 Pre-conditioning of human MdDCs with soluble CD40L dampens IL-1β production. Human MdDCs were incubated for 16 hours with varying concentrations of monomeric ($sCD40L_1$) or multimeric ($sCD40L_m$) soluble CD40L, before stimulation with 1µg/ml SAA **(a)**, m.o.i. 50 UV-killed *Klebsiella* **(b)** or left unstimulated **(c)** for 24 hours. Supernatants were then analysed by ELISA for IL-1β and IL-12p40 levels. Data are representative of experiments performed using cells derived from 2 different donors. Points show the mean of duplicates +/- SEM.

The ability of sCD40L to mimic the effect iNKT cells in pre-conditioning MdDCs led us to study whether, like iNKT cells, inhibition was mediated at the level of pro-IL-1β. Analysis by immunoblotting revealed

that, consistent with the effect of iNKT cells, sCD40L reduced the accumulation of pro-IL-1β in SAA and *Klebsiella* stimulated cells **(Fig. 4.3.3)**.

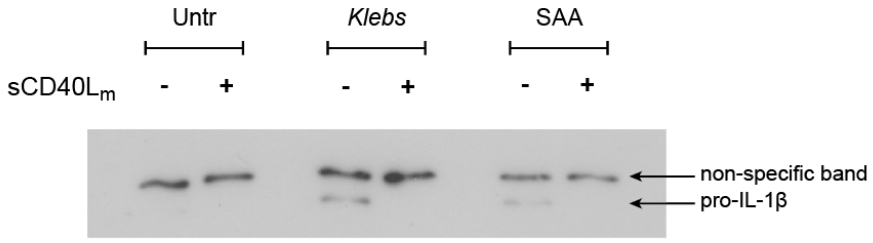

Fig. 4.3.3 sCD40L inhibits pro-IL-1β synthesis. Human MdDCs were pre-conditioned using 1μg/ml of soluble, multimeric CD40L for 14 hours, before further stimulation with 1μg/ml SAA or UV-killed *Klebsiella atlantae* (m.o.i. 50) for 24 hours. Cell lysates were then analysed by immunoblotting. Data are representative of over 3 independent experiments, each performed using cells derived from a different donor.

We have thus demonstrated that sCD40L, when used in place of basal autoreactive iNKT cells, can reproduce the inhibitory effect on IL-1β production, at the level of pro-IL-1β. As a result, we explored whether blocking CD40L on iNKT cells could rescue IL-1β synthesis, by including a neutralising antibody against CD40L during the iNKT cell-DC pre-conditioning step. Surprisingly, we were unable to rescue IL-1β production, although we observed a minor, but statistically insignificant trend towards increased IL-1β secretion, in the presence of the antibody **(Fig. 4.3.4a)**. The functionality of the antibody was verified by successfully neutralisation of the effect of recombinant sCD40L on IL-1β release **(Fig. 4.3.4b)**. In addition, we were able to block the secretion of IL-12p40 and IL-12p70 by MdDCs following iNKT cell activation by α-GalCer **(Fig. 4.3.4c, d)**, a process previously described to be CD40-dependent [283].

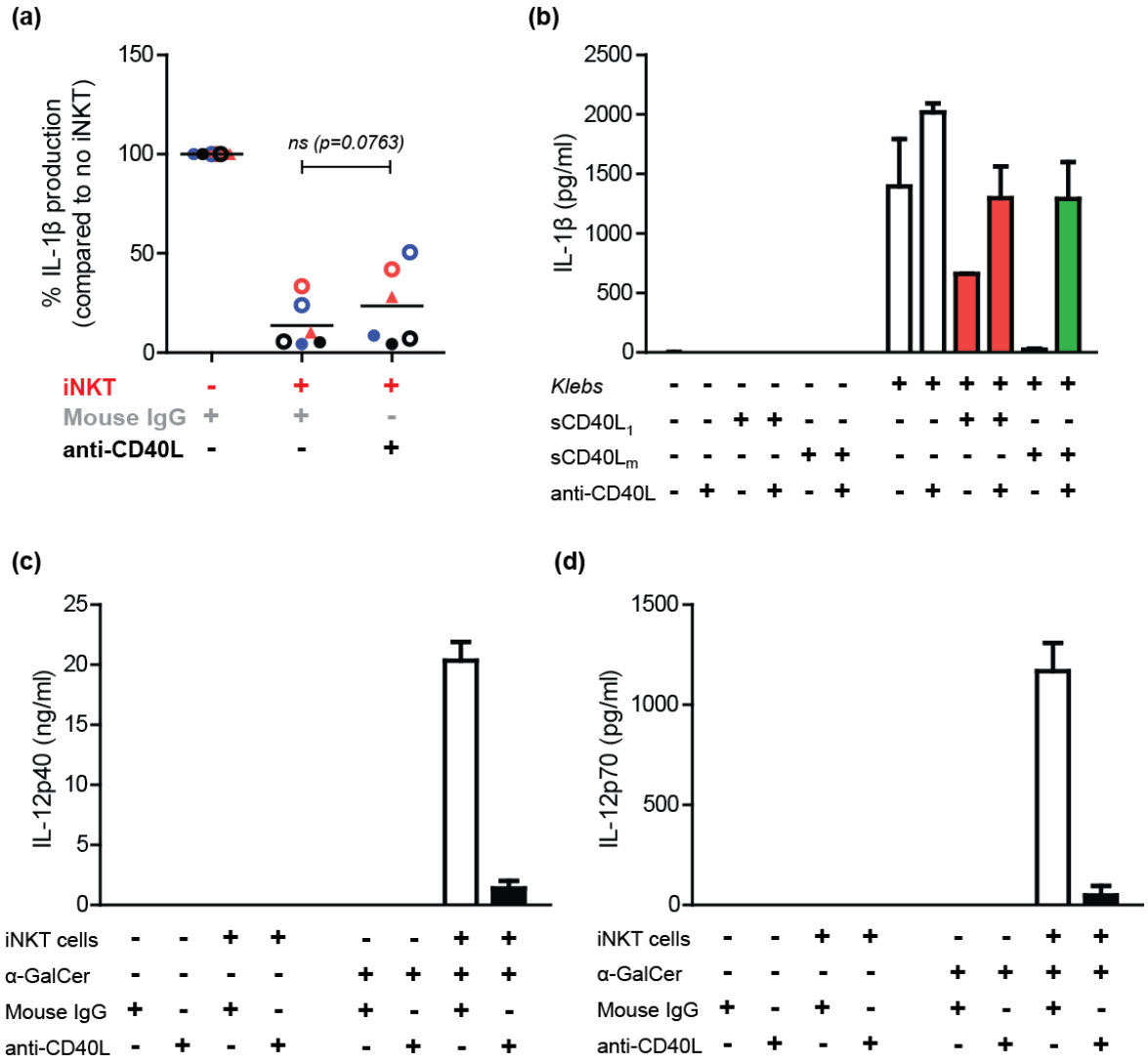

Fig. 4.3.4 Addition of anti-CD40L blocking antibody fails to rescue IL-1β production. **(a)** Human MdDCs were conditioned with iNKT cells in the presence of anti-CD40L blocking antibody or mouse IgG isotype control (10μg/ml) for 14 hours before stimulation with UV-killed *Klebsiella atlantae* for 24 hours. Cell supernatants were analysed by ELISA for IL-1β production. Results are presented as % maximum IL-1β production (with 100% as the "DC without iNKT cells" condition). Each set of data points represents one donor, and each colour represents one iNKT cell line. Statistical analysis was performed using Student's t test. **(b)** Human MdDCs were pre-conditioned with 1μg/ml soluble CD40L (monomer - $sCD40L_1$ or multimer - $sCD40L_m$) for 16 hours in the presence or absence of 10μg/ml anti-CD40L blocking antibody. Cultures were then stimulated for 24 hours with UV-killed *Klebsiella atlantae*. **(c, d)** Human MdDCs were cultured with iNKT cells at a ratio of 3:1 for 14 hours in the presence of 2ng/ml α-GalCer, with or without 10μg/ml anti-CD40L blocking antibody. Cell supernatants were then analysed by ELISA. (b-d) Data are representative of experiments performed using cells from at least 2 different donors. Bars show the mean of duplicates +/- SEM.

Although blockade of CD40-CD40L interactions did not have a significant effect in rescuing IL-1β production, the ability of sCD40L to accurately reproduce the effect of iNKT cells suggested that

CD40L may act in parallel with other factors. Therefore, we proceeded to investigate the role of soluble factors in the iNKT cell-mediated reduction of IL-1β synthesis.

4.4 Modulation of IL-1β production by iNKT cell-derived cytokines

Initially, we tested whether iNKT cells could still exert their inhibitory effect on MdDCs in the absence of cellular contact. As initial activation of iNKT cells by self-ligands on MdDCs is contact-dependent, we were unable to use a transwell system. As an alternative, we generated "basal autoreactivity supernatant", by co-incubating iNKT cells with MdDCs, mimicking the pre-conditioning step. Any soluble factors released by iNKT cells during pre-conditioning would therefore be retained. As a control, "DC supernatant" was generated by harvesting the supernatant of MdDCs cultured alone for the same length of time.

Basal autoreactivity supernatant was used to pre-condition fresh MdDCs in place of iNKT cells; this was followed by SAA or *Klebsiella* stimulation, as before, to induce IL-1β synthesis. Similar to iNKT cells, basal autoreactivity supernatant was also able to reduce IL-1β production **(Fig. 4.4.1a, b)**, implying that soluble factors derived from iNKT cells are also able to pre-condition MdDCs in the same way. Analysis by immunoblotting confirmed that basal autoreactivity supernatant dampened IL-1β by reducing pro-IL-1β **(Fig. 4.4.1c)**, again reproducing the effects of iNKT cells.

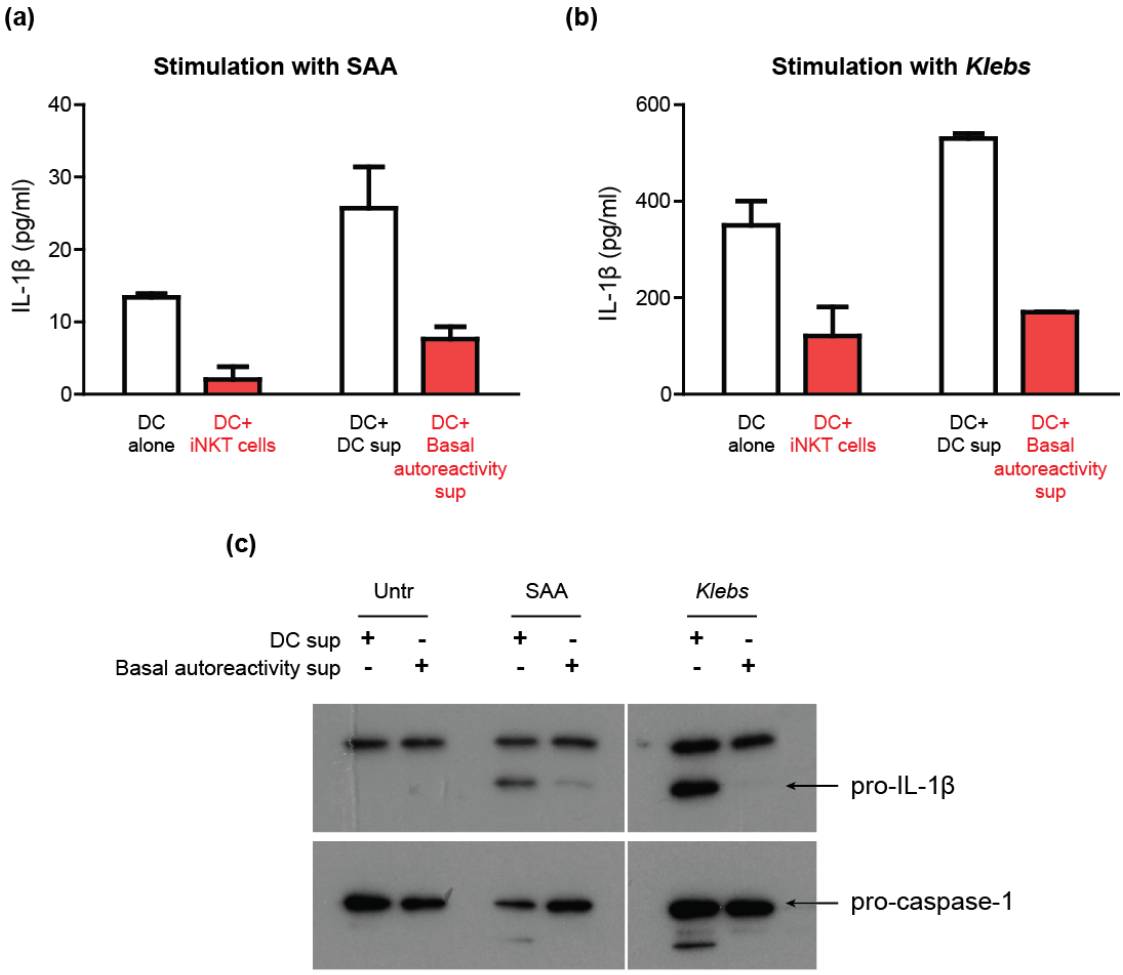

Fig. 4.4.1 Soluble factors secreted during iNKT-DC pre-conditioning can dampen IL-1β production. Human MdDCs were pre-conditioned with iNKT cells, DC supernatant or basal autoreactivity supernatant (at 2:1 ratio with complete medium) for 14 hours before stimulation with 1μg/ml SAA **(a)** or m.o.i. 50 UV-killed *Klebsiella atlantae* **(b)**. DC supernatant was generated by incubating human MdDCs in complete medium for 14 hours, and basal autoreactivity supernatant was generated by incubating human MdDCs with iNKT cells at a ratio of 3:1 for 14 hours. Data are representative of more than 3 independent experiments, with bars showing the mean of duplicates +/- SEM. **(c)** Human MdDCs were pre-conditioned using DC or basal autoreactivity supernatant (at 2:1 ratio with complete medium) for 12 hours before stimulation with SAA or *Klebsiella* as above. Cell lysates were analysed by immunoblotting, with pro-caspase-1 used as a loading control.

Having established the ability of soluble factors to exert the same inhibitory effect, we continued by analysing the composition of the supernatant, which was generated as a result of basal recognition. As already discussed, the dampening of IL-1β is dependent on iNKT cell-DC interaction in the absence of exogenous stimulation or lipid. It has been demonstrated that basal autoreactivity leads to production of GM-CSF and IL-13, whilst activation by α-GalCer results in secretion of IFN-γ, IL-4 and IL-2, in addition to boosting IL-13 and GM-CSF [216]. Similar to this study, we observed minimal

levels of IFN-γ and IL-4, but appreciable levels of IL-13 and GM-CSF in basal autoreactivity supernatants **(Fig. 4.4.2)**.

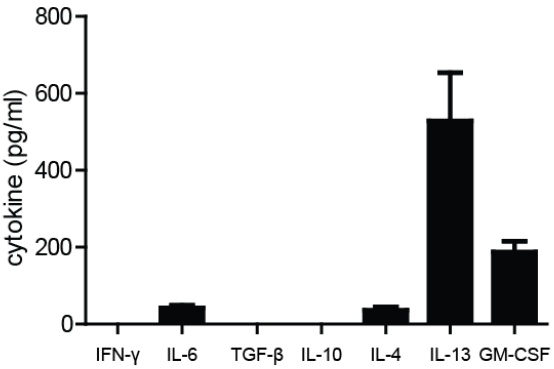

Fig. 4.4.2 IL-13 and GM-CSF are present in basal autoreactivity supernatants. iNKT cells were co-incubated with human MdDCs at a ratio of 1:3 for 14 hours before supernatants were tested for the presence of various cytokines by ELISA. The data are representative of experiments done with at least 3 iNKT cell lines. Data bars show the mean of triplicates +/- SEM.

The detection of IL-13 led us to investigate whether this cytokine could modulate IL-1β in MdDCs. We showed that both recombinant IL-13 and IL-4 could decrease IL-1β elicited by both SAA and UV-killed *Klebsiella* **(Fig. 4.4.3a, b)**. However, in accordance with published reports [286], IFN-γ boosted IL-1β production. These findings were also extended by immunoblotting analysis; we were able to show that, similar to iNKT cells and sCD40L, IL-13 reduces IL-1β production at the level of pro-IL-1β induction **(Fig. 4.4.3c)**.

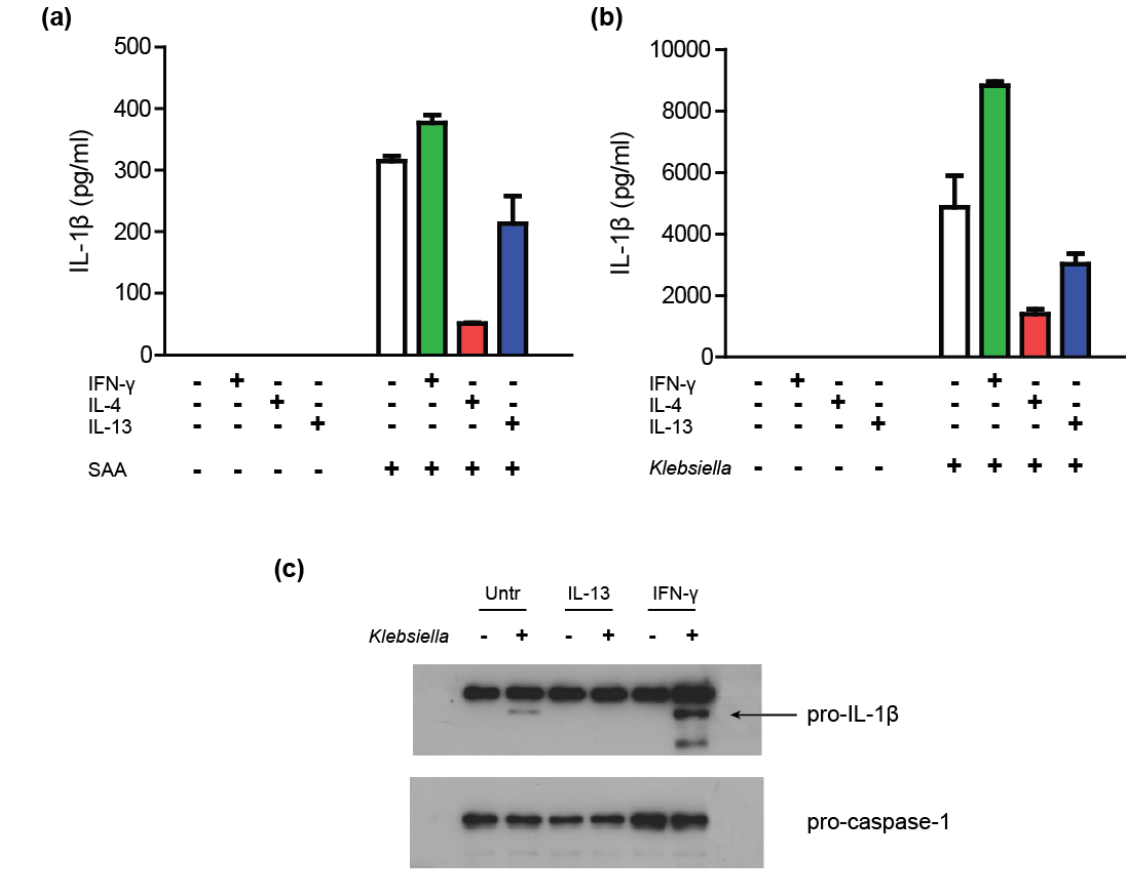

Fig. 4.4.3 Cytokines elicited by iNKT cell-DC crosstalk modulate IL-1β production from DCs. Human MdDCs were pre-conditioned with 1ng/ml of the indicated cytokines for 16 hours before stimulation with SAA at 1μg/ml or UV-killed *Klebsiella atlantae* at an m.o.i. of 50. Cell supernatants were analysed by ELISA **(a)** and immunoblotting **(b)**. Data are representative of at least 2 independent experiments, each performed using cells derived from different donors. Bars show the mean of duplicates +/- SEM.

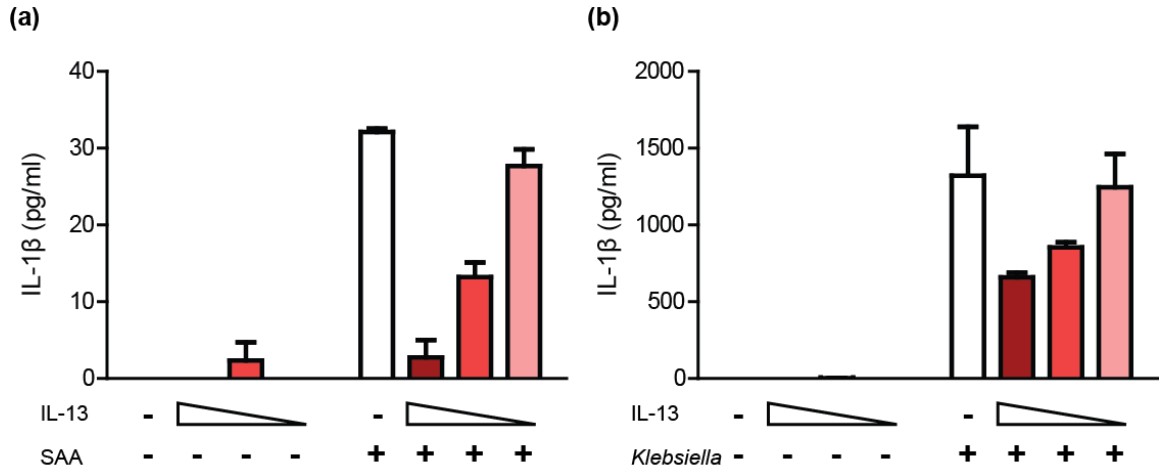

Fig. 4.4.4 Recombinant IL-13 conditions DCs to release lower levels of IL-1β upon subsequent stimulation. Human MdDCs were pre-conditioned with 10, 1 or 0.1ng/ml recombinant human IL-13 for 14 hours before stimulation with SAA (1μg/ml) or UV-killed *Klebsiella atlantae* (m.o.i. 50) for 24 hours. Supernatant was then analysed by ELISA. Data are representative of 2 experiments, with bars showing the mean of duplicates +/- SEM.

Given the inhibitory effect of IL-13 together with its presence in basal autoreactivity supernatant, we further examined its role in modulating of IL-1β production. First, we showed that the inhibitory effect of IL-13 could be titrated, with decreasing levels of IL-1β as IL-13 concentration increased **(Fig. 4.4.4)**. It should be noted that the levels of IL-13 we detected from basal autoreactivity supernatant, in all experiments performed, were around 800pg/ml, within the range of our titration curves.

Next, we proceeded to investigate the signals inducing IL-13 release from iNKT cells. As both basal autoreactivity and α-GalCer recognition are dependent on engagement of lipid-bound CD1d by the invariant TCR, we blocked this cognate interaction by the addition of an anti-CD1d blocking antibody. We were able to inhibit the secretion of IFN-γ by iNKT cells in the presence of α-GalCer **(Fig. 4.4.5a)**, as well as reduce the levels of IL-13 produced, both as a result of basal recognition and α-GalCer activation **(Fig. 4.4.5b)**. However, blockade of CD1d did not completely abolish IL-13 production. This is especially interesting in the case of basal autoreactivity, suggesting a CD1d-independent mechanism of IL-13 secretion. This could be an artefact arising from the protocol by which iNKT cells are restimulated and perpetuated *in vitro*, giving a low level of continuous activation.

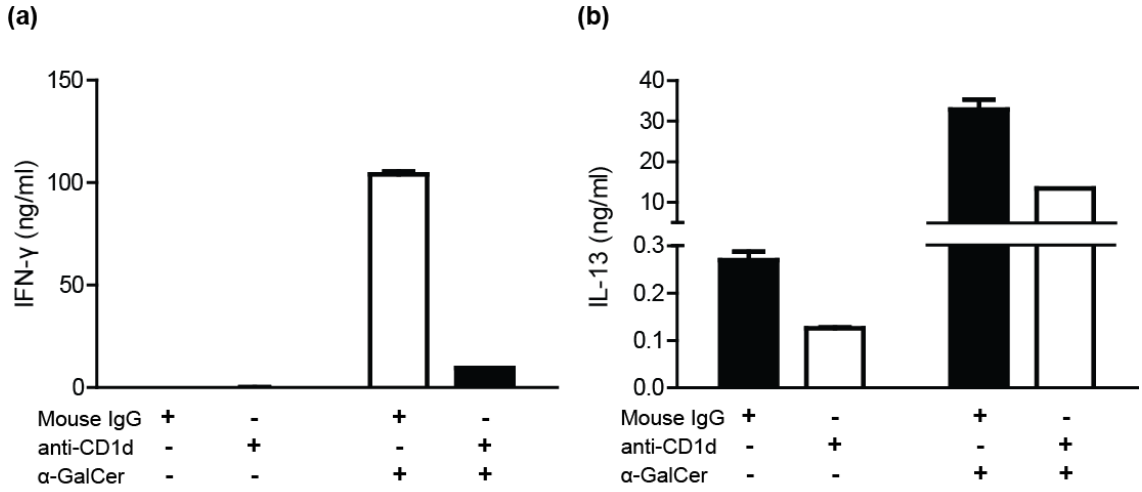

Fig. 4.4.5 CD1d blockade reduces IL-13 secretion from iNKT cells. Human MdDCs were co-incubated with iNKT cells at a ratio of 3:1 for 14 hours, in the presence of a mouse IgG isotype control or anti-CD1d blocking antibody (10μg/ml) and α-GalCer (2ng/ml). Supernatant was then analysed for levels of IFN-γ and IL-13. Data are representative of 3 independent experiments. Data bars show the mean of duplicates +/- SEM.

Having established that IL-13 could act similarly to iNKT cells to inhibit IL-1β, we performed experiments to block IL-13 activity and rescue IL-1β production. **Fig. 4.4.6** shows the rescue of IL-1β

production in most donors upon addition of a neutralising anti-IL-13 antibody to the basal autoreactivity supernatant. These data show that IL-13 plays an important part in the inhibitory activity of basal autoreactivity supernatant.

These experiments, taken together, demonstrate that iNKT cell-DC basal crosstalk results in a specific activation state, characterised by IL-13 secretion. This IL-13 can pre-condition MdDCs to inhibit IL-1β secretion upon later stimulation, and blockade of this pathway rescues IL-1β production.

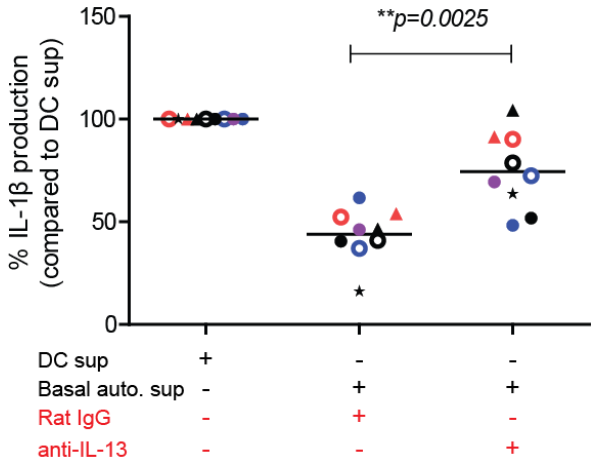

Fig. 4.4.6 IL-13 blockade rescues IL-1β dampening by basal autoreactivity supernatant. Human MdDCs were pre-conditioned with DC supernatant or basal autoreactivity supernatant (generated as previously described) in the presence of anti-IL-13 neutralising antibody or rat IgG isotype control (both at 10μg/ml). Data are presented as % IL-1β production, with 100% set as the "DC pre-conditioned with DC supernatant" condition. Each set of symbols represents one individual experiment, with each colour representing one iNKT cell line. Statistical analysis was performed using Student's t test.

4.5 Combined activity of cell-associated and secreted factors mediates IL-1β inhibition by iNKT cells

To this point, we have shown that both CD40L on the iNKT cell membrane and IL-13 secreted by iNKT cells can reproduce the inhibition of IL-1β production from MdDCs. We therefore hypothesised that pre-conditioning of MdDCs was dependent on both of these pathways, in this manner explaining our inability to rescue IL-1β production when CD40L blockade was used alone **(Fig. 4.3.4)**.

Having observed an inhibitory effect on IL-1β by sCD40L and IL-13 in isolation, we examined the effect of using both factors together to reduce IL-1β synthesis. We titrated the amount of sCD40L used to condition MdDCs, in the absence or presence of 1 or 10ng/ml recombinant IL-13. Cells were then stimulated, as before, with SAA or UV-killed *Klebsiella*. As we have already observed, the inhibitory effect of sCD40L increased with concentration, with the multimer having a stronger effect than the monomer **(Fig. 4.5.1a, b left panels)**. In the presence of IL-13 alone, we also observed a decrease in IL-1β. However, we found that $sCD40L_m$ and IL-13, when used in combination, had an additive effect in dampening IL-1β release; this was especially pronounced in the case of *Klebsiella* stimulation **(Fig. 4.5.1b)**. This would suggest that iNKT cells achieve a greater inhibitory action on IL-1β by engaging both pathways. We also verified that $sCD40L_m$ and IL-13, as with iNKT cells, did not inhibit IL-12p40 induction; on the contrary IL-12p40 levels were boosted by sCD40L **(Fig. 4.5.1a, b right panels, c)**. This experiment demonstrates that the cooperation between sCD40L and IL-13 is selective, as IL-1β is inhibited whereas IL-12p40 is enhanced.

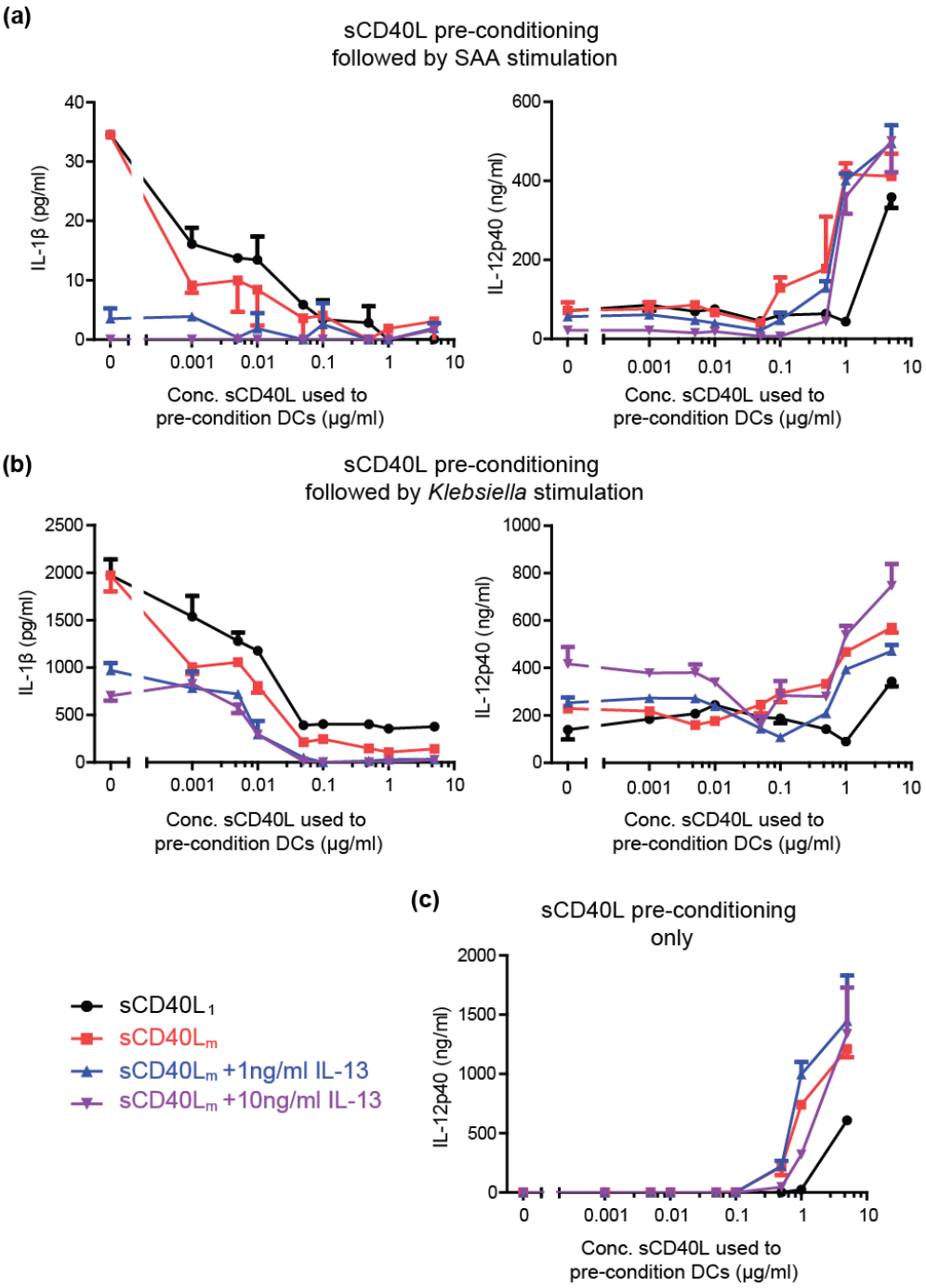

Fig. 4.5.1 Soluble CD40L and recombinant IL-13 have additive effects on IL-1β dampening. Human MdDCs were pre-conditioned with varying concentrations of soluble CD40L (monomeric or multimeric) with or without 1 or 10ng/ml recombinant IL-13 for 14 hours. 1μg/ml SAA **(a)** or UV-killed *Klebsiella* **(b)** were added to stimulate IL-1β production for 24 hours. Alternatively, DCs were left unstimulated following the pre-conditioning step **(c)**. Data are representative of 2 independent experiments, performed with cells from 2 different donors. Data points show the mean of duplicates +/- SEM.

We then proceeded to test our hypothesis that iNKT cells condition MdDCs using both CD40L on the cell surface and secreted IL-13 to reduce IL-1β. We co-incubated MdDCs with iNKT cells whilst blocking these two pathways using neutralising antibodies, either separately, or together, before

inducing IL-1β synthesis. The summary of all independent experiments performed is shown in **Fig. 4.5.2**. As described earlier, blockade of CD40L alone did not rescue IL-1β production. Although we have shown that neutralising IL-13 can rescue IL-1β when basal autoreactivity supernatant is used, addition of anti-IL-13 alone was also insufficient to rescue IL-1β in the presence of iNKT cells. However, when both CD40-CD40L and IL-13 signalling were blocked, we observed a statistically significant increase in IL-1β release.

This crucial experiment demonstrates that iNKT cells mediate dampening of IL-1β via a two-pronged mechanism. iNKT cells interact with MdDCs via their basal autoreactivity, and CD40L on iNKT cells engages CD40 on MdDCs. The same basal activation also induces IL-13 secretion, and these two factors then have an additive effect on inhibiting IL-1β production, with iNKT cells utilising both cell-contact and soluble factor dependent mechanisms to exert this anti-inflammatory state.

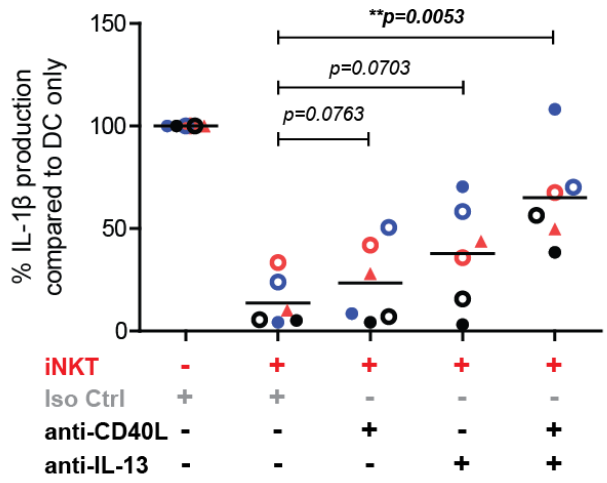

Fig. 4.5.2 Blockade of both CD40L and IL-13 rescues IL-1β dampening by iNKT cells. Human MdDCs were pre-conditioned with iNKT cells at a ratio of 3:1 in the presence of anti-CD40L blocking antibody, anti-IL-13 blocking antibody, or both, with each at 10μg/ml. Alternatively, isotype controls to both antibodies (mouse and rat IgG) were used. Data are presented as % maximum IL-1β production, with 100% set to the "DC alone" condition. Each symbol represents one experiment, and each set of symbols of the same colour represents experiments performed using the same iNKT cell line. Statistical analysis was performed using Student's t test (two-tailed, paired).

4.6 Molecular mechanisms of iNKT cell-mediated IL-1β inhibition

We have thus far established that iNKT cells condition MdDCs to produce less IL-1β when subsequently stimulated, using CD40L- and IL-13-dependent pathways. We also observed that this inhibition was exerted at the level of pro-IL-1β induction, the first step in IL-1β synthesis.

We then proceeded to examine the molecular pathways involved in mediating this phenotype. Given that pro-IL-1β was being reduced, we investigated whether levels of the *Il1b* transcript were also affected. To do this, we pre-conditioned MdDCs, as before, with iNKT cells, or $sCD40L_m$, basal autoreactivity supernatant and IL-13, the factors we have identified to be responsible. UV-killed *Klebsiella* was then added to stimulate IL-1β production, and the accumulation of *Il1b* mRNA followed by real-time PCR **(Fig. 4.6.1)**. We found that the *Il1b* transcript peaked at 6 hours after stimulation, with levels reaching up to 20,000 times that of resting cells, depending on the donor from which the

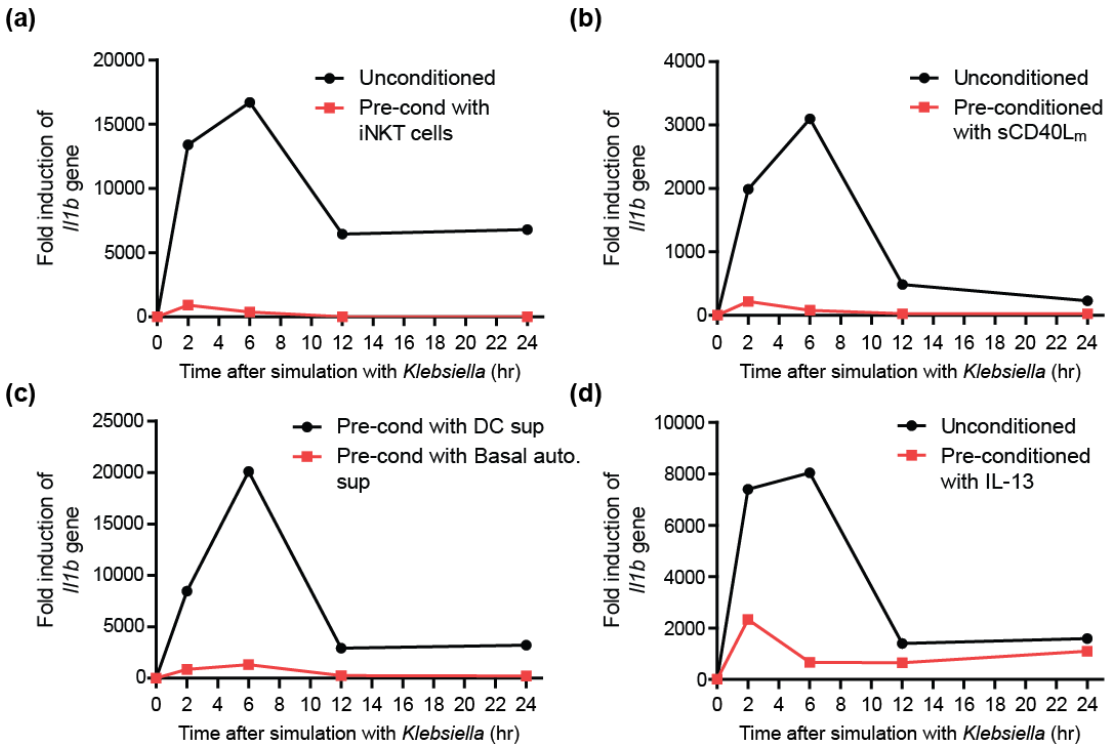

Fig. 4.6.1 iNKT cells decrease the accumulation of *Il1b* mRNA in human MdDCs. Human MdDCs were pre-conditioned with **(a)** iNKT cells (ratio 3:1), **(b)** sCD40L (multimer, 1μg/ml), **(c)** basal autoreactivity supernatant (ratio 2:1 with complete medium) or **(d)** recombinant IL-13 (1ng/ml). UV-killed *Klebsiella atlantae* (m.o.i. 50) was then added and cellular mRNA content analysed by real-time PCR at various timepoints. For (a) MdDCs were separated after *Klebsiella* stimulation by CD2 negative selection, using magnetic beads. Cycle numbers were normalised to the *Gapdh* housekeeping gene, before $\Delta\Delta C_T$ values calculated relative to unstimulated (Time 0hr) samples. Data are representative of at least 2 independent experiments in each case.

MdDCs were derived. Strikingly, MdDCs which were pre-conditioned by iNKT cell signals showed a dramatic decrease in the upregulation *Il1b* mRNA after bacterial stimulation, thus suggesting that the inhibitory effect of iNKT cells is exerted at the mRNA level.

A defect in the ability of MdDCs to upregulate *Il1b* transcript could be explained by two possible mechanisms – a reduction in the transcription of the *Il1b* gene or changes in the stability of the transcript. Whilst the two are not mutually exclusive, we decided to focus on investigating the former possibility – modulation of *Il1b* transcription.

Transcription of *Il1b* is dependent on the activity of the NF-κB family of transcription factors, with activation of NF-κB downstream of TLRs, or IL-1R1 itself, required for *Il1b* gene activation [73]. The canonical pathway of NF-κB activation culminates in the ubiquitinylation and degradation of the IκB proteins, which hold the NF-κB p50 and p65 activatory subunits in an inactive state in the cytoplasm. Upon release from IκB, p50 and p65 can move into the nucleus to drive the expression of NF-κB-dependent genes [17]. Among the many biochemical studies which have been done on the *Il1b* promoter, Zhang and colleagues identified an initial phase of *Il1b* transcription which is dependent on IκB degradation and p65 binding to the *Il1b* enhancer region [76]. Despite the involvement of other transcription factors such as PU.1, IRF4, and AP-1, NF-κB is described as being critical in driving the transcription of the *Il1b* gene [74].

Following these observations, we designed experiments to investigate whether, upon iNKT cell pre-conditioning, activation of the NF-κB pathway is perturbed. We chose, as a readout, the degradation of IκBα, with normal activation of NF-κB signalling characterised by a rapid degradation of this protein, before feedback mechanisms restore it back to starting levels. This pattern of degradation and resynthesis was indeed what we observed upon bacterial stimulation of unconditioned MdDCs **(Fig. 4.6.2)**. Noticeably, pre-conditioning MdDCs with basal autoreactivity supernatant and IL-13, stimuli which we have shown to be provided by iNKT cells, reduced the degradation of IκBα. An even more striking effect was observed when $sCD40L_1$, or $sCD40L_1$ with basal autoreactivity supernatant (completely mimicking the iNKT cell signals), were used. In all cases, although slight degradation of IκBα was observed, pre-conditioned MdDCs retained some level of IκBα protein. With the addition of $sCD40L_1$, levels of IκBα at 30 minutes after *Klebsiella* stimulation were still higher than that of unconditioned MdDCs at 0 minutes. These experiments indicate that pre-conditioning of MdDCs by

iNKT cells can decrease the activation of the NF-κB signalling pathway, with this inhibition exerted at or above the level of IκBα degradation.

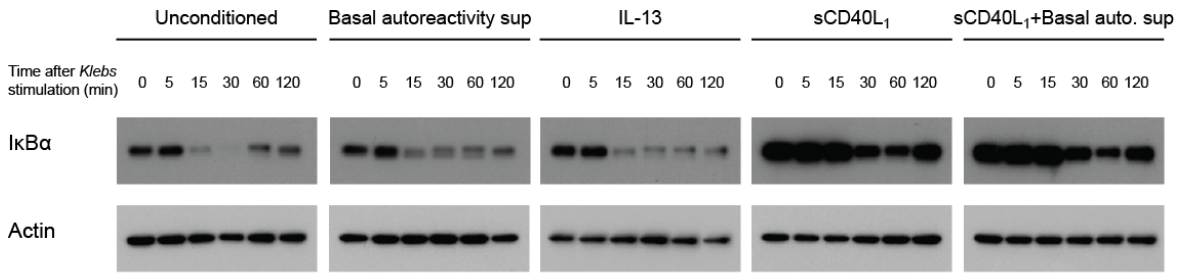

Fig. 4.6.2 Pre-treatment associated with iNKT cell conditioning reduces IκBα degradation upon stimulation of the NF-κB pathway. Human MdDCs were pre-conditioned using the conditions as indicated, before UV-killed *Klebsiella* was added at an m.o.i. of 50. Basal autoreactivity supernatant was used at a ratio of 2:1 with complete medium, IL-13 at 1ng/ml and $sCD40L_1$ at 1μg/ml. Cell lysates were harvested at various timepoints after *Klebsiella* stimulation and analysed by immunoblotting. Data are representative of 3 independent experiments.

Having observed a reduction in the strength of NF-κB signalling, we then investigated whether negative regulators of the NF-κB pathway were upregulated by iNKT cell-DC crosstalk. Upon co-incubation with iNKT cells, we observed the induction of the ubiquitin editor A20 in MdDCs, with levels increasing with the duration of interaction **(Fig. 4.6.3a)**. Furthermore, treatment of MdDCs with $sCD40L_1$, basal autoreactivity supernatant or IL-13 also upregulated A20 **(Fig. 4.6.3b)**. These observations imply that pre-conditioning of MdDCs by iNKT cells is able to upregulate A20, a negative regulator of NF-κB signalling. This, in turn, primes the DC to respond to subsequent TLR signalling with decreased levels of NF-κB activation, thereby dampening their synthesis of pro-IL-1β.

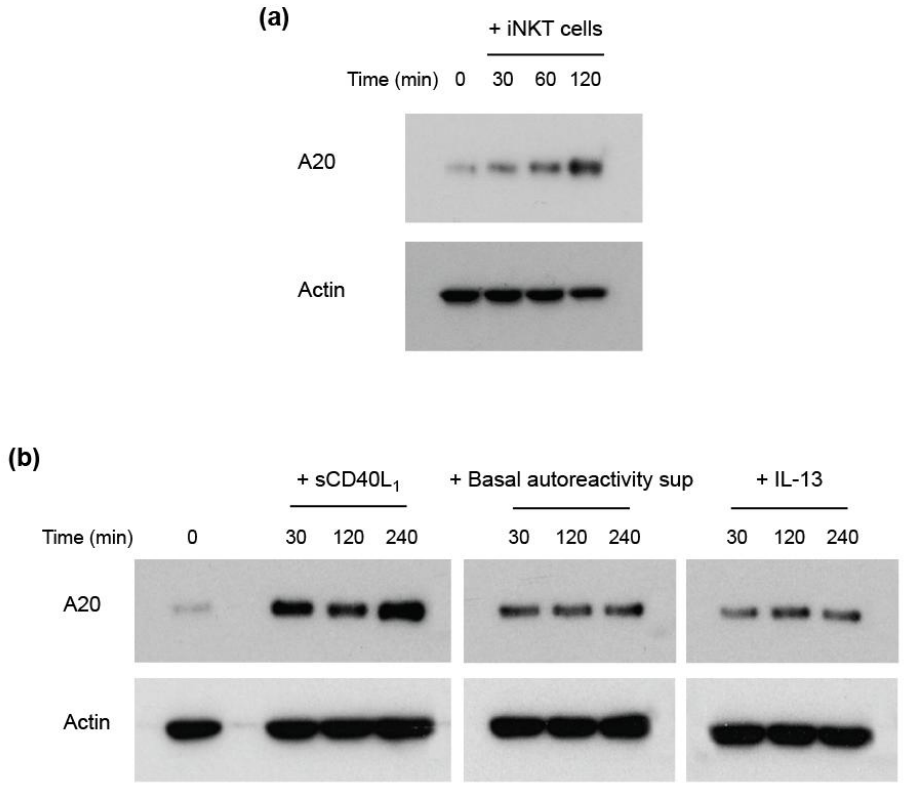

Fig. 4.6.3 iNKT cell-DC interaction results in upregulation of A20 in DCs. (a) Human MdDCs were cocultured with iNKT cells at a ratio of 4:1 for the indicated timepoints. MdDCs were then isolated by CD2 negative selection using magnetic beads. Cells were lysed and analysed by immunoblotting. **(b)** MdDCs were incubated for various lengths of time with the indicated stimuli (sCD40L$_1$ at 1µg/ml, basal autoreactivity supernatant at ratio of 1:1 with complete medium and IL-13 at 1ng/ml) before lysis of cells and analysis by immunoblotting. Data are representative of 3 independent experiments performed using cells from different donors.

4.7 Discussion and future perspectives

4.7.1 The dual role of iNKT cells

We have shown that iNKT cells are able to interact with MdDCs via their basal autoreactivity, and in doing so, condition MdDCs to produce reduced levels of IL-1β upon subsequent stimulation. We observed that this was dependent on both CD40L-CD40 signalling from iNKT cell to DC, and also on the secretion of IL-13 from iNKT cells. These pathways reduce the transcription of the *Il1b* gene by decreasing NF-κB signalling and inducing A20, a negative regulator of NF-κB signalling.

This anti-inflammatory property of iNKT cells therefore adds to our understanding of how iNKT cells shape the immune response. Indeed, iNKT cells are thought to play a key role in modulating immunity, as they have both pro- and anti-inflammatory properties, not least by their hallmark dual production of IL-4 and IFN-γ upon activation by α-GalCer [217]. However, the question remains how iNKT cells are able to perform this dual role. We have shown that the type of iNKT cell stimulation plays a crucial role in determining whether IL-1β is subsequently dampened. We showed that stimulation at the level of basal autoreactivity was required, whereas stimulation with α-GalCer failed to condition MdDCs in the same way. Although we have not formally investigated its role, we observed high levels of IFN-γ secreted from iNKT cells upon α-GalCer stimulation, but not during basal recognition. Whilst α-GalCer stimulation also induced much increased levels of IL-13 **(Fig. 4.4.5b)**, this was nonetheless unable to dampen IL-1β production, perhaps pointing to IFN-γ as a dominant factor in this case. Given the ability of recombinant IFN-γ to boost IL-1β production (**Fig. 4.4.3** and [286]), further investigation into its role in our system would be of interest. Our data therefore highlights the requirement for a weak stimulus to engage iNKT cells during the pre-conditioning step. This could therefore translate physiologically to iNKT cell autoreactivity against immature, unstimulated APCs resulting in an anti-inflammatory outcome, whereas stimulation of iNKT cells by matured APCs presenting exogenous lipids or increased levels of natural ligand activating a pro-inflammatory response.

The scenario we have posited would also imply that the timing of interaction, whether iNKT cells interact with DCs before or after maturation, is also important. Maturation of DCs with TLR ligands drastically increases their capacity to activate iNKT cells, via upregulation of self ligand-CD1d complexes and also by the stimulatory action of cytokines secreted upon maturation, IL-12 and IL-18 [236, 289]. In our experiments, iNKT cells are coincubated with immature MdDCs in a pre-

conditioning step 12-14 hours before addition of SAA or UV-killed bacteria. The timing and microenvironment in which the iNKT cells are activated are therefore key; iNKT cells are not able to dampen IL-1β secretion if added to DCs at the same time as or shortly after TLR stimulation (data not shown). Physiologically, this could equate to iNKT cells being important in dampening inflammation and preventing exacerbated immune responses in the absence of infection, but boosting adaptive immunity with their pro-inflammatory properties when they encounter stimulated APCs. This model is in agreement with another report describing a similar dichotomy in mouse iNKT cells, which, upon encountering immature myeloid DCs, drive tolerogenic maturation, whereas interaction with LPS-stimulated DCs results in pro-inflammatory DC maturation [290].

4.7.2 iNKT cells and conventional T cells

The adaptive immune response has been described to be able to modulate IL-1β production by influencing the activity of the inflammasome. Guarda *et al.* reported the ability of mouse activated and memory T cells to dampen the inflammasome, thereby switching off inflammatory responses at the late stages of the immune response [285]. The authors also demonstrated that CD40L and other ligands of the TNF superfamily were able to reproduce the effect of T cells. Recently, another group has described the ability of human activated memory T cells to downregulate inflammasome activity, although this was found to be at the level of P2X7R signalling, upstream of NLRP3 activation [291]. Both studies use a similar protocol of pre-conditioning to inhibit IL-1β production. The disparity between our data and the observation that murine T cells and CD40L downregulate IL-1β production at the level of the inflammasome may be due to species-specific differences. Meanwhile, the human study focused on monocytes as the APC, which can have greatly different responses and properties to DCs. Nevertheless, our observations highlight a unique property of iNKT cells amongst the T cell family, confirming their distinctive ability to shape the immune response.

The ability of iNKT cells to dampen IL-1β production could also play a unique role *in vivo*. Whereas activated and memory T cells would only appear at the later stages in infection, iNKT cells are constitutively present, especially in appreciable numbers in the lymphoid organs and the liver. This implies that, while conventional T cell-mediated reduction of IL-1β is probably part of a negative feedback mechanism to shut down inflammatory responses, iNKT cells could reduce IL-1β levels

directly from the early stages, potentially playing a key role in preventing overzealous inflammatory responses and immunopathology.

We have described that iNKT cell interaction with MdDCs via basal recognition results in activation of CD40-CD40L signalling and IL-13 secretion. Whilst conventional activated T cells express CD40L on the cell surface, IL-13 production, together with IL-4, is limited to Th2 helper T cells. Given the mechanism which we propose, with inhibition dependent on CD40L and IL-13, it would be interesting to examine whether *in vitro* T cell activation in the presence of Th2 skewing conditions could produce activated, Th2 helper T cells which can inhibit IL-1β production in DCs in the same way iNKT cells.

4.7.3 The role of CD40L in inhibiting IL-1β production

We have, perhaps surprisingly, described an anti-inflammatory role for CD40L during the action of iNKT cells. Traditionally, the CD40-CD40L pathway has been described to provide positive signals for DC maturation. Signalling downstream of CD40 is able to activate the NF-κB pathway, inducing DC maturation and cytokine production [180]. However, the induction of IL-10, an anti-inflammatory cytokine, has also been documented [193]. In this report, the authors found that weak CD40 signals induced IL-10, whilst stronger signals resulted in IL-12. Our titrations of sCD40L also yielded a similar conclusion **(Fig. 4.3.2)**, with weak CD40 stimulation (0.1 to 1μg/ml of $sCD40L_1$ and 0.1μg/ml of $sCD40L_m$) unable to induce IL-12p40 production, but still inhibiting IL-1β. These conditions best mimic the strength of CD40 signalling during our conditioning of MdDCs. We would therefore hypothesise that weak CD40 signals are provided by iNKT cells during the pre-conditioning of MdDCs, which are able to inhibit IL-1β production, but unable to induce production of IL-12p40.

The ability of CD40 signalling to activate the NF-κB pathway also places importance on the strength of the signal in our experimental system. Since IL-1β is also driven by NF-κB, powerful CD40 signalling could induce the production of IL-1β, as has been reported [292]. Indeed, in our experiments stimulation of MdDCs with greater than 5μg/ml of multimeric sCD40L was able to directly induce IL-1β production in some donors (data not shown). The inhibitory effect of iNKT cells which we observe therefore relies on the induced CD40 and NF-κB signalling being below the threshold required to drive *Il1b* transcription.

On the other hand, weak NF-κB activation during pre-conditioning of MdDCs by sCD40L was shown to upregulate the negative factor A20 **(Fig. 4.6.3b)**. We demonstrated this using 1μg/ml $sCD40L_1$, which is insufficient to drive IL-12 expression. A20 induction is NF-κB-dependent to establish a negative feedback mechanism and re-establish homeostasis [22]. We have therefore shown that a weak CD40 signal, not enough to induce pro-inflammatory cytokine production, is still sufficient to drive NF-κB-dependent A20 expression.

This "touching" of the CD40-CD40L pathway by iNKT cells may then be responsible for establishing an anti-inflammatory state in the DC, with subsequent inhibition of the NF-κB pathway.

4.7.4 The role of soluble factors in inhibiting IL-1β production

In addition to CD40L, we demonstrated that iNKT cells secrete soluble factors which are capable of recreating the inhibitory effect. We then went on to show the involvement of IL-13, with blockade of IL-13 rescuing IL-1β production in the presence of basal autoreactivity supernatants.

IL-13 is a Th2 cytokine with important roles in hypersensitivity reactions, antibody responses and immunity against gastrointestinal nematodes, all of which are well documented. The IL-13 receptor is a heterodimer made up of the IL-4Rα chain together with IL-13Rα1, and is identical to the type II IL-4 receptor [293]. IL-13R signalling culminates in phosphorylation of the transcription factor STAT6 [294]; the number of interaction partners and target genes directed by STAT6 continues to grow, with some directly relevant to the experimental system which we have described.

Both IL-13 and IL-4 have been described to be able to inhibit the induction of IL-1β [295, 296], although most of this work has been done on human monocytes, and rarely has this been shown in human MdDCs. Interestingly, IL-4 and IL-13 have also been shown to inhibit NF-κB activation, in a STAT6-dependent manner [84, 293, 297]. Indeed, IL-13 has been reported to able to inhibit NF-κB activation by preserving IκB levels upon stimulation [85], an observation which we have reproduced in MdDCs. However, despite reports of STAT6 being able to directly interact with NF-κB components [294], the exact molecular mechanisms mediating these effects remain unclear. Rather more intriguing is our observation that IL-13 can induce A20 upregulation. There are several accounts suggesting that IL-13 stimulation can lead to NF-κB translocation into the nucleus [298], and one laboratory demonstrating the upregulation of A20 *in vivo* following recombinant IL-13 administration

[299]. However, most of these experiments were performed in non-haematopoietic cells. In our experiments, the upregulation of A20 in MdDCs by basal autoreactivity supernatants and IL-13 was weaker than that observed with sCD40L, perhaps reflecting the relative abilities of IL-13 and sCD40L to influence NF-κB signalling. Further investigation is required to elucidate whether A20 upregulation is indeed the mechanism through which IL-13 decreases IκBα degradation, or whether this is mediated by IL-13 signalling via an alternative pathway.

The inhibition of IL-1β by iNKT cells required their interaction with DCs via basal autoreactivity, inducing the release of IL-13 but not IFN-γ. We reduced the release of IL-13 from iNKT cells by blocking the cognate interaction between the invariant TCR and lipid-CD1d using an anti-CD1d blocking antibody. Interestingly, blockade of CD1d was unable to rescue IL-1β inhibition by iNKT cells, despite the antibody reducing IFN-γ and IL-13 (data not shown). Noticeably, however, we were unable to completely abolish IL-13 secretion **(Fig. 4.4.5b)**. We attributed this to a low level of CD1d-independent induction of IL-13, due to the continuous stimulation of iNKT cells during their maintenance in culture. Indeed, culture of iNKT cells alone (without MdDCs) also yielded amounts of IL-13 comparable with the CD1d-independent levels (data not shown). This, together with the constitutive expression of CD40L in our iNKT cells, could account for the inability of CD1d blockade to rescue IL-1β production. However, we cannot exclude the possibility that an alternative, CD1d-independent mechanism is responsible. In fact, others have reported CD1d-independent activation and effects of iNKT cells [253, 289, 300]. However, most of these reports point to cytokine-mediated iNKT cell activation as the mechanism for bypassing CD1d recognition, with IL-12 and IL-18 derived from APCs being especially significant.

In addition to IL-4 and IL-13, many other cytokines have been reported to be able to modulate IL-1β production [301, 302]. Recently, much focus has been on the ability of the type I interferons to dampen IL-1β production. IFN-α and IFN-β were shown, in an *in vitro* system, to be able to downregulate inflammasome activity directly, and also reduce pro-IL-1β synthesis through the induction of IL-10 [303]. This was confirmed in a *Mycobacterium tuberculosis* infection model, where defects in type I IFN signalling led to increased pro-IL-1β in pulmonary DCs and inflammatory monocytes [304]. These observations may be important in further exploring the role of soluble factors in the inhibition of IL-1β by iNKT cells. Whilst we were unable to detect significant levels of IL-6, IL-10

or IFN-β in our basal autoreactivity supernatants (data not shown), other cytokines may be present which cooperate with IL-13 in conditioning MdDCs. This would also account for the observation we made that IL-13 blockade in basal autoreactivity supernatants, whilst resulting in rescue, did not restore IL-1β levels back to 100%.

4.7.5 Modulation of NF-κB signalling by iNKT cells

In investigating the molecular mechanisms of the inhibitory effect on IL-1β, we observed a reduction in the accumulation of the *Il1b* transcript, which was associated with a reduction in IκBα degradation.

Interestingly, where $sCD40L_1$ was used to pre-condition MdDCs, we observed a general increase in the levels of IκBα in the cell. The gene encoding IκBα is under the control of NF-κB itself [20], setting up an negative feedback loop to terminate NF-κB signalling. We can speculate that low level CD40 stimulation in MdDCs, either by iNKT cells or low concentrations of $sCD40L_1$, weakly activates the NF-κB pathway. This activation is insufficient to drive pro-inflammatory cytokine transcription, but results in the upregulation of negative factors, like IκBα and A20. This generates a tolerogenic state in the MdDCs, which then respond to subsequent stimulation with SAA or bacteria with reduced NF-κB activation and pro-IL-1β synthesis.

This proposed mechanism for establishing an inhibitory state after initial stimulation is reminiscent of endotoxin tolerance, first described by Paul Beeson, who observed that repeated injection of typhoid vaccine in rabbits led to progressively weakened febrile responses induced by the vaccine. Reports in the literature routinely identify IL-1β as one of the cytokines which fail to be induced during endotoxin tolerance [305]. In the literature, the consensus is that several mechanisms cooperate to bring about this refractory condition, with downregulation of pro-inflammatory signalling, upregulation of anti-inflammatory cytokines and epigenetic changes all playing a key role.

Significantly, elevated levels of inhibitors of TLR and NF-κB signalling, including IκBα, have been reported in endotoxin tolerant macrophages and monocytes [306]. This parallels our observation following stimulation with iNKT cells. In addition, changes in the profile of the NF-κB response have been described, with endotoxin tolerant cells showing a bias towards p50 homodimers, away from the p50/p65 heterodimers associated with pro-inflammatory gene transcription [307]. Elevated levels of RelB have also been described, responsible for maintaining increased IκBα expression [87]. This shift

in the NF-κB pathway allows for subsequent activation to drive alternative gene transcripts, whilst suppressing those associated with the inflammatory response. Finally, RelB has also been shown to induce chromatin remodelling at the *Il1b* promoter, thus preventing p65 binding [86]. These observations were extended by a report showing an additional role for IκBα in maintaining this closed chromatin state [308]. Thus far, we have only investigated the modulation of NF-κB signalling by testing IκBα and A20 levels. The induction of these two proteins by iNKT cells is likely to be one of various mechanisms by which a tolerant phenotype is imprinted onto MdDCs during pre-conditioning. Therefore, it will be important to test whether these other pathways are also employed by iNKT cells to reduce IL-1β production in MdDCs.

4.7.6 Selectivity in NF-κB signalling

The reduction in NF-κB signalling in MdDCs upon iNKT cell pre-conditioning also raises the question of how this process selectively affects IL-1β, but not IL-12. Speculatively, since NF-κB is a family of 5 transcription factors which can form homo- and heterodimers, selectivity may come from the requirement of different genes for different NF-κB family transcription factors. Significantly, c-Rel deficiency has been demonstrated to have profound effects on IL-12 production, with both IL-12p35 and IL-12p40 (*Il12a* and *Il12b* respectively) requiring c-Rel for full transactivation [309, 310]. By contrast, IL12p40 levels were only modestly affected in p65-deficient macrophages, with overexpression of c-Rel, but not p65, able to restore IL-12p40 production in IL-10-treated macrophages [311]. On the other hand, *Il1b* transcription is thought to be driven primarily by p65, complexed with p50 in a heterodimer [73]. In this way, the different requirements of *Il12b* and *Il1b* for transcription suggest that they can be differentially regulated. Further investigation is required to examine how iNKT cells can differentially affect IL-1β and IL-12 production; for example, how the increase in IκBα in our system preferentially dampens *Il1b* transcription.

The potential involvement of other signalling pathways, and other transcription factors, should also be considered. The large number of reports identifying putative transcription factor binding sites in the promoters of both *Il1b* and *Il12b* makes the picture considerably more complex. However, in line with the absolute requirement of NF-κB activation for *Il1b* transcription, its pharmacological inhibition abolishes IL-1β production [258]. *Il12b* transcription, however, is affected to a lesser extent by the same treatment, whilst inhibition of p38MAPK can lead to a substantial defect in IL-12p70 production

[312]. Mechanistically, p38MAPK was shown to be responsible for the phosphorylation of histone H3 and therefore opening of the chromatin structure in several cytokine promoters, including *Il12b* [313]. This raises the possibility that iNKT cells could also induce selective epigenetic changes at certain cytokine promoters.

These observations from the literature add another layer of complexity in the interpretation of our results. iNKT cells could conceivably affect the recruitment and activity of certain NF-κB family members, selectively modulate certain signalling pathways, or both. As a result, much further work is required to elucidate the molecular mechanisms by which iNKT cells selectively inhibit IL-1β production.

4.7.7 Future perspectives

We have shown that iNKT cells are able to dampen IL-1β secretion by MdDCs, and highlighted the importance of CD40L and IL-13 in mediating this process. In addition to what has been discussed above, several questions arise directly from these results.

Blockade of both CD40L and IL-13 rescued IL-1β, but did not restore its production back to levels seen with unconditioned DCs **(Fig. 4.5.2)**. There remains the possibility that additional factors are involved in mediating the inhibitory effect. We have already discussed the role other soluble factors may play in our experimental system; other membrane-bound ligands of the TNF superfamily, such as OX40L, RANKL and 41BBL, could also be important. In fact, soluble forms of these proteins have already been shown have the same effect as CD40L with respect to IL-1β production [285]. Investigation of these molecules on iNKT cells could therefore be revealing.

Following iNKT cell conditioning, we have only examined the levels of IκBα and A20 to investigate the effect of iNKT cells on the NF-κB pathway. Direct analysis of NF-κB transcription factor activity (for example by EMSA) would provide greater insight into the molecular mechanism of IL-1β inhibition, as well as selectivity between different cytokines. In addition, distinguishing between the actions of different members of the NF-κB family would be important in clarifying which pathways are modulated by iNKT cells.

So far, we have investigated the interaction between iNKT cells and human MdDCs. Preliminary data from our laboratory, confirmed in a recent report [291], suggest that the response of monocytes to

iNKT cell conditioning results in the opposite effect, an increase in pro-IL-1β production. As well as delving into the molecular mechanism of this effect, and how it differs from that of MdDCs, investigation of other IL-1β-producing cell types, such as macrophages, neutrophils and epithelial cells would allow us to build a better picture of how iNKT cell modulation of IL-1β is coordinated *in vivo*. Examination of a larger panel of pro- and anti-inflammatory cytokines, and how their synthesis can be influenced by iNKT cell conditioning, would also add to our knowledge of how iNKT cells can modulate the immune response.

Thus far, we have considered the activity of human iNKT cells in an *in vitro* system. In the next chapter, we will address the physiological relevance of these experiments using an *in vivo* model of infection. By comparing the phenotypes of WT or *Jα18*$^{-/-}$ (iNKT cell-deficient) mice, we can begin to clarify the role of iNKT cells in dampening inflammation *in vivo*.

CHAPTER 5

Suppression of Influenza Virus-induced Pulmonary Inflammation by Invariant Natural Killer T cells

Chapter 5: Suppression of Influenza Virus-induced Pulmonary Inflammation by Invariant Natural Killer T cells

We have established that iNKT cells can decrease the production of IL-1β from human MdDCs upon stimulation with SAA or whole bacteria. We now proceed to investigate the *in vivo* significance of this observation, using an influenza virus infection model.

Severe influenza infection has been shown to induce a pneumonia-like disease both in humans and mice, characterised by leukocytic influx and inflammation-associated tissue destruction. IL-1β and inflammasome activation are important inducers of the early innate response to influenza, with IL-1R1 signalling crucial in the recruitment of neutrophils into the lung.

In this chapter, we will examine the role played by iNKT cells in the early inflammatory response to influenza virus. By comparing the phenotypes of infected WT and *Jα18*$^{-/-}$ mice, we can observe the effect of iNKT cell-deficiency on the induction of IL-1β and associated inflammation during severe pulmonary infection. Using this experimental system, we will be able to confirm the physiological role played by iNKT cells during infection, and add to our understanding of their dual role in immunobiology.

5.1 *In vitro* influenza A infection induces IL-1β production from human monocyte-derived DCs

Having shown that iNKT cells are able to suppress the production of IL-1β elicited by bacteria and SAA, we wanted to extend our observations to investigate IL-1β production and modulation in the context of viral infection. In order to do this, we infected human MdDCs *in vitro* with the influenza A virus A/Puerto Rico/8/34 (PR8), which has been shown to be able to directly induce IL-1β production by activating TLR7 (signal 1) and the NLRP3 inflammasome (signal 2) [120]. We observed, by ELISA, the release of IL-1β into the supernatant of infected cells, with higher concentrations of cytokine secreted with increasing multiplicities of infection (m.o.i.) **(Fig. 5.1a)**. In addition to IL-1β, we also observed the release of a range of pro- and anti-inflammatory cytokines **(Fig. 5.1b-d)**, confirming the ability of influenza A virus (IAV) to elicit and modulate the immune response. Importantly, staining by propidium iodide confirmed that cell vitality was comparable between infected and uninfected cells (data not shown).

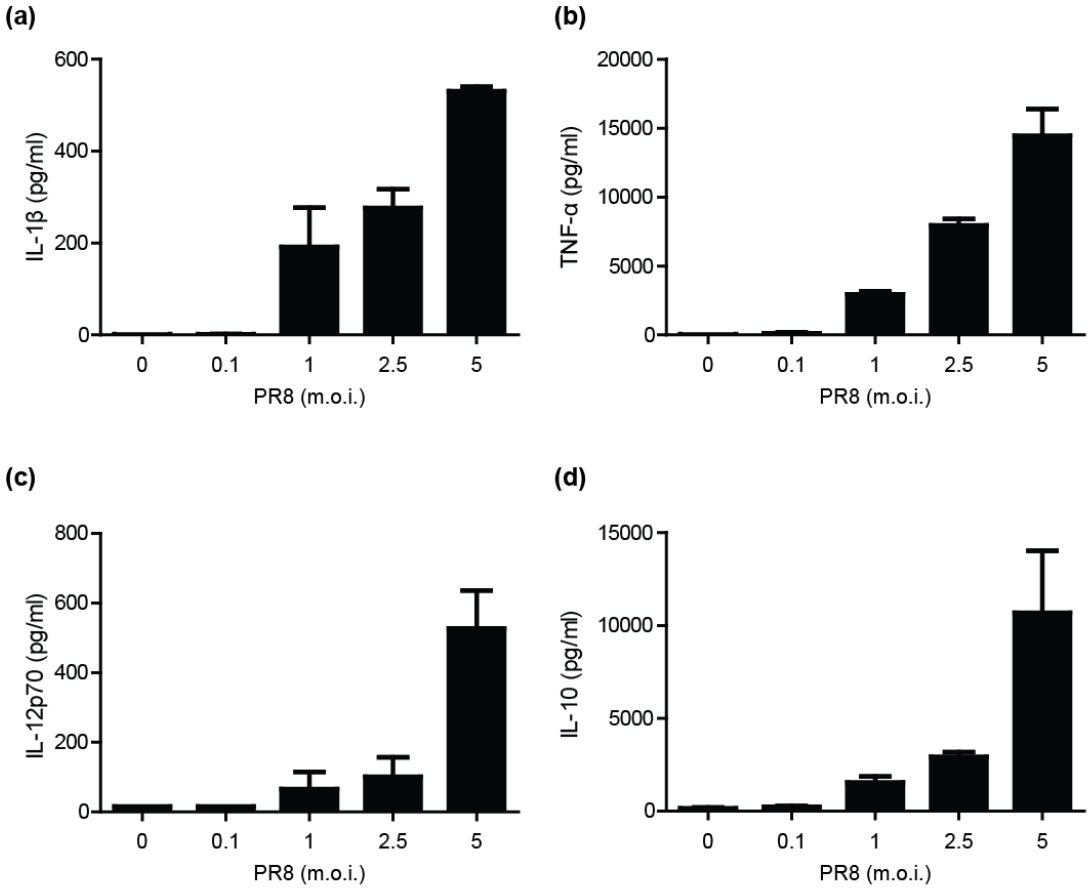

Fig. 5.1 PR8 influenza virus induces a panel of pro- and anti-inflammatory cytokines in a dose-dependent manner. Human MdDCs were infected with live PR8 influenza virus at increasing m.o.i. 24 hours later, cell supernatants were harvested and analysed for a panel of cytokines by ELISA. Data are representative of 2 independent experiments. Data bars depict the mean of triplicates +/- SEM.

5.2 iNKT cells decrease IL-1β secretion induced by influenza A virus *in vitro*

Having established the ability of influenza virus to elicit the production of IL-1β, we next investigated whether influenza-induced IL-1β could be modulated by iNKT cells, as we observed with bacterial and non-microbial stimuli. First, we investigated the ability of PR8 to induce crosstalk between MdDCs and iNKT cells. We pre-conditioned MdDCs by co-incubating with iNKT cells in the absence of exogenous glycolipids, hence allowing iNKT cells to interact with MdDCs via their basal autoreactivity. We were able to show that after pre-conditioning, infection with PR8 fosters the ability of MdDCs to activate iNKT cells. This was detected by the induction of IFN-γ release upon addition of PR8 **(Fig. 5.2a)**. Next, we examined whether iNKT cell-conditioning modulates PR8-induced IL-1β production. Following pre-conditioning, stimulation with live PR8 virus elicited a lower level of IL-1β secretion than observed in unconditioned DCs **(Fig. 5.2b)**, thus confirming our data with bacterial and non-microbial stimulation. In addition, analysis of cell lysates from these experiments showed that the decrease of IL-1β production by iNKT cells was mediated at the level of signal 1, with the induction of pro-IL-1β significantly impaired after pre-conditioning with iNKT cells **(Fig. 5.2c)**. Interestingly, we also observed inhibition of signal 2, as detected by an inhibition of caspase-1 cleavage. However, it has been shown that, as well as inducing pro-IL-1β production, a robust signal 1 is also required for priming of the NLRP3 inflammasome [98], which would account for our observed decrease in signal 2 as well as signal 1.

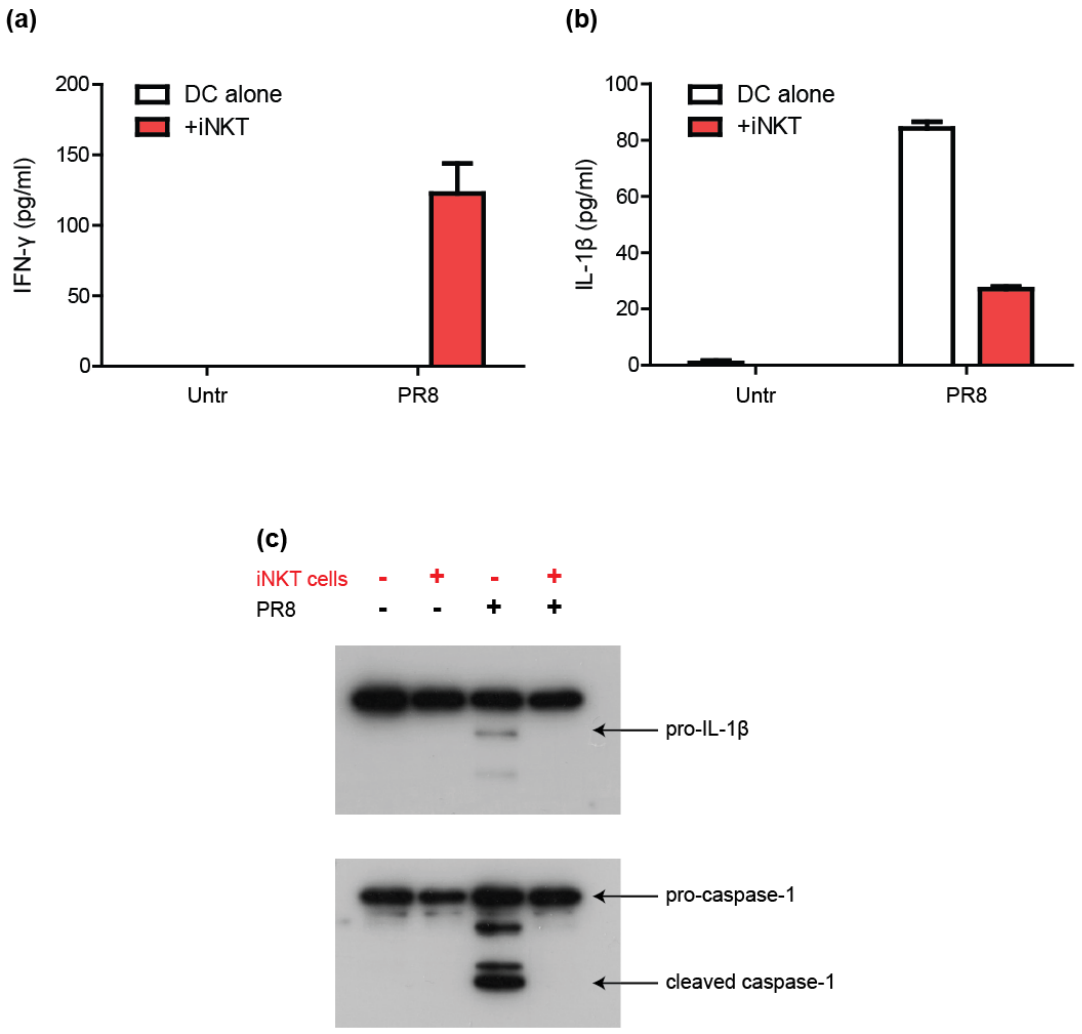

Fig. 5.2 iNKT cells dampen IL-1β release from PR8-infected DCs. Human monocyte-derived DCs were co-incubated with iNKT cells for 14 hours at a ration of 3:1. The cells were then infected with live PR8 at an m.o.i. of 5, with supernatant collected and analysed by ELISA after 24 hours for IFN-γ **(a)** and IL-1β levels **(b)**. In addition, cell lysates were analysed by immunoblotting **(c)**. Data are representative of 2 independent experiments. Bars show the mean of triplicates +/- SEM.

5.3 iNKT cell-deficient animals exhibit greater morbidity during influenza A infection

After showing that iNKT cells could decrease IL-1β production from MdDCs *in vitro*, we moved to an *in vivo* infection system in order to establish the physiological significance of this phenomenon. To do this, we used the *Jα18*$^{-/-}$ mouse strain, which are deficient in iNKT cells due to the lack of the Jα18 gene segment used in the invariant TCR [314]. In our experiments, we infected both WT and *Jα18*$^{-/-}$ animals with 1.8x10^{6} pfu of PR8 virus. In the absence of iNKT cells, our laboratory and others have reported greater morbidity and lower survival rates in *Jα18*$^{-/-}$ mice as compared with the WT when infected with IAV [210, 230]. We confirmed these reports by measuring the weight loss of WT and *Jα18*$^{-/-}$ mice after PR8 infection **(Fig. 5.3)**. In line with these previous reports, we found that *Jα18*$^{-/-}$ animals lost more weight than WT mice, indicating a poorer disease outcome and giving iNKT cells a protective role in our IAV infection model. Interestingly, we did not detect weight loss in the WT mice despite infection with IAV. This discrepancy with other reports could be due to differences in the exact viral dose used and in the quality of each stock of IAV, with our infections giving less severe disease in WT animals.

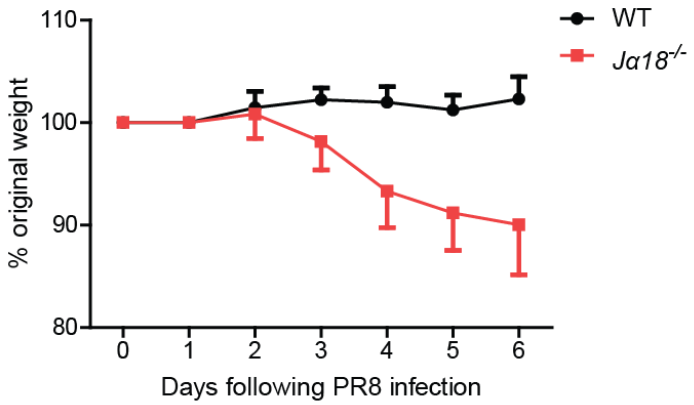

Fig. 5.3 iNKT cell-deficient animals exhibit greater morbidity during PR8-influenza infection. WT or *Jα18*$^{-/-}$ mice were administered with 1.8x10^{6} pfu PR8 virus intranasally. Mice were weighed daily and % original weight calculated. (n=7) Data points show the mean +/- SEM.

5.4 iNKT cells suppress IL-1β production during *in vivo* influenza A virus infection

Having demonstrated that *Jα18*$^{-/-}$ mice are more susceptible to IAV infection, we started to investigate the role of iNKT cells in modulating *in vivo* IL-1β. **Fig. 5.3** shows that *Jα18*$^{-/-}$ mice begin to lose weight at day 3, an early stage in infection before the appearance of influenza-specific T cell responses. This implicates the innate response in the difference between WT and *Jα18*$^{-/-}$ mice which we observe at this timepoint. In addition, IL-1β levels have been shown to peak in the lungs 3 days after infection, becoming undetectable by day 6 ([131] and data not shown). Therefore we decided to examine *in vivo* IL-1β levels at day 3 of infection. This was done by washing the airways of infected animals and uninfected controls by broncho-alveloar lavage (BAL), with the resulting BAL fluid (BALF) analysed by ELISA **(Fig. 5.4a)**. We observed a modest induction of IL-1β in the lungs upon infection of WT mice, but, significantly, we observed higher levels of IL-1β in the lungs of infected *Jα18*$^{-/-}$ animals, suggesting that iNKT cell-deficiency removes inhibitory mechanisms which would normally limit IL-1β production in the lungs of WT animals. In addition, we tested, by ELISA, the levels of IL-1β in the serum of the same animals **(Fig. 5.4b)**. Interestingly, we observed no significant difference between infected WT and *Jα18*$^{-/-}$ animals in terms of IL-1β levels in the serum, suggesting that iNKT cells are important in restraining local IL-1β production at the site of infection, whereas systemic levels of IL-1β remain unaffected.

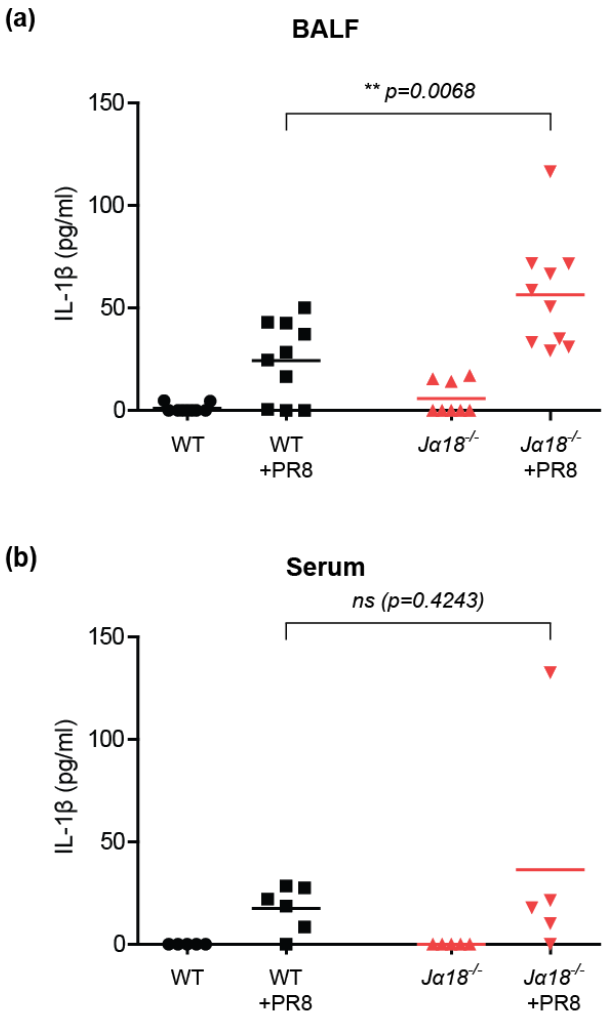

Fig. 5.4 iNKT cell-deficient animals exhibit increased IL-1β levels in the lungs upon infection with PR8 influenza virus. WT or *Jα18*$^{-/-}$ animals were infected with 1.8x10^{6} pfu of PR8 influenza by intranasal instillation. Uninfected animals were given an equivalent volume of PBS. 3 days after infection, BAL fluid **(a)** and serum via tail vein bleed **(b)** were collected, before being analysed by ELISA for IL-1β levels. Data shown are a summary of all animals included in 3 independent experiments. Statistical analysis was performed using Student's t test (two-tailed, unpaired).

5.5 iNKT cells restrain pro-IL-1β induction in lung DCs during influenza A infection

Following our *in vitro* data in human MdDCs showing a negative effect on IL-1β production following their interaction with iNKT cells, we wanted to investigate whether IL-1β induction by DCs *in vivo* could be similarly modulated during infection. In order to do this, we decided to examine the expression of IL-1β in lung DCs during IAV infection. We sorted lung DCs from WT or *Jα18*$^{-/-}$ animals, uninfected or infected with PR8 virus for 3 days, by FACS, using the CD11c^{+}F4/80^{-} phenotype to distinguish lung tissue DCs from macrophages (which are F4/80^{+}) [315]. Expression of IL-1β was then analysed by immunoblotting **(Fig. 5.5a)**. We found that, in WT animals, levels of pro-IL-1β did not increase significantly at day 3 after infection. However, we detected a dramatic increase in the levels of intracellular pro-IL-1β at the same time point in *Jα18*$^{-/-}$ mice. This suggests that, in line with elevated IL-1β levels in the BALF of *Jα18*$^{-/-}$ mice, *in vivo* iNKT cell-deficiency relieves inhibitory mechanisms on pro-IL-1β induction. This, in turn, leads to an increase in IL-1β production during early time points in PR8 infection.

As well as analysing pro-IL-1β levels at the protein level, we proceeded to investigate the modulation of *Il1b* transcript levels in lung DCs during IAV infection **(Fig. 5.5b)**. Mirroring our immunoblotting data, we found only a modest induction of the *Il1b* gene by real-time PCR at day 3 after IAV infection in WT animals, whereas in *Jα18*$^{-/-}$ animals we observed significantly higher levels of the *Il1b* transcript. These *in vivo* data therefore complement our previous *in vitro* work, by identifying iNKT cells as suppressors of IL-1β production. As well as identifying a physiological relevance for our observations, our IAV infection model also then allowed us to investigate the downstream consequences of IL-1β modulation by iNKT cells.

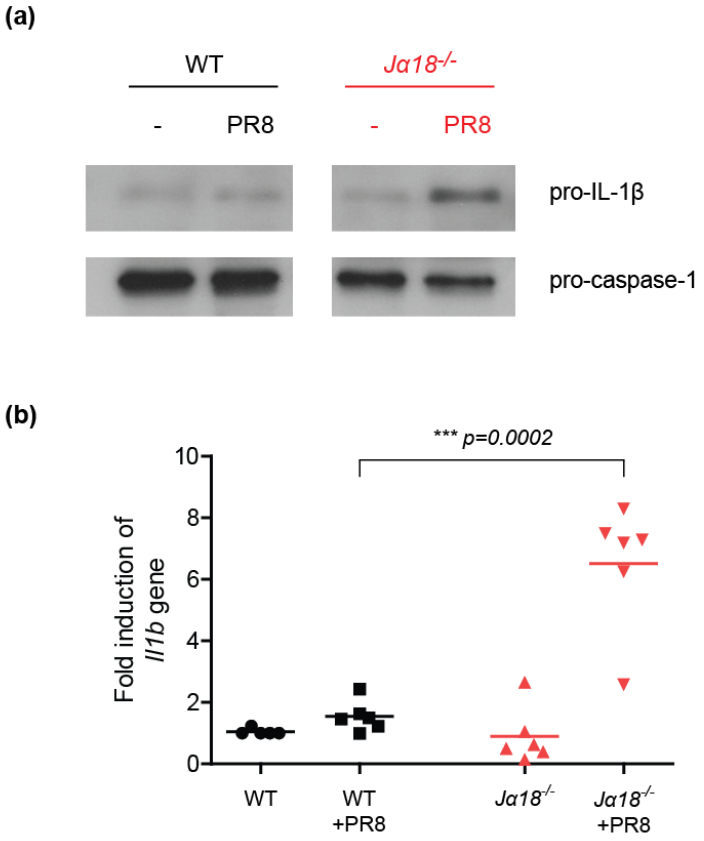

Fig. 5.5 Lung DCs from PR8-infected *Jα18*$^{-/-}$ mice have higher levels of pro-IL-1β than their WT counterparts. Animals were infected with 1.8x10^6 pfu of PR8-influenza as previously described. 3 days after infection, single cell suspensions were prepared from the lung before DCs (identified as CD11c$^+$F4/80$^-$ cells), were sorted by FACS. Cells were then lysed and analysed by immunoblotting **(a)**, where cells were pooled from 3 animals per group and data is representative of 3 independent experiments. Pro-caspase-1 was used as a loading control. Sorted DCs were also analysed by real-time PCR **(b)**, were the data shown includes all animals from 2 independent experiments and statistical analysis was performed using Student's t test.

5.6 Poorer disease outcome in *Jα18*$^{-/-}$ animals is associated with more severe IL-1-mediated neutrophilia

Our finding of more severe disease after IAV infection in *Jα18*$^{-/-}$ mice correlated with higher IL-1β levels. Crucially, this observation was made at an early time point in infection (day 3), before appreciable levels of antibody and T cell responses are detected. This therefore led us to investigate whether the poorer disease outcome in iNKT cell-deficient animals may be due to differences in the innate immune system and modulation of the inflammatory response in the lungs. Firstly, we observed significant hepatisation of the lungs in IAV infected *Jα18*$^{-/-}$ mice, but not WT mice (data not shown). Seen as large areas of acute exudation giving a red, liver-like gross appearance, lung hepatisation is characteristic of an acute, lobar pneumonia-like disease affecting the deep lung tissue. As a consequence of this observation, we went on to investigate more quantifiable aspects of the inflammatory state of IAV infected WT and *Jα18*$^{-/-}$ animals. To do this, we examined the neutrophilia within the lungs of these mice, as neutrophils, as well as being recruited early during infection with important roles in the inflammatory process, have also been described to be key mediators of immunopathology during influenza infection [316].

We first confirmed that neutrophilia during the early phase of IAV infection is dependent on IL-1R1 signalling, as has been described [178]. To do this, we infected WT and *Il1r1*$^{-/-}$ mice with the same dose of PR8 as before and analysed lung neutrophilia at day 3. Total neutrophil count was ascertained using the total number of cells in the BALF (counted by haemocytometer) together with the percentage of $CD11b^{+}Ly6G^{+}$ cells as analysed by flow cytometry. Confirming the previous report, we were unable to detect significant increases in neutrophil numbers in the BALF of *Il1r1*$^{-/-}$ animals at this time point, whereas in WT animals we observed a significant neutrophil influx **(Fig. 5.6a)**. This demonstrates that neutrophilia in the lungs during the early phase of PR8 infection, at least up to day 3, is dependent on IL-1R1 signalling. This therefore places IL-1α and IL-1β as important components in the early inflammatory response to IAV. As a control, we observed that the induction of IL-1β in the BALF was still present in *Il1r1*$^{-/-}$animals **(Fig. 5.6b)**, showing that these animals, despite having no neutrophilia, were nevertheless infected with IAV.

We then proceeded to compare lung neutrophil influx in WT and *Jα18*$^{-/-}$ mice upon IAV infection. In line with the observed lung hepatisation and gross pathology in *Jα18*$^{-/-}$ animals, neutrophil counts were significantly higher in iNKT cell-deficient mice as compared with WT mice **(Fig. 5.6a)**.

Taking all these data into account, we therefore conclude that, in WT animals, iNKT cells are responsible for restraining IL-1β production in the lungs during severe IAV infection. This was observed in the airways (BALF) as well as in lung tissue DCs. The inhibition of IL-1β production also serves to limit IL-1R1-dependent neutrophilia in the lungs. In the absence of iNKT cells, lung IL-1β levels are elevated, leading to more severe neutrophilia and therefore acute immunopathology, with an acute pneumonia-like disease, leading to greater morbidity during PR8 infection. We therefore describe, for the first time, a novel anti-inflammatory role of iNKT cells during IAV infection, which is nevertheless protective due to its inhibition of severe immunopathology.

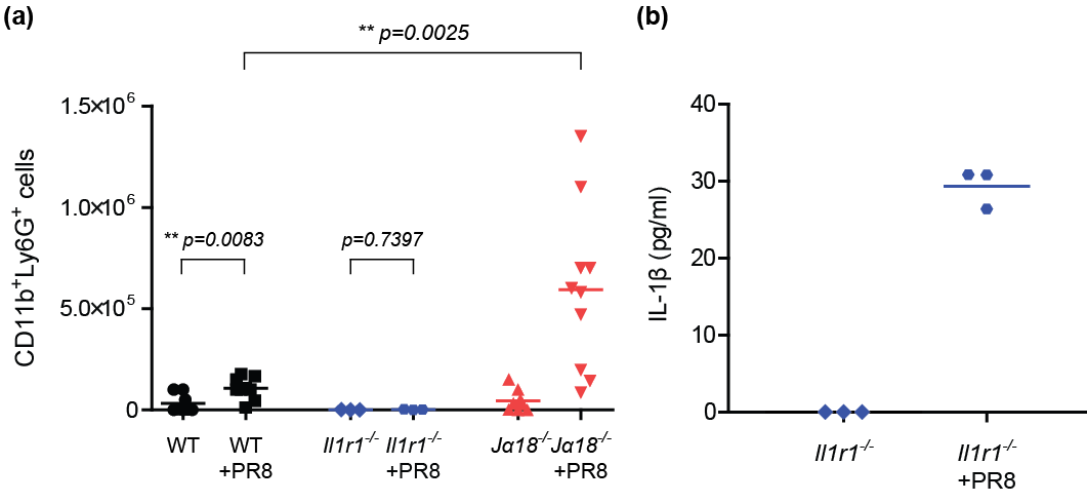

Fig. 5.6 More severe IL-1R1-dependent neutrophilia in iNKT cell-deficient animals infected with PR8 influenza. **(a)** WT, *Il1r1*$^{-/-}$ or *Jα18*$^{-/-}$ animals were infected with 1.8x10^6 pfu PR8 virus and BALF analysed for the number of neutrophils (CD11b$^+$Ly6G$^+$) at day 3. This was calculated using the total number of cells in the BALF (counted by haemocytometer) and the percentage of CD11b$^+$Ly6G$^+$ cells (by flow cytometry). Each data point represents one animal. **(b)** As a control to verify infection, BALF from uninfected and infected *Il1r1*$^{-/-}$ animals were analysed for IL-1β levels by ELISA.

5.7 The role of iNKT cells in induction of influenza A virus-specific T cell responses

In the literature, the association of iNKT cells with influenza virus infection has mostly been described as a pro-inflammatory one, with iNKT cells being important for generating protective influenza-specific adaptive immunity and abolishing mechanisms which suppress influenza-specific T cell responses [210, 317]. However, we have shown an anti-inflammatory role for iNKT cells in the early stages of IAV infection. In the context of the dual nature of iNKT cells with both pro- and anti-inflammatory potential, we investigated whether iNKT cells could influence the IAV-specific adaptive response in the same animal model.

To do this, we analysed the T cell response in WT or *Jα18*$^{-/-}$ animals infected with lower titres of PR8 than in our previous experiments, such that survival rates were higher with 100% recovery from the initial severe disease, even in iNKT cell-deficient mice. At day 20 after infection, we restimulated splenocytes with live PR8 virus or an MHC class I-restricted peptide from the IAV NP protein, and used IFN-γ release as detected by ELISA as a readout for the presence of IAV-specific T cell responses **(Fig. 5.7)**. The ovalbumin-derived class I-restricted peptide SIINFEKL and PMA with ionomycin were used in the restimulations as negative and positive controls respectively. We found lower levels of IFN-γ release in splenocytes from infected *Jα18*$^{-/-}$ mice than WT mice when restimulated with PR8 virus, suggesting that iNKT cells are important in inducing an effective T cell response against the virus. Interestingly, when the class I-restricted NP peptide was used as a stimulus for restimulation, we observed an equal level of IFN-γ release from WT and *Jα18*$^{-/-}$ splenocytes. One explanation of this apparent discrepancy is that CD4^{+} T cell responses, which would only be restimulated by whole virus after processing and cross-presentation, are more dependent on iNKT cells than CD8^{+} T cell responses. Nevertheless, we have shown that, despite an anti-inflammatory role of iNKT cells in suppressing the innate immune response during the early stages of IAV infection, at later time points adaptive immune responses remain either unaltered (in the case of CD8^{+} responses) or are boosted (in the case of CD4^{+} responses) by the presence of iNKT cells.

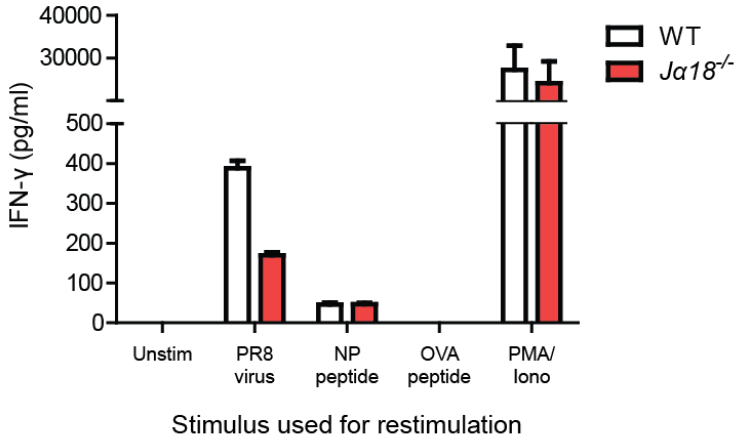

Fig. 5.7 Analysis of influenza-specific T cell responses in WT and iNKT cell-deficient animals. WT or *Jα18*$^{-/-}$ animals were infected with 0.9x10^6 PR8 influenza virus. 20 days later, animals were sacrificed and 1x10^6 splenocytes restimulated with the stimuli as shown. 72 hours after restimulation, cell supernatants were harvested and analysed for IFN-γ secretion by ELISA. PR8 virus used for restimlation was added at 10^6 pfu, NP peptide (ASNENMETM) and OVA peptide (SIINFEKL) at 20μM, and PMA and ionomycin at 1μg/ml. Data bars represent the mean of quadruplicates +/- SEM.

5.8 Modulation of sterile pulmonary inflammation by iNKT cells

During infection, both local and systemic levels of acute phase proteins increase dramatically. These include SAA, whose concentration in the serum can increase by up to 3 orders of magnitude into the mg/ml range. In PR8 infection, increases in SAA levels can be detected both in the BALF and serum (data not shown). Extending our results showing increased lung IL-1β levels in *Jα18*$^{-/-}$ animals, we induced sterile inflammation in the lung by instilling SAA into the airways intranasally. We and others have previously shown that this method of SAA administration induces the release of IL-1β into the airways (**Fig. 3.8a**, [275]). In line with our IAV infection data, we found that BALF IL-1β levels were significantly higher in *Jα18*$^{-/-}$ mice than WT animals **(Fig. 5.8)**. This confirms, in another *in vivo* model, that iNKT cell deficiency results in increased IL-1β levels in the lungs, again giving iNKT cells an anti-inflammatory role in inhibiting IL-1β production.

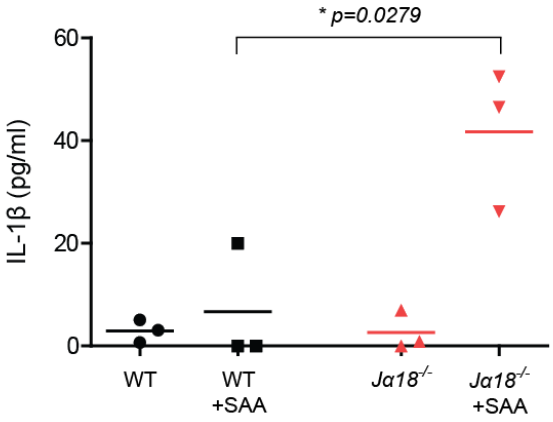

Fig. 5.8 iNKT cell-deficient mice have increased IL-1β levels in the lung following intranasal administration of SAA. WT or *Jα18*$^{-/-}$ mice were instilled with 10μg SAA per mouse. 4 hours later, animals were sacrificed and BALF harvested, and analysed by ELISA. Data are representative of 3 independent experiments, each with n≥3.

5.9 Discussion and future perspectives

5.9.1 Induction of bioactive IL-1β by influenza virus

We have confirmed, in an *in vitro* system, that IAV infection of human MdDCs can directly induce pro-IL-1β production together with its cleavage into the bioactive cytokine. Interestingly, influenza virus is able to provide both signals 1 and 2 for bioactive IL-1β production. However, the exact mechanism of activation of both pro-IL-1β induction and inflammasome activation has only recently been elucidated. The ability of immune cells to recognise viral RNA by TLR7 and RIG-I [318, 319] and activate signalling pathways culminating in NF-κB activation provided important insights into the mechanism of influenza virus-induced signal 1. Through the use of various knockout mice, a number of groups were then able to confirm, both *in vitro* and *in vivo*, that influenza virus activates the NLRP3 inflammasome to induce IL-1β cleavage [320, 321]. This was expanded upon in elegant experiments performed by Ichinohe and colleagues [120], who showed, at least in mouse BMMs and BMDCs, that recognition of viral RNA in the endosomes by TLR7 is the activating signal for pro-IL-1β induction. The authors also found that IAV fusion and replication were required to stimulate the NLRP3 inflammasome and subsequent IL-1β cleavage. They identified the virally-encoded M2 protein as a key protein in stimulation of NLRP3. M2 is a proton channel which is essential in the replication cycle of IAV. Firstly, it deacidifies endosomes carrying virus particles which are entering the cell. This equilibration of the pH of virus-bearing endosomes allows the uncoating of the virus and subsequent injection of the viral genome into the cytoplasm [322]. Secondly, M2 is also required at a later stage of viral replication by neutralising the pH of the trans-Golgi network. This alters the conformation of haemagglutinin molecules on newly-formed virions in order for the virus to bud efficiently from intracellular organelles [322]. In the context of inflammasome activation, proton efflux from the Golgi apparatus, induced by M2, was found to be responsible of NLRP3 activation by influenza virus.

Our finding that human DCs also respond to influenza virus by releasing bioactive IL-1β raises the question of whether a similar mechanism of IL-1β production is operating within human cells. Our recent understanding of the exact molecular mechanisms of IL-1β induction by IAV may open up new therapeutic avenues for modulating lung inflammation during influenza infection. As M2 is crucial for in the viral replication cycle and in NLPR3 inflammasome activation, disruption of this protein's biological activation could, in one strike, target both viral replication and reduce inflammation.

However, amantadine and rimantadine, the only two M2-targeting antiviral drugs licensed for use, are now no longer recommended in severe influenza infection as many circulating influenza viruses have already developed drug resistance [323].

5.9.2 The importance of IL-1β during influenza virus infection

The observation that IAV could induce the production of bioactive IL-1β from infected cells suggests that IL-1β plays an important role in anti-influenza immunity. With the discovery that IAV-induced IL-1β cleavage is dependent on the NLRP3 inflammasome [321], the role played by IL-1β during influenza infection could be dissected using animals deficient in NLRP3, ASC and caspase-1. Three independent reports all identify the inability of NLRP3, ASC and caspase-1 deficient animals to secrete IL-1β into the BALF in response to IAV infection [131, 324, 325]. All these studies identified the clearance of viral loads late in infection (7-10 days after infection) as being dependent on the NLRP3 inflammasome complex and/or IL-1R1 signalling. Despite these reports each highlighting different mechanisms (decreased inflammatory cytokine production, increased epithelial damage and decreased T cell and antibody responses respectively), all three groups identified components of the NLRP3 inflammasome as being crucial in anti-influenza immunity. Interestingly, during low-dose IAV infection, mixed bone marrow chimera experiments revealed that caspase-1 expression was only required in the haematopoietic compartment for IL-1β secretion into the alveolar space (BALF) [131], showing that immune cells are responsible for producing bioactive IL-1β in this model.

Detection of lung IL-1β levels has largely been done three days after infection, as this timepoint coincides with the peak in IL-1β production. However, given the effect of NLRP3 deficiency on affecting control of virus persistence 7-10 days after infection, it would be important to examine the role of IL-1β at later time points. We have preliminary observations that at day 6 post-infection, BALF IL-1β is no longer detected, although lung-infiltrating neutrophil numbers continue to increase, especially in the absence of iNKT cells (data not shown). This may point to alternative mechanisms which take over control of pulmonary inflammation after the initial spike in IL-1β levels. Indeed, a gradual increase in lung neutrophil numbers has been demonstrated in *Il1r1*$^{-/-}$ mice after day 5 [178].

5.9.3 Innate immunopathology during influenza virus infection

One detrimental effect of IL-1β production during influenza infection is its potential to initiate an inflammatory cascade which could lead to severe immunopathology. Indeed, histological and pathological analyses in several species, including humans, suggest that influenza-induced mortality is associated with pulmonary immunopathology, rather than uncontrolled viral dissemination [326]. Therefore a fine balance in the inflammatory response, between limiting viral replication and excessive tissue damage, is required.

During the first few days of influenza infection, a 'cytokine storm' is induced, wherein the host attempts to mount a powerful immune response to control the infection. A spike in the levels of various pro-inflammatory cytokines, including TNFα, IL-1α, IL-1β, IL-6 and IL-17, are accompanied by a dramatic increase in the levels of macrophage and neutrophil-recruiting chemokines such as MIP-1α, MCP-1 and KC (or CCL3, CCL2 and CXCL1, respectively) [327]. Subsequent studies in various knockout and depletion models suggested that TNFα, IL-6 and MCP-1 blockade were unable to increase survival [328], although the contribution of these factors to overzealous inflammation, perhaps playing a redundant role with other pro-inflammatory cytokines, cannot be excluded. Analysis of the early stages of IAV infection in *Il1r1*$^{-/-}$ animals has revealed a defect in the recruitment of inflammatory cells into the lungs, specifically neutrophils [178], an observation which we have confirmed. Although *Il1r1*$^{-/-}$ mice were less able to clear the virus later on, one striking observation was markedly decreased morbidity and lung immunopathology in the first three days of infection. Another report, using *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ animals, also observed decreased neutrophil and monocytic DC numbers at the same time point [325]. These data suggest that, at least in models where animals are infected with a high dose of virus, IL-1β is responsible for recruiting cells which are detrimental to the host due to an exacerbated inflammatory response. Certainly, IL-1R1 signalling has been shown to be important in inducing emergency granulopoiesis, which expands the pool of neutrophil precursors in the bone marrow to replenish effectors which differentiate and enter the site of infection [161].

Here, we have used neutrophilia in the lungs during IAV infection as one indicator of the inflammatory response. Like pro-inflammatory cytokines, neutrophils appear to play a dual role during influenza infection, with some reports indicating that neutrophils are important in host defence, by clearing infected and dying cells, and controlling viral dissemination. This is supported by reports where depletion of neutrophils led to greater weight loss and viral load [329, 330]. However, increasing

evidence points to the neutrophil population as mediators of excessive inflammation. Neutrophils release potent anti-microbial enzymes such as myeloperoxidases and form neutrophil extracellular traps (NETs) which become entangled in alveoli and areas of tissue damage, further contributing to lung dysfunction [331]. Some models of influenza infection showing decreased neutrophil infiltration have been associated with lower mortality and more efficient viral clearance, thus also suggesting that neutrophils can play a detrimental role [332].

Considering the importance of IL-1β and neutrophil recruitment into the lungs in the early stages of influenza infection, and the fine balance required to control virus replication but limit immunopathology, our observation that iNKT cells can limit IL-1β production gives us a novel insight into the complex regulatory network which coordinates the inflammatory process.

5.9.4 iNKT cells in influenza virus infection – pro- or anti-inflammatory?

Our finding that iNKT cells play an anti-inflammatory, yet protective, role early in infection adds to the complexity of iNKT cell biology, particularly in the context of influenza infection. Most early reports investigating the role of iNKT cells in IAV infection have found a pro-inflammatory and protective role for this population. Whilst activation of iNKT cells *in vivo* by injection of α-GalCer enhances antiviral immunity and improves survival with an increase in early polymorphonuclear infiltrates in the lungs [317], iNKT cells can also interact with MDSCs to abolish their suppressive activity and restore influenza-specific T cell responses [210]. In addition, vaccination against IAV antigens adjuvanted with α-GalCer boosts antiviral immunity during subsequent challenge [333]. In this model, iNKT cell numbers following α-GalCer instillation increased in the nasal tissues and iNKT cells were found to associate with DCs and produce IL-4, which was key in inducing mucosal immunity via the production of secreted IgA. Furthermore, iNKT cells have also been suggested to promote cell-mediated immunity during IAV infection by secreting IFN-γ to boost the antiviral activity of NK and $CD8^+$ T cells [334].

Our observation that iNKT cells dampen IL-1β production in the lung, thus giving iNKT cells an anti-inflammatory role, is in line with a recently emerging picture where iNKT cells were found to be protective by limiting pulmonary inflammation. Infection of *Jα18*$^{-/-}$ animals with H3N2 IAV resulted in a decreased $CD8^+$ T cell response in the draining lymph nodes but, interestingly, increased pneumopathy [335]. Analysis at day 4 after infection revealed increased infiltration of neutrophils and

macrophages in the lungs. In addition, Kok and colleagues have shown that *Jα18*$^{-/-}$ mice have a greater inflammatory infiltrate at early stages of high dose, lethal H1N1 IAV infection [230]. However, this experimental system focused on inflammatory monocytes as the main constituent of the cellular infiltrate, and the authors were unable to detect appreciable numbers of neutrophils in the lung. The disparity between our model, where neutrophilia was detected, and this report could be due to the use of different doses of virus. In our system, a sublethal dose for the WT is used, and WT mice suffer from less severe disease and weight loss than the high dose model. Nevertheless, these papers are consistent with our observation that iNKT cells can limit the accumulation of innate cells in the lungs, particularly at the early stages of acute IAV infection. These data suggest that iNKT cells play an important physiological role in limiting innate pulmonary immunopathology during the early stages of influenza infection.

5.9.5 Consequences for the adaptive response

The anti-inflammatory role iNKT cells we describe in this influenza model needs to be rationalised in the context of reports describing the ability of iNKT cells to boost adaptive immunity to various infections and model antigens [243]. Indeed, our laboratory has shown that iNKT cells can enhance IAV-specific T cell responses by abolishing the suppressive activity of MDSCs to remove their inhibitory effect [210]. Although we have not investigated the role of MDSCs in our experiments, we have confirmed that splenocytes from PR8-infected *Jα18*$^{-/-}$ mice produce less IFN-γ than WT mice when restimulated with live PR8. Importantly, these data were generated using the exact same virus as our studies on IL-1β levels and neutrophilia, strongly suggesting that iNKT cells can play a dual role in influenza immunity.

The increased induction in IL-1β levels in *Jα18*$^{-/-}$ mice after SAA administration complements unpublished data, also from our laboratory, showing higher levels of neutrophilia in these mice than WT animals. Whilst this is consistent with our observations in IAV infection, it will be of interest to analyse the phenotype of these cells. De Santo and colleagues have demonstrated the ability of SAA to expand the neutrophil population *in vivo* [62]. Importantly, SAA also induces IL-10 production in these neutrophils, with this polarising effect reversed by iNKT cells. In our IAV infection model, it will therefore be important to analyse the expanded neutrophil population in *Jα18*$^{-/-}$ mice, and to test

whether these cells are able to produce IL-10. This will add another level of complexity in the role of iNKT cells *in vivo* during infection.

5.9.6 The dual action of iNKT cells *in vivo*

Crucially, many reports describing iNKT cell activity *in vivo* report the interaction of iNKT cells with other cell types to mediate their pro- or anti-inflammatory effect. These range from lung DCs [334] and MDSCs [210] to plasmacytoid DCs in the pancreas [249]. Therefore, whether iNKT cell activity results in a pro- or anti-inflammatory outcome could conceivably depend on the nature of their crosstalk with other cell types – including the affinity, timing and location of the interaction. In addition, these variables are likely to change depending on the severity of influenza infection, whether the experimental model is characterised by a mild or highly active inflammatory response, and the time point at which animals are analysed.

As we have mentioned previously, the affinity of interaction between iNKT cells and APCs has the potential to shape the nature of iNKT cell activation, with basal autoreactivity likely to result in IL-1β dampening in DCs. Whereas autoreactive recognition at a basal level on APCs induces the release of GM-CSF and IL-13 without significant upregulation of iNKT cell activation markers CD69 and CD25, addition of α-GalCer leads to greatly enhanced activation, characterised by increases in CD69 and CD25 expression and the additional production of high levels of IFN-γ and IL-4 (confirmed by [216]). We have shown that influenza infection can promote the ability of APCs to activate iNKT cells and induce IFN-γ release, and others have reported the ability of iNKT cells to directly lyse infected monocytes in a CD1d-dependent manner [230]. Whilst the exact identity of the glycolipid(s) recognised in this case remains unclear, as with most viral infections capable of activating iNKT cells, it is likely to be host-derived. The ability of influenza-infected DCs to elicit IFN-γ production already implies that DC-iNKT cell crosstalk in this system is stronger than the level of basal autoreactivity. However, these *in vitro* experiments were performed using a high titre of IAV and purified populations of cells to ensure efficient interaction. Therefore, the nature of iNKT cell-DC interactions during *in vivo* IAV infection remains unknown, and further investigation is required to elucidate the affinity of this crosstalk.

With the affinity of iNKT cell-APC recognition playing such a crucial part in determining the subsequent cytokine profile of iNKT cells, the timing and location of this interaction then become important

considerations in interpreting the *Jα18*$^{-/-}$ phenotype. iNKT cells have been shown, via intravital imaging techniques, to crawl in liver sinusoids and touch Kupffer cells even in the absence of infection [228]. Gumperz and colleagues also showed that while iNKT cell-DC interaction via basal autoreactivity was short-lived, addition of α-GalCer induced arrest of iNKT cells and lengthened interaction [216]. This brings about the possibility that iNKT cell interaction with tissue-resident APCs (such as DCs and macrophages) in the uninfected, unstimulated, state could occur continuously, at the basal autoreactive level. During subsequent influenza infection, this then leads to a decrease in pro-IL-1β in iNKT cell-preconditioned lung APCs. However, if APCs are not pre-conditioned and their interaction with iNKT cells occurs after PRR activation during infection (eg. in the draining lymph node), crosstalk between activated APCs and iNKT cells would lead to a stronger affinity CD1d-TCR interaction and the release of IFN-γ and IL-4, which are important in driving the influenza-specific T and B cell responses. Therefore, depending on the timing and location of the interaction, iNKT cells could be activated differentially, suppressing IL-1β and innate infiltration in the lung tissue, whilst enhancing adaptive immune responses in the mediastinal lymph node. This could also explain the differences in IL-1β modulation by iNKT cells in the BALF and serum, with inhibition of IL-1β only seen in the infected tissue. In addition, our observation of decreased splenic T cell responses in *Jα18*$^{-/-}$ animals despite increased IL-1β levels in the lung could also be due to differential effects of iNKT cells depending on physiological location.

5.9.7 Further questions and future perspectives

In summary, we have shown that iNKT cell deficiency leads to increased IL-1β levels in the lung during PR8 IAV infection, and that this leads to exacerbated neutrophilia which is associated with increased morbidity. However, various questions still remain which will be of interest in further investigation.

Firstly, it will be important to investigate which cell type(s) interacts with iNKT cells in the lungs. Although we show an increase of intracellular IL-1β in lung tissue DCs and that PR8 virus can induce iNKT cell crosstalk with DCs, we should not exclude the potential of iNKT cells to influence the response of other key producers of IL-1β in the lung, especially macrophages and epithelial cells. Epithelial cells are the first cell type infected during influenza infection, and have been shown to express inflammasome components [336]. Although non-haematopoietic cells were shown to not be

responsible for IL-1β production into the alveolar space in low-dose IAV infection [131], our experiments utilise a higher dose of virus. Considering the massive disruption of epithelial architecture during high dose PR8-induced inflammation, epithelial cells could be major producers of IL-1β in the lungs during influenza infection in our model. In fact, a protective role for iNKT cells has been posited, where DC-iNKT cell interaction induces the release of IL-22 from iNKT cells, which rescues lung epithelial cells from mortality [337]. Considered together with a recent report describing CD1d expression on lung epithelium and their ability to crosstalk with iNKT cells [338], it would be interesting to investigate whether disrupting iNKT cell-epithelium interactions would reproduce the *Jα18*$^{-/-}$ phenotype early in infection.

Secondly, despite the overt phenotype we observe in iNKT cell-deficient animals, the number of iNKT cells in the lungs, especially in the absence of infection, remains low, at 1-2% of $CD3^+$ cells in the uninfected lung, increasing to 3-4% during IAV infection [317]. The question therefore arises whether this small population is sufficient to condition APCs in the lungs, or whether lung iNKT cells cooperate with iNKT cell populations at other anatomical sites. Further work will be required to elucidate whether iNKT cell-APC crosstalk which we have hypothesised occurs in the lungs or, rather, at another anatomical site where iNKT cells are more abundant (eg. liver or spleen), before cells then migrate into the lung tissue during early IAV infection.

In addition, it would be interesting to extend our observations to other models of infection, at other anatomical sites. IL-1β levels and neutrophilia have already been reported to be elevated in the lung during *Legionella pneumophila* infection of iNKT cell-deficient mice [255]. Whether this anti-inflammatory action of iNKT cells is limited to the lung, or can extend to infections at other mucosal and non-mucosal sites, would be an important question to address. Also, given the role of IL-1β-associated inflammation in various autoimmune diseases, the ability of iNKT cells to dampen inflammation could be harnessed to develop novel strategies to alleviate the symptoms of these disorders.

CHAPTER 6

Synthesis

Chapter 6: Synthesis

6.1 Summary of results

In this thesis we have focused on the pro-inflammatory cytokine IL-1β, identifying a novel mechanism for its induction via SAA, before investigating its modulation by iNKT cells. Through these studies, we have extended our knowledge of the interplay between IL-1β and the acute phase response, and elucidated a physiological mechanism by which inflammation could be controlled in the tissues during infection. Using our *in vivo* model of influenza virus infection, we have also established an experimental system which can be extended in the future to delve into the mechanisms by which iNKT cells can modulate the immune response.

In chapter 3, we demonstrated that SAA, an acute phase protein, was able to induce the production of bioactive IL-1β in both human and mouse myeloid cells by signalling through TLR2 and activating the NLRP3 inflammasome. We also observed the ability of SAA to induce IL-1β secretion and IL-1R1-dependent neutrophilia *in vivo*, validating the physiological relevance of this experimental system.

Previous work in our laboratory has demonstrated that SAA is able to induce crosstalk between iNKT cells and neutrophils; in chapter 4, we extended this finding to human MdDCs before proceeding to investigate the effect of this interaction on IL-1β production. We used a pre-conditioning system, based on the basal autoreactivity of iNKT cells, to model the interaction between iNKT cells and immature APCs before the onset of infection and TLR ligation. We found that iNKT cells were able to exert an anti-inflammatory effect, dampening the secretion of IL-1β via weak activation of the CD40 pathway and the secretion of the anti-inflammatory cytokine IL-13.

These results were then corroborated in chapter 5 using an *in vivo* infection model, where we infected WT or iNKT cell-deficient mice with influenza A virus. We demonstrated that the absence of iNKT cells led to increased levels of IL-1β in the lungs, as well as increased neutrophil recruitment, which was associated with increased morbidity. This suggested that iNKT cells play a protective role during influenza virus-induced pulmonary inflammation, dampening the influx of inflammatory cells.

These studies have identified iNKT cells as important modulators of the innate response by affecting the production of IL-1β, a key cytokine in inflammation. The ability of SAA to induce IL-1β, as well as

other pro- and anti-inflammatory cytokines, could also allow it to shape the immune response. Through studying their effects on IL-1β, we have therefore revealed novel pathways by which immunity is coordinated during the early stages of infection, and these findings may have important implications in our understanding of autoinflammatory conditions and their therapeutic intervention.

6.2 Discussion – Dichotomy in the immune response

In this thesis, we have focused on the mechanisms by which inflammation can be modulated. Taking into account our experimental data as a whole, it is clear that each element of the immune response we have analysed (SAA, iNKT cells and IL-1β) have dual effects, potentially leading to pro- or anti-inflammatory effects in the case of SAA and iNKT cells, and mediating protective immunity or detrimental pathology in the case of IL-1β. We can hypothesise that this dichotomy is responsible for maintaining the delicate balance between raising an effective immune response and preventing immunopathology, and in this final discussion we will consider this concept in a physiological context, as well as identify areas where further investigation is warranted.

6.2.1 Interplay between SAA and IL-1β

Our finding that SAA can provide both signals for bioactive IL-1β synthesis places this acute phase reactant in a positive-feedback loop that amplifies inflammatory signals. Given that serum SAA levels can increase to the mg/ml range during infection [56], this suggests SAA may have an important role in transmitting a pro-inflammatory signal to sites of the body distant from the infected tissues. SAA is induced by IL-6 and IL-1 [59]; although SAA is mainly produced by hepatocytes, its secretion by myeloid cells and tumours has also been documented [57, 58]. Thus, IL-1β could also increase local concentrations of SAA, which may be important in immunomodulation at sites of infection or tumour growth. SAA and IL-1β therefore participate in a complex network of signals which coordinate the immune response (summarised in **Fig. 6.1**).

We have shown that, as well as IL-1β, SAA has the ability to induce the production of various pro- and anti-inflammatory cytokines, including TNF-α, IL-6, IL-10 and TGF-β **(Fig. 3.3)**. This suggests that SAA is important in shaping the inflammatory response, initially driving inflammation but subsequently shutting down innate immunity to prevent excessive tissue damage. Indeed, preliminary analysis of the gene transcripts induced by SAA shows that *Il1b* mRNA accumulates rapidly, whereas the *Il10* transcript peaks at a much later time point (unpublished observations from our laboratory). This implies a temporal control over the induction of cytokines, initially with the production of pro-inflammatory molecules before a later wave of anti-inflammatory cytokines. Kinetic analysis of a wider panel of pro- and anti-inflammatory factors would be important to confirm this hypothesis.

In addition, whether SAA levels in the body are transiently or chronically elevated may be important. Significantly, our laboratory has demonstrated that SAA can induce the differentiation of immunosuppressive, IL-10-producing neutrophils [62]. Clinically, chronically high levels SAA in the body during late-stage melanoma were also shown to correlate with the accumulation of these MDSCs. Given that IL-1β and chronic inflammation are also associated with MDSC expansion and carcinogenesis [165, 168], prolonged elevation of SAA levels may contribute to an inflammatory milieu which supports tumorigenesis. In addition, SAA may also induce MDSC accumulation to establish a suppressive environment which inhibits the anti-tumour adaptive response.

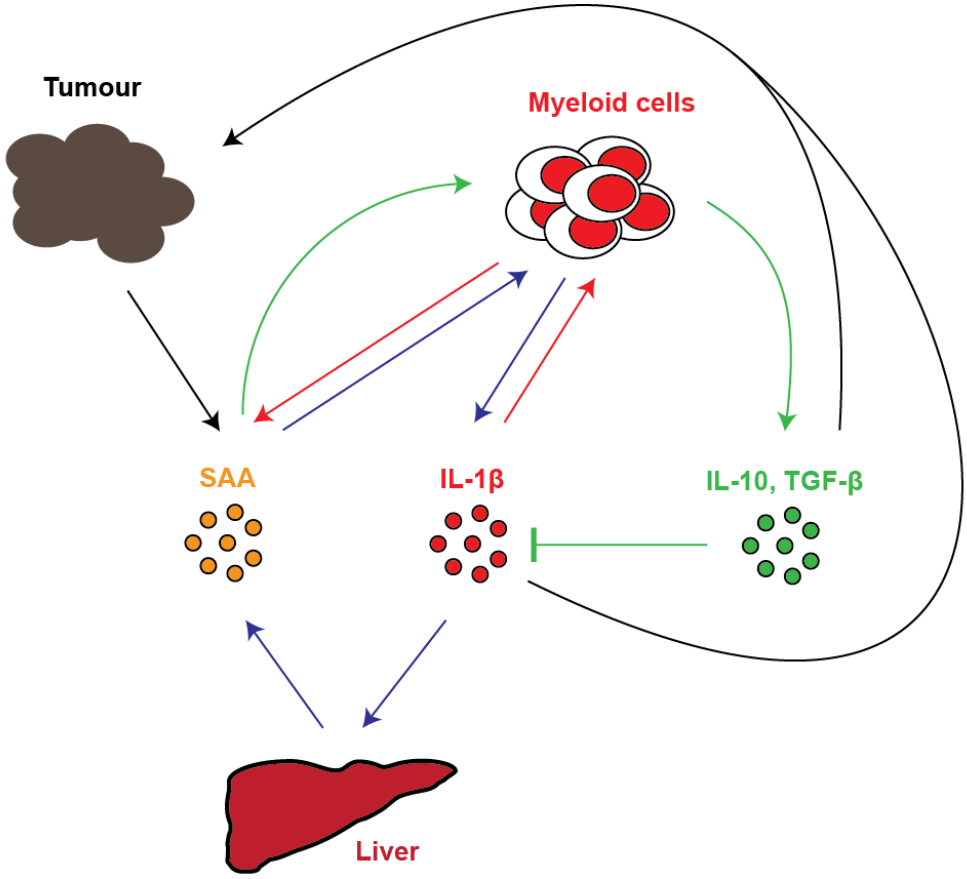

Fig. 6.1 Interactions of SAA and IL-1β. SAA and IL-1β reciprocally induce each other's synthesis in myeloid cells and hepatocytes, establishing a self-amplifying inflammatory loop which can also lead to tumour development. In addition, SAA can induce anti-inflammatory cytokines such as IL-10 and TGF-β, which can inhibit further IL-1β production. These cytokines are also important for establishing an immunosuppressive environment, abolishing anti-tumour responses.

The ability of SAA to signal through various different cell surface receptors [56] is consistent with its pleiotropic role in the immune system (summarised in **Fig. 1.3**). More detailed understanding of each SAA signalling pathway may allow the development of therapeutic agents which specifically target certain effects. For example, blockade of pro-inflammatory cytokine production may be beneficial in treating inflammatory diseases, whereas inhibiting SAA-induced IL-10 secretion may be an effective complement to anti-tumour therapies. In this way, a better understanding of the molecular mechanisms underpinning the effects of SAA may allow us to harness its multiple roles to manipulate the immune system.

6.2.2 iNKT cells as modulators of the immune response

We have identified a novel, anti-inflammatory role of iNKT cells in their ability to dampen IL-1β secretion from DCs. This finding adds to the complex biology of iNKT cells, which can mediate both pro- and anti-inflammatory functions [217]. We have also hypothesised that the activation strength of the iNKT cell, and therefore the timing of the interaction with APCs, are crucial in determining the outcome of the iNKT cell response.

We demonstrated that weak CD40 signals, whilst able to pre-condition MdDCs to release lower levels of IL-1β, are unable to directly induce cytokine production **(Fig. 4.3.2)**. This is consistent with the interaction between iNKT cell and DC at a basal autoreactive level, where interaction leads to IL-1β inhibition in the absence of IL-12 production **(Fig. 4.2.1, 4.2.2)**. A weak signal through the semi-invariant TCR is also important in inducing an IL-13-dominated cytokine profile from the iNKT cell, which we have also shown to be involved in their anti-inflammatory effect **(Fig. 4.4)**. We can therefore conclude that a weak iNKT cell-APC interaction results in an anti-inflammatory outcome, whereas stronger iNKT cell activation (for example, by α-GalCer) leads to the induction of pro-inflammatory factors such as IFN-γ **(Fig. 4.2.6)**, which overrides the inhibitory signal. We have used this system as a model for the crosstalk between iNKT cells and APCs in the resting state, an interaction demonstrated by Lee and colleagues to be continuously occurring *in vivo* [228]. This interaction will therefore be important in the tissues by modulating the response of APCs upon detection of pathogens, with decreased secretion of IL-1β leading to the prevention of exacerbated inflammation and immunopathology.

We have also shown that the absence of iNKT cells leads to dysregulation of this system in an *in vivo* influenza infection model, resulting in increased morbidity **(Fig. 5.3)**. This therefore supports our hypothesis, that iNKT cell-interaction with APCs is responsible for restraining the inflammatory response upon infection. However, further investigation is needed to determine the location and nature of this interaction in the resting state. With few iNKT cells in the lung in the absence of infection, it is conceivable that the pre-conditioning of APCs may occur at another anatomical site before they migrate into the lungs. In addition, the interaction of iNKT cells with other cell types which release IL-1β should be investigated, to fully understand how iNKT cells can coordinate inflammation. The ability of iNKT cells to affect pro-IL-1β production, the first step in IL-1β biosynthesis, allows iNKT cells to

affect IL-1β even in cells such as neutrophils, which can cleave pro-IL-1β in an inflammasome-independent manner [143]. The ability of iNKT cells to modulate the plasticity of neutrophils [62] also warrants further investigation in our experimental model. It will be important to determine whether the increased accumulation of neutrophils in the lungs in *Jα18*$^{-/-}$ animals is also associated with changes in the phenotype of these cells, especially with regard to IL-10-producing capacity, and if this correlates with increased lung pathology.

However, the dual role of iNKT cells is apparent given their ability to also enhance innate responses in the lungs. iNKT cells have been shown to directly recognise *Mycobaterium tuberculosis*-infected macrophages, with this strong activation signal inducing the secretion of IFN-γ and mediating innate control of bacterial replication [339]. During established, active tuberculosis infection, however, iNKT cell numbers were shown to be decreased, together with increased levels of acute phase proteins [340]. This correlation therefore suggests that the presence of iNKT cells is associated with decreased levels of inflammation.

In addition, the role of iNKT cells in autoinflammatory diseases with a strong association with IL-1β should be investigated. For example, recombinant IL-1Ra (anakinra) has high clinical efficacy in the treatment of rheumatoid arthritis, and the potential for harnessing iNKT cells to dampen inflammation in this disease should be considered. Indeed, there are reports of iNKT cell activation prior to disease onset being protective in collagen-induced arthritis [341, 342], with increased IL-10 production demonstrated to be important in these models. Furthermore, the ability of iNKT cells to dampen pro-IL-1β secretion could be beneficial in the case of the NLRP3-associated diseases (CAPS). As well as the potential for novel therapies, study of iNKT cell biology in the context of these diseases will be important in identifying additional mechanisms by which they can perform an anti-inflammatory role *in vivo*.

Despite dampening IL-1β levels, interaction between iNKT cell and DC was shown not to affect the production of IL-12 **(Fig. 4.2.2)**. By selectively affecting IL-1β and not IL-12, iNKT cells can achieve a dual role – dampening inflammation but also promoting the development of Th1 adaptive immunity. This was directly demonstrated in our influenza infection model, where we showed decreased T cell recall responses in *Jα18*$^{-/-}$ animals **(Fig. 5.7)**, despite increased pulmonary inflammation earlier in infection.

Our model of iNKT cell-pre-conditioning was extended by the observation that weak interaction with iNKT cells can induce the upregulation of negative factors such as IκBα and A20, important molecules in switching off canonical NF-κB signalling **(Fig. 4.6)**. This is consistent with our hypothesis that iNKT cells can interact with and weakly stimulate APCs via their basal autoreactivity, in doing so inducing the expression of negative regulators without stimulating cytokine production or DC maturation. However, the question remains how iNKT cells can selectively affect the production of certain cytokines, and further analysis of the molecular mechanisms responsible for this inhibitory effect will allow us to understand how iNKT cells can perform their dual function. This may lead to the possible development of clinical strategies to selectively enhance the pro- or anti-inflammatory effects of iNKT cells, to boost vaccine efficacy or relieve the symptoms of autoimmunity.

6.2.3 The dual role of IL-1β during influenza virus infection

We and others have identified the importance of balance in IL-1-mediated responses, to effectively establish immunity whilst avoiding immunopathology. In the context of our influenza virus model, we have demonstrated the latter, that dysregulated IL-1β production in the absence of iNKT cells is associated with increased neutrophil influx and morbidity **(Fig. 5.6)**. Indeed, excessive pulmonary immunopathology has been identified as a major cause of mortality in pandemic influenza [326]. Whilst high levels of IL-1β appear to be detrimental during the inflammatory phase of the infection, a requirement for IL-1R1 signalling has been demonstrated for an effective adaptive response to the virus [131, 324, 325]. The levels of IL-1β required during influenza infection therefore need to be carefully regulated; low enough to prevent overzealous inflammation initially, but sufficient to raise protective T cell responses. In addition, the exact experimental model used is likely to be important in determining whether IL-1β is protective. Using higher doses of virus would lead to more severe inflammation, and in these cases we can speculate that elevated IL-1β levels would be detrimental; whereas in milder cases of infection, the induction of higher levels of IL-1β would be required to enhance the induction of protective T and B cell immunity.

6.2.4 A model of immunomodulation by iNKT cells

In this thesis, we have described various mechanisms of immunomodulation, where cytokine- and cell-mediated interactions play important roles in fine-tuning the immune response. **Fig. 6.2** summarises the model which we have put forward, identifying iNKT cells as key mediators of both innate and

adaptive immunity, with their response dependent on activation strength and timing. As well as extending our knowledge of iNKT cell biology, these findings may be important in the development of therapies to manipulate the immune response, during diverse pathologies including infection, autoimmunity and cancer.

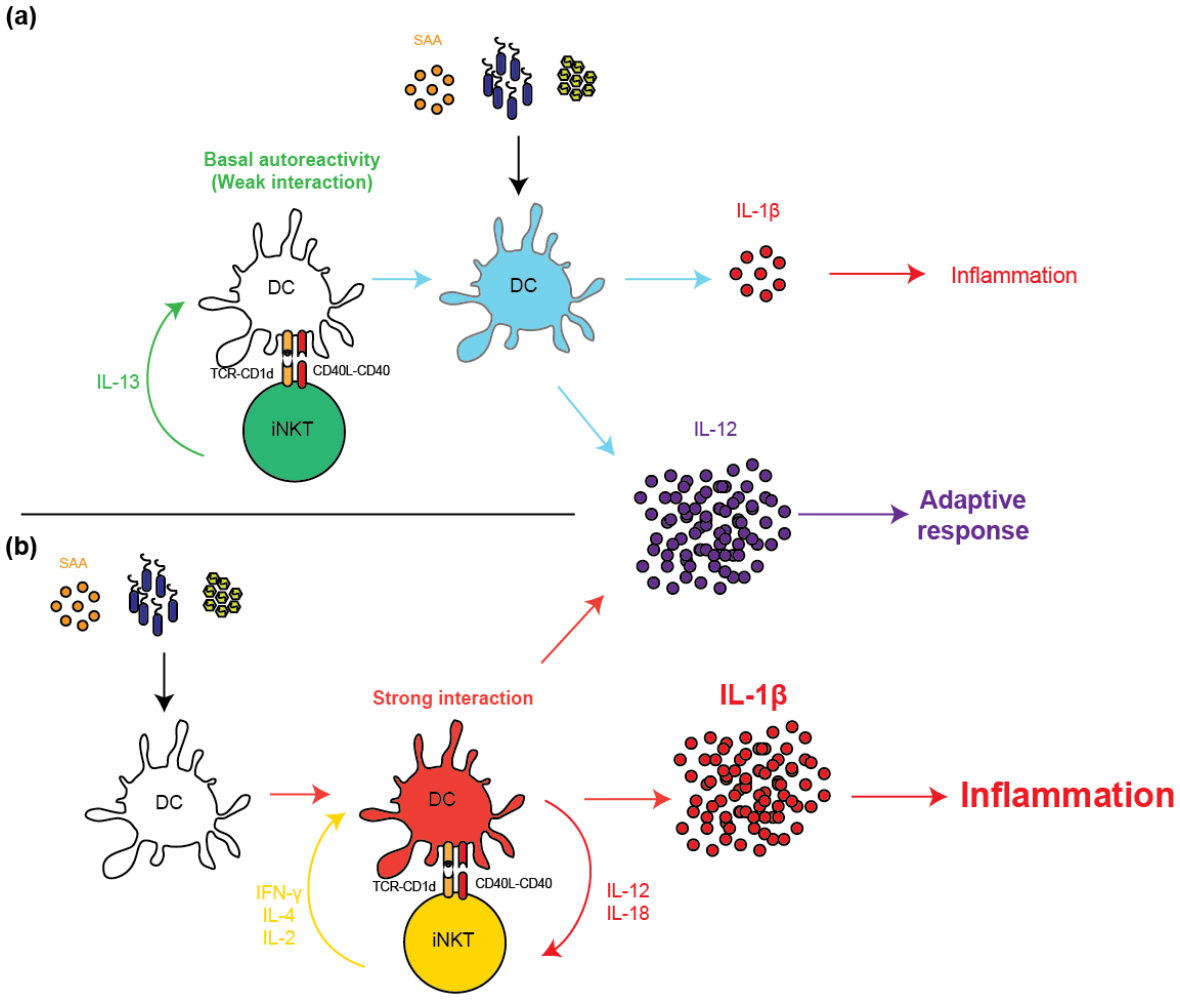

Fig. 6.2 The dual role of iNKT cells. **(a)** Pre-conditioning of DCs with iNKT cells prior to maturation leads to decreased production of IL-1β and restraining of inflammation. **(b)** iNKT cell interaction with TLR-ligand matured DCs leads to a strong interaction and the secretion of high levels of IL-1β with the potential to cause immunopathology. In both cases, secretion of IL-12 by DCs induces an effective adaptive response.

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