ABSTRACT

The Influence of Invariant Natural Killer T Cells on Myeloid-derived Suppressor Cell Generation and Function

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Trinity 2011

The absence of invariant Natural Killer T cells (iNKT cells) in mice infected with Influenza A virus (flu) has previously been shown to augment the expansion of Myeloid-derived suppressor cells (MDSCs), a bone marrow derived population that powerfully suppress the development of viral and tumor immune responses. Moreover, iNKT cell adoptive transfer into flu-infected mice has been shown to abolish the expansion and flu-induced suppressive activity of the MDSCs in a CD1d- and CD40-dependent manner. However, the mechanisms by which this relatively small subset of T cells influence myelopoiesis and MDSC differentiation remain largely unknown.

In this manuscript we firstly better define the MDSCs found in flu-infection as IL-10-secreting neutrophils that can suppress T cell proliferation. We then go further to show that the flu-induced ability to suppress T cells is acquired as early as the level of the Granulocyte-Macrophage Progenitors (GMPs) in the bone marrow and that iNKT cells can not only abrogate the suppressive activity of the IL-10-secreting neutrophils in the periphery but also that of the GMPs by a direct CD1d-dependent GCSF-mediated crosstalk.

MDSC expansion has previously been shown to be associated with the expression of the myeloid-related protein S100A9, and the mechanism of action of granulocytic-MDSCs shown to be ARG1-dependent. We built upon both these findings to show that iNKT cells influence the expansion and function of the MDSCs in part by regulating S100A9 and ARG1 expression. Following this we then showed for the first time that the acute phase protein Serum Amyloid A (SAA), shown to increase during flu-infection, has a dual reciprocal role: having the ability to up-regulate S100A9 and ARG1 in myeloid cells and differentiate IL-10-secreting suppressive neutrophils, while simultaneously facilitating the ability of the MDSCs to crosstalk with iNKT cells in a CD1d-dependent GCSF-mediated manner to abrogate the SAA-induced suppressive activity.

All together the data highlights the complexity of the immune response and the role iNKT cells play in influencing the differentiation of MDSCs during demand-driven myelopoiesis. More importantly however, it further affirms that research into harnessing the immunomodulatory capacity of iNKT cells remains an exciting prospect in bolstering future vaccination strategies and should continue to be pursued.

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ACKNOWLEDGEMENTS

No words can express how grateful I am for the unbelievably life-altering, character building, stimulating and enjoyable experience I have had at Oxford. To name each person who has touched my life in some way over the last four to five years would be near impossible, however there are some key figures along the path toward the DPhil that have been pillars in the process and must get specially mentioned.

Firstly I have to extend a heartfelt thanks to Dr. Carmela De Santo (Carmen), for making absolutely everything in this project possible. Carmen has not only taught me mostly all the techniques needed for this project but also helped me on a daily basis conceptually and technically. I cannot thank her enough.

Secondly, I would like to thank Enzo for his steady motivation and support in navigating this project and making the most of my time in his lab. It's truly been wonderful. And to all the lab members for the support and kind words, a genuine warm thanks.

To our collaborators, Sarah, Natalia and Helen from the Jacobsen Group, Nicholas from the Vyas Group at the WIMM, and Dr. Babak Baban at the Peter Medawar Building of Pathogen Research, University of Oxford, many many thanks.

Of course, my existence at Oxford was made entirely possible through the Rhodes Trust, to whom I'll be forever indebted. The support provided by the Trust and the Rhodes community went way beyond financial; to everyone in the community who walked beside me and stood behind me, and for the many fond memories that we created together, a million thanks.

To all the staff and friends at Lincoln College, where I spent the majority of my time outside of the lab, you were family away from home; thanks for the support and great times.

And finally to my family and wife Christie, for helping me to cope in the most stressful of times, thanks from the bottom of my heart.

CHAPTER 1

BACKGROUND

1.1 Bone Marrow Haematopoietic Stem Cells and Progenitors

Bone marrow haematopoietic stem cells (HSCs) are functionally defined by their ability to self-renew and to differentiate into all mature blood cells[1]. The first report of the clonal origin of haematopoietic cells was by Becker et al in 1963[2]. Since that time it has been shown that the progeny of HSCs progressively lose self-renewal capacity and become restricted to one lineage[3]. In the last two decades HSCs and lineage-restricted progenitors have been characterised using the differential expression of cell surface markers and functional readout colony-forming unit (CFU) assays[4]. However, despite the hematopoietic system being arguably the best characterised among all tissues, much remains unknown regarding the points at which hematopoietic stem cell-derived progenitors commit to each of the various lineages and the mechanisms modulating haematopoiesis.

1.2 The Hierarchy of Haematopoiesis

Models of the haematopoietic tree and lineage restrictions have constantly been altered over time as progeny have been sequentially identified. Morrison and Weissman in 1994, followed by Osawa et al in 1996, characterised the HSCs in the bone marrow as being within the $Lin^{-}Sca\text{-}1^{hi}c\text{-}Kit^{hi}$ (LSK) population[5,6]. Additional markers, including CD34 (known to

mark a fraction of HSCs and progenitors[6]), Flt3 (a tyrosine kinase receptor), Flk-2, Thy1.1 and CD11b (also known as αM-integrin, or Mac-1) were subsequently used to further subdivide the LSK population into long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs)[7,8]. Neither ST-HSCs nor MPPs can dedifferentiate into LT-HSCs under any circumstances yet tested and the latter is only capable of self-renewing for two weeks[9] (**Figure 1**).

As subdivisions of the HSCs were being characterised, downstream progeny were also being identified. In 1997, Kondo et al published on a common lymphoid progenitor (CLP), identifying it as being $Lin^{-}Sca\text{-}1^{lo}c\text{-}Kit^{lo}IL\text{-}7R\alpha^{+}$ and showing *in vitro* potential for B cell, T cell and NK cell differentiation[10]. The discovery of a complementary clonogenic common myeloid progenitor (CMP) was described soon thereafter as a $Fc\gamma R^{lo}CD34^{+}$ within a $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}IL\text{-}7R\alpha^{-}$ population[11]; by using the CD34 and Fcγ receptor-II/III markers, CMPs were reported to be cleanly isolatable by fluorescence-activated cell sorting (FACS). Further, methylcellulose CFU assays containing a cocktail of steel factor (Slf), Flt-3 ligand, (FL), IL-11, IL-3, granulocyte/macrophage-colony stimulating factor (GM-CSF), erythropoietin (Epo) and thrombopoietin (Tpo) demonstrated that more than 90% of these sorted single $Fc\gamma R^{lo}CD34^{+}$ cells formed myeloid colonies, including CFU-Mix, Burst-forming units-erythroid (BFU-E), CFU-Megakaryocyte (CFU-Meg), CFU-MegE, CFU-granulocyte/macrophage (CFU-GM), CFU-G and CFU-M[11].

Additionally, Akashi et al noted that FcγR^{hi}CD34^{+} cells sorted from the Lin^{-} Sca-1^{-}c-Kit^{+}IL-7Rα^{-} population only gave rise to colonies of granulocytes and/or macrophages and were therefore called granulocyte/macrophage lineage restricted progenitors (GMPs). Similarly, FcγR^{lo}CD34^{-} sorted cells formed colonies exclusively of megakaryocytes and/or erythrocytes and were therefore termed megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs)[11]. Consistent with these findings, adoptive transfer of CMPs, MEPs and GMPs separately into lethally irradiated mice revealed reconstitution with *in vivo* populations that mirrored the *in vitro* results.

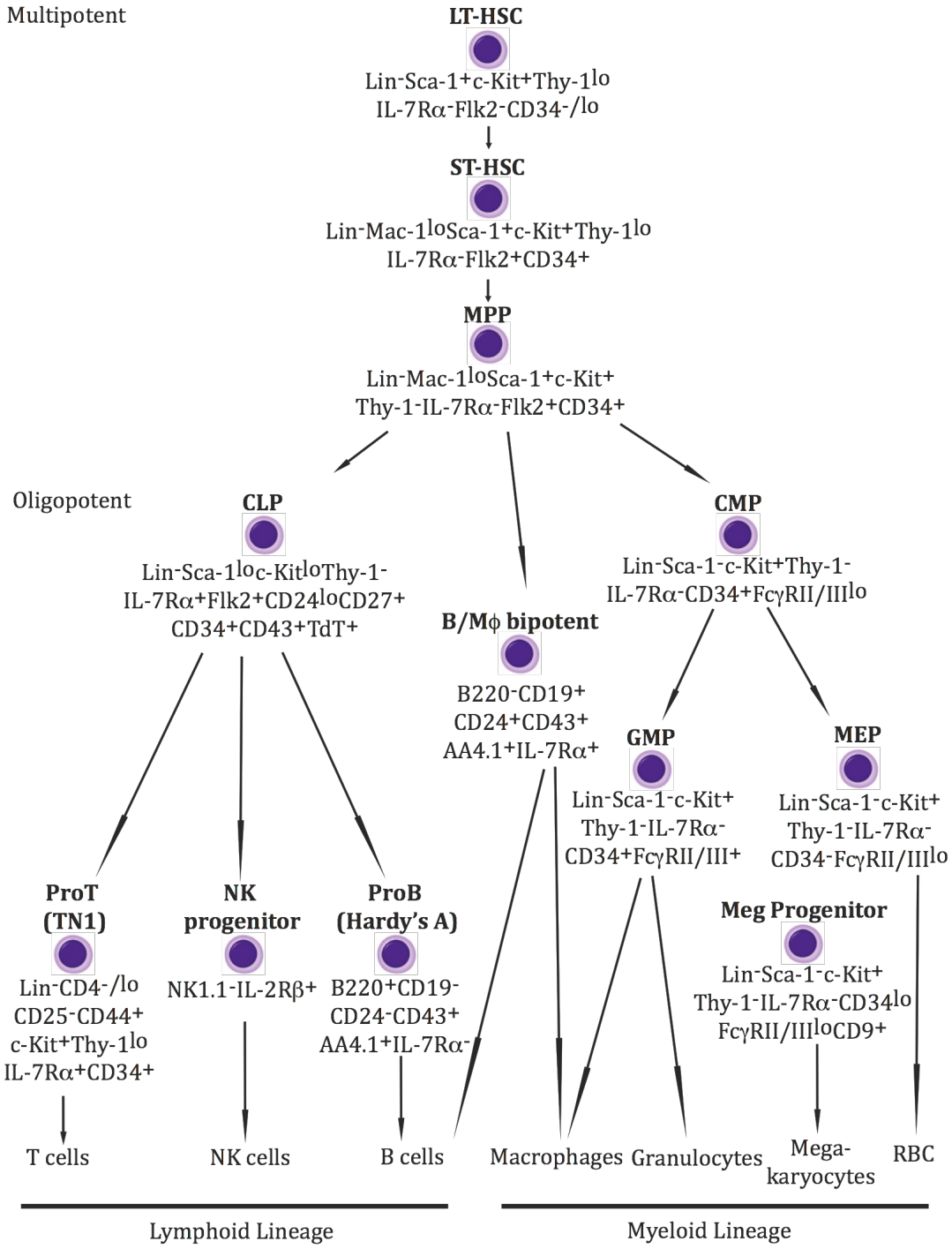

Multipotent
LT-HSC
Lin-Sca-1+c-Kit+Thy-1lo
IL-7Rα-Flk2-CD34-/lo
ST-HSC
Lin-Mac-1loSca-1+c-Kit+Thy-1lo
IL-7Rα-Flk2+CD34+
MPP
Lin-Mac-1loSca-1+c-Kit+
Thy-1-IL-7Rα-Flk2+CD34+
Oligopotent
CLP
Lin-Sca-1loc-KitloThy-1-
IL-7Rα+Flk2+CD24loCD27+
CD34+CD43+TdT+
B/Mϕ bipotent
B220-CD19+
CD24+CD43+
AA4.1+IL-7Rα+
CMP
Lin-Sca-1-c-Kit+Thy-1-
IL-7Rα-CD34+FcγRII/IIIlo
GMP
Lin-Sca-1-c-Kit+
Thy-1-IL-7Rα-
CD34+FcγRII/III+
MEP
Lin-Sca-1-c-Kit+
Thy-1-IL-7Rα-
CD34-FcγRII/IIIlo
ProT
(TN1)
Lin-CD4-/lo
CD25-CD44+
c-Kit+Thy-1lo
IL-7Rα+CD34+
NK
progenitor
NK1.1-IL-2Rβ+
ProB
(Hardy's A)
B220+CD19-
CD24-CD43+
AA4.1+IL-7Rα-
Meg Progenitor
Lin-Sca-1-c-Kit+
Thy-1-IL-7Rα-CD34lo
FcγRII/IIIloCD9+
T cells
NK cells
B cells
Macrophages
Granulocytes
Mega-
karyocytes
RBC
Lymphoid Lineage
Myeloid Lineage

Figure 1: Conceptual Haematopoietic tree in adult mice. Indicated cell populations have been prospectively isolated by phenotypic, functional, and for the more mature cells, gene expression profile homogeneity. Not all of the linear relationships in this figure have been proven. Long-term haematopoietic stem cells (LT-HSC) self renew for life, whereas downstream short-term (ST)-HSC self renew for six to eight weeks, and multipotent progenitors (MPP) for two weeks; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor *(figure modified from Kondo et al Annu. Rev. Immunol. 2003)*

Recently, intermediate progenitors have been more precisely characterised with the use of gene expression signatures. *In vitro* and *in vivo* differentiation potentials of cells have been matched to a cascade of lineage-affiliated gene expression signatures, primed in HSCs and differentially propagated in the lineage-restricted progenitors[12,13]. In 2007 Pronk et al used global gene expression signatures and hierarchical analysis to support the isolation of subsets of myeloerythroid progenitors within what was previously defined as the pure CMP population. In short, using the differential expression of CD150 and Endoglin, Pronk identified a Pre-GM and a Pre-MegE progenitor within the $Fc\gamma R^{lo}CD34^{+}$ population[12], described

by Akashi as just a population of CMPs (These findings have not been taken into account in Figure 1).

1.3 Myeloid-derived Suppressor Cells – Misfits in the Myelopoietic tree

Many stages in myelopoiesis downstream of GMPs can be identified based on phenotype and morphology, and lower haematopoietic hierarchy has been structured in part by these characteristics (**Figure 2**). However, in the last decade there has been extensive literature identifying a phenotypically heterogeneous group of immature cells within the myeloid lineage based on their common ability to suppress T cell responses in various pathological conditions. Reflecting the wide-ranging data on what morphology and markers characterize these cells, the most precise name accepted to date is "Myeloid-derived Suppressor cells" (MDSCs).

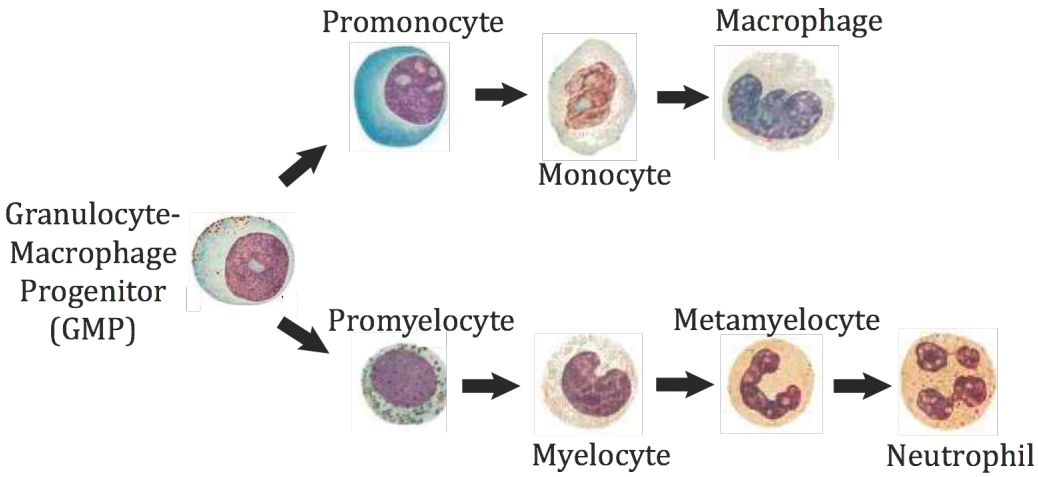


Figure 2: The differentiation of the Granulocyte-Macrophage Progenitor (GMP) into the monocytic and neutrophilic lineages; *Modified from Rosenbauer et al, Nature Reviews Immunology 7, 105-117 (February 2007)*[14]

With the main commonality of MDSCs being their suppressive activity (and not phenotype), MDSCs have yet to be directly placed into the haematopoietic phylogenetic tree. However, there are a few MDSC surface markers worthy of note. In mice, MDSCs co-express Gr1 (a myeloid-cell lineage differentiation antigen) and CD11b (also known as αM-integrin)[15]; whereas in humans, MDSCs are characteristically $CD11b^{+}CD14^{-}$, express the common myeloid marker CD33, but lack the expression markers of mature myeloid and lymphoid cells and of the MHC class II molecule HLA-DR[16,17].

In 2001 Almond et al further defined MDSCs by isolating suppressive Lin^{-} $HLA\text{-}DR^{-}CD33^{+}$ cells from patients with head and neck squamous cell carcinoma (HNSCC) and analyzing them with electron microscopy. Histologically, all the MDSCs appeared to be immature cells along the myeloid lineage, specifically having the morphology of immature granulocytes and monocytes[17]. They also demonstrated that apart from having the ability to suppress T cell proliferation, about one third of these immature myeloid cells (IMCs) could differentiate into macrophages and dendritic cells (DCs) in the presence of the appropriate cytokines *in vitro*[17]. With many studies of other tumour types and infection models supporting Almond et al's findings the current prevailing dogma is that MDSCs are a population of activated suppressive IMCs that have also been prevented from fully differentiating into mature cells[18].

The ability to modulate the differentiation, proliferation, survival and commitment of haematopoietic stem cells and progenitors is critical to the

establishment of a proper defence against invading pathogens, tumours and the maintenance of homeostasis. Consequently, much investigation is currently underway into the factors that control the differentiation, expansion and activation of MDSCs as well as the regulation of haematopoiesis on a whole.

1.4 Regulation of Haematopoiesis

The differentiation of HSCs and progenitor cells depends on the integration of signals from the haematopoietic cells themselves, other cellular and extracellular components of the bone marrow microenvironment, and a variety of soluble factors[1]. Integrating these stimuli through an intricate network of complex but finely tuned regulatory pathways culminates in the expression of specific genes for the appropriate differentiation of cells in response to stress conditions[1].

1.4.1 Haematopoiesis Regulation by Cytokines

More than twenty soluble haematopoietic regulators have been identified to date. They arrive from the circulation or are produced locally to act on HSCs and haematopoietic progenitors in a paracrine, juxtacrine, or autocrine manner[19,20]. Of these, the colony-stimulating factors have been widely studied and found to be essential to haematopoiesis, particularly for the myeloid lineage. They include interleukin 3 (IL-3, also known as multi colony-stimulating factor)[21], the granulocyte-macrophage colony-stimulating factor (GM-CSF), the macrophage colony-stimulating factor (M-

CSF) and the granulocyte colony-stimulating factor (G-CSF). Though these factors show some redundancy, by being able to exert overlapping functions on specific cells, they have also been shown to have significant functional pleiotropy, exhibiting a wide array of functions on various tissues and cells.

CSFs have been shown to functionally modulate activation, degranulation, cytotoxicity, adhesion and chemotaxis of various populations of mature blood cells and their precursors[22-24]. However CSFs are better known and defined by the instructive signals they exert on HSCs and progenitors for survival, proliferation, differentiation and more recently, instructing haematopoietic lineage choice[25]. The latter concept was confirmed in 2009 by Rieger et al using bio-imaging approaches that allow continuous long-term observation of individual differentiating mouse HPCs to demonstrate the ability of GCSF and MCSF to influence the intracellular lineage commitment machinery of GMPs[25].

CSFs, however, do not act alone. They are part of a well-orchestrated network of cytokines that are either produced locally in the microenvironment of the bone marrow or arrive through the circulation[26]. These include interleukin-6 (IL-6), leukemia inhibitory factor (LIF), IL-11 and oncostatin M (OSM), each specialized for cellular differentiation control, in balance with several inhibitors of hematopoietic development, such as tumour necrosis factor (TNF), transforming growth factor β-1 (TGFβ1), interferons (IFNs) α/β and γ, IL-4, IL-10 and IL-13[26]. The signal transduction pathways of these cell-extrinsic cytokines converge to

modulate the pattern of gene expression of the haematopoietic cells culminating in an overall biological response.

1.4.2 Cytokines Driving MDSC Expansion and Activation

Cytokines are also implicated in aberrant haematopoiesis. A good example of this is the accumulation of MDSCs in a host due to cytokine-induced arrest of differentiation, expansion and activation of myeloid precursors. Multiple publications have shown that an altered cytokine milieu associated with acute and chronic infections[27-30], polymicrobial sepsis[31], trauma[32], and tumour microenvironments results in the systemic expansion of IMCs with immunosuppressive activity. Cytokines highlighted include vascular endothelial growth factor (VEGF)[33], prostaglandins[34], stem-cell factor (SCF)[35], M-CSF, IL-6[36], and GM-CSF[37]; each seen to be activating pathways that converge on Janus kinase (JAK) – STAT3, causing increased survival and expansion of IMCs, and a partial block in their differentiation into mature myeloid cells.

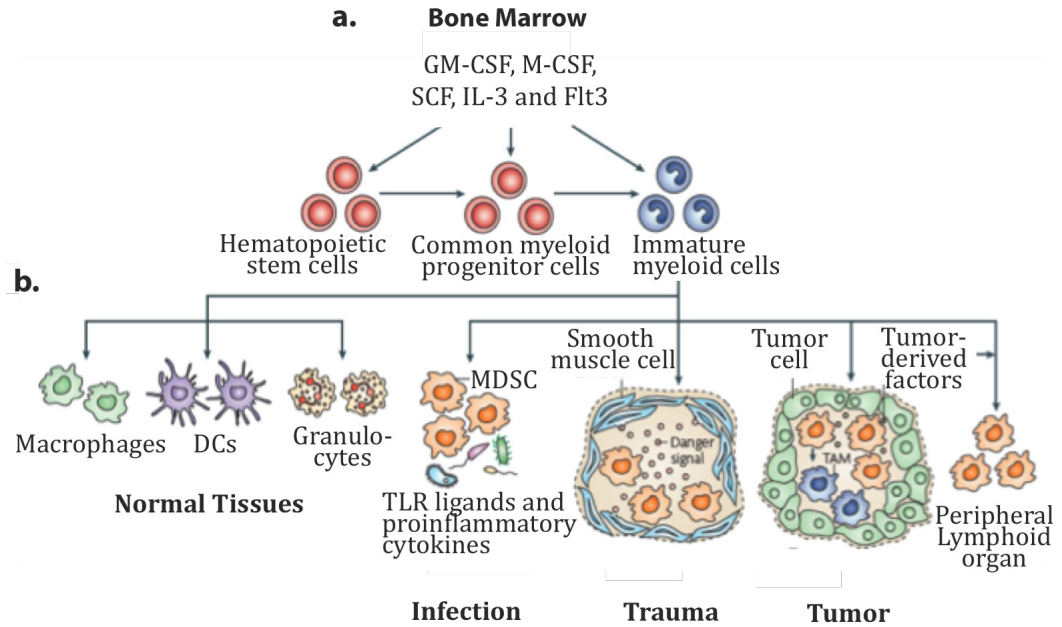


Figure 3: The Generation of MDSCs – (a) Immature myeloid cells (IMCs), by convention, is a collective term for cells down stream of the common myeloid progenitor but upstream of terminal differentiation in normal myelopoiesis. Differentiation depends on the integration of signals, including that from soluble factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage CSF (M-CSF), stem-cell factor (SCF), interleukin-3 (IL-3) and FMS-related tyrosine kinase 3 (FLT3)**; (b)** Factors produced in certain infections, trauma, sepsis and tumour microenvironments promote the accumulation of IMCs at the sites of pathology and secondary lymphoid tissue, prevent their differentiation and activate an immunosuppressive function, at which point they are referred to as myeloid-derived suppressor cells (MDSCs). They may also differentiate into Tumour Associated Macrophages (TAMs) within the tumour microenvironment, which have a distinctly different phenotype and function from MDSCs. *(figure modified from Gabrilovich et al, Nature Reviews March 2009)*

STAT3 is arguably the main transcription factor regulating MDSCs expansion. It is postulated that the increased survival and proliferation of myeloid progenitors associated with STAT3 activation is probably due to up-regulation of B-cell lymphoma XL, cyclin D1, MYC and survivin[18]. More recently, STAT3-dependent up-regulation of the expression of the S100 calcium-binding protein A9 (S100A9) in myeloid progenitors has also been implicated in preventing their differentiation and promoting MDSCs expansion[38].

The cytokine-induced immunosuppressive ability of MDSCs has been shown to be due to IFNγ-mediated up-regulation of inducible nitric oxide synthase (iNOS) in IMCs through signal transducer and activator 1 (STAT1) activation[39-42], as well as a TGF-β, IL-4 and/or IL-13-mediated up-regulation of Arginase I (ARGI) through STAT6 activation[42-45]. In sepsis, ARG1 and iNOS are also up-regulated in MDSCs by a TLR-MyD88-NFkB dependent mechanism[31]. The reducing of levels of arginine in the microenvironment

and production of peroxynitrates and reactive oxygen species by iNOS and ARG1, have been highlighted as the MDSCs' main mechanisms of suppressing T-cell proliferation and antigen-specific T-cell responses.

Though there is detailed literature on MDSCs in infection and trauma models, the majority of the cytokines shown to drive MDSC expansion have been identified in tumour models. Table 1 outlines some of the tumours and respective cytokines associated with the expansion and activation of MDSCs.

Table 1: Soluble factors implicated in the expansion of MDSCs in cancer

Cytokine	*Murine Tumour Model*	*Human Tumour*
IL-1β	Fibrosarcoma[46], mammary carcinoma[47]	Undetermined
IL-6	Mammary carcinoma[36]	Undetermined
IL-10	Colon Carcinoma[48], melanoma[48]	Undetermined
IL-12	Colon carcinoma[49]	Undetermined
IL-13	Colon carcinoma[40,45], Fibrosarcoma[40,45], mammary adenocarcinoma[40], lymphoma[40]	Undetermined
VEGF	Sarcoma[33,50], melanoma[51], lymphoma[51], lung carcinoma[51]	Renal cell cancer[52], pancreatic cancer[53]
TGFβ	Colon carcinoma[45], Fibrosarcoma[45], mammary adeno-carcinoma[54], Lewis lung carcinoma[55]	Undetermined
IFNγ	Mammary adenocarcinoma[40,56], Fibrosarcoma[40], colon carcinoma[40], lymphoma[40]	Undetermined
C5a	Cervical cancer[57]	Undetermined
Prostaglandins	Mammary carcinoma[34], lung	Undetermined

	cancer[58], colon cancer[59]	
Gangliosides	Neuroblastoma[60]	Undetermined
CCL2	Lewis lung carcinoma[61], MethA sarcoma[61], melanoma[61,62]	Undetermined
CXCL5, CXCL12	Mammary adenocarcinoma[54]	Undetermined
MMP9	Colon carcinoma[63], Lewis lung carcinoma[63], mammary carcinoma[64]	Undetermined
GM-CSF	Lewis lung carcinoma[65], colon carcinoma[66], mammary carcinoma[66,67], lymphoma[37,66]	Head and neck cancers[68], Melanoma[69,70]
G-CSF	Lewis lung carcinoma[61], MethA sarcoma[61], melanoma[61]	Undetermined
M-CSF	Sarcoma[71], mammary carcinoma[71]	Renal carcinoma cell lines[72]
SCF	Colon carcinoma[35]	Undetermined
S100A8 and S100A9	Colon carcinoma[38], lymphoma[38], Fibrosarcoma[38] and mammary carcinoma[73]	Undetermined

Key: C5a, complement component 5a; CCL2, CC-chemokine ligand 2; CXCL, CXC-chemokine ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage CSF; IFNγ, interferon-γ; IL, interleukin; M-CSF, macrophage CSF; MDSCs, myeloid-derived suppressor cells; MMP9, matrix metalloproteinase 9; ND, S100A, S100 calcium-binding protein A; SCF, stem cell factor; TGFβ, transforming growth factor-β; VEGF, vascular endothelial growth factor.

It is important to reiterate that cytokine-signals are not the only regulatory mechanisms modulating the differentiation of haematopoietic progenitors. A convergence of central and peripheral signals from diverse cellular components, including that from stromal cells and T cells, are also integral to haematopoiesis regulation. The role of T cell is discussed further below.

1.4.3 The Role of T cells in Myelopoiesis

There is extensive evidence supporting the potential role of T cells in modulating haematopoiesis. The body of work ranges from the involvement of T cells in haematopoietic reconstitution after bone marrow

transplantation (BMT) to their contribution to haematopoietic control in response to infections.

Animal models have suggested a role for CD4 cells in bone marrow engraftment after BMT by highlighting the dependency of complete post-transplantation recovery on major histocompatibility complex (MHC) class II expression[74]. These studies supported previous data from other experimental models which used mixed radiation chimeras to show that bone marrow engraftment is dependent on the presence of T cells syngeneic to the graft[75]. Both Kaufman and Ho et al decided to extend these results to the clinical setting and demonstrated that there is an increased risk of graft failure in T-cell depleted allogeneic BMT[76,77]. Further, results from subsequent clinical studies, which involved patients under non-myeloablative regimen, indicated a T-cell donor chimerism higher than 90% was required for donor cell engraftment to occur[78,79].

Several models of infection have shown that T cells activated in the context of the immune response produce a diverse set of cytokines related to haematopoietic regulation, including IL-3, IL-4, IL-5, IL-6, IL-13, IL-17, GM-CSF and Oncostatin M (OSM)[80-87]. They each have been associated with either normal haematopoietic homeostasis or the up-regulation of the innate immune response in controlling pathogen dissemination during bacterial and/or helminthic infections[87-90]. It is worth noting however, that the effects of T cells on haematopoiesis have also been shown in response to both polyclonal[91] and syngeneic[92] stimulation in the absence of infection.

Monteiro et al sought to investigate the precise role of T cells in haematopoiesis regulation and found that T-cell deficient mice had severely defective terminal differentiation of myeloid progenitors. Monteiro demonstrated a direct correlation between the numbers of $CD4^+$ T cells in the bone marrow and granulocyte constitution in the virtual complete absence of $CD8^+$ cells. Specifically, HSCs gave rise to committed myeloid progenitors in the absence of activated BM $CD4^+$ T cells, however the ability of these cells to complete their differentiation program and give rise to mature cells was impaired. Consequently, immature cells accumulated in the bone marrow while leucopenia was observed in the periphery. Of note, monocyte generation was preserved while the granulocyte lineage was strongly affected in these athymic mice. Notably, myelopoiesis was restored in the presence of activated $CD4^+$ but not $CD8^+$ T cells in the bone marrow[93].

It has previously been shown that bone marrow T cells make up only 1-3% of the BM microenvironment and have an activated phenotype with a semi-diverse repertoire of Vβ chains[94-96]. Consistent with this, Monteiro et al's data indicated that haematopoiesis maintenance requires the presence of BM T-helper cells activated by cognate antigen. In short, transgenic mice carrying T-cell receptors specific only for an ovalbumin peptide presented in the context of a specific class II molecule ($I\text{-}A^d$) (DO11.10 $RAG^{-/-}$), if not stimulated by their cognate antigen, showed very few $CD3^+$ $CD69^+$ cells in the bone marrow and the same deficiency in myeloid differentiation as athymic mice. Systemic priming of the transgenic mice with ovalbumin

increased the frequency of BM activated $CD4^+$ T cells and restored normal myelopoiesis[93].

Restoring the defect in terminal differentiation of myeloid progenitors in both the T-cell deficient mice and in the transgenic mice with inactivated T cells was suggested to be dependent on cytokines produced by activated T cells and not on direct cell-cell interaction. This was demonstrated by isolating myeloid progenitors from nude mice, and showing that they responded to specific growth factors used in soft agar cultures (colony-forming unit assays) and reached terminal differentiation[93]. However, it is known that any cytokine production by T cells is dependent on TCR engagement. So although the progenitor differentiation in response to soluble factors does not depend on cellular contact with T cells, the production and secretion of the cytokines by the T cells does[93].

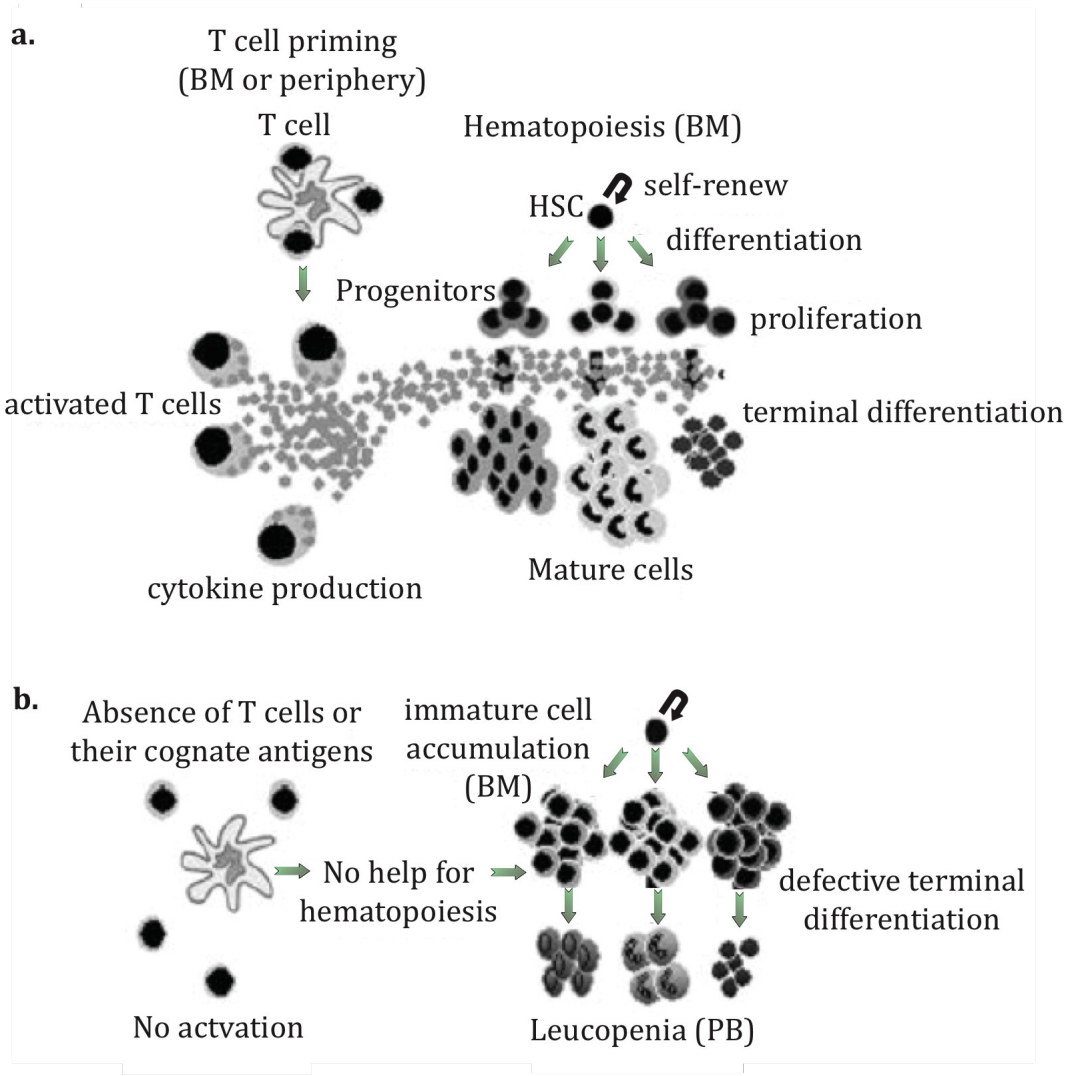

a.
T cell priming
(BM or periphery)
T cell
Hematopoiesis (BM)
HSC
self-renew
differentiation
Progenitors
proliferation
activated T cells
terminal differentiation
cytokine production
Mature cells
b.
Absence of T cells or
their cognate antigens
immature cell
accumulation
(BM)
No help for
hematopoiesis
defective terminal
differentiation
No actvation
Leucopenia (PB)

Figure 4: Model for $CD4^+$ T cell-dependent normal haematopoiesis. (a) In the presence of antigen-primed $CD4^+$ T cells, cytokines are secreted and terminal differentiation of haematopoietic committed progenitors and precursors is efficient; **(b)** When $CD4^+$ T cells are absent or their cognate antigens are not available, haematopoiesis is defective, resulting in immature cell accumulation in the bone marrow or leucopenia in the peripheral blood. *Modified from Monteiro et al, Blood 105 (4), 1484-1491 (2005)*

Following the evidence that haematopoiesis regulation involved $CD4^+$ T cells, others sought to investigate further T-cell subset populations. One particular immunoregulatory T-cell shown to play a significant role in modulating haematopoiesis is the invariant Natural Killer T cell (iNKT cell)[97], discussed further below.

1.5 Natural Killer T cells – a regulatory subset of T cells

Natural Killer T (NKT) cells are a unique population of cells that express a T-cell receptor (TCR) restricted by a class I MHC-like molecule called CD1d. Two subdivisions of NKT cells have been identified thus far: (i) invariant NKT cells (also known as Type I NKT cells or iNKT cells) and (ii) diverse NKT cells (also known as Type II NKT cells or dNKT cells)[98-100].

1.5.1 Activation of iNKT cells

iNKT cells make up the vast majority of the NKT population. They have been named "invariant" because they express a distinctive semi-variant TCR composed of a canonical strictly conserved α chain (Vα24Jα18 in humans and Vα14Jα18 in mice)[101-103] paired with a limited repertoire of β chains (Vβ11 in humans and Vβ2, Vβ7 and Vβ8.2 in mice). Unlike the conventional $CD4^+$ and $CD8^+$ T cells that recognize peptides, the TCR of iNKT cells recognizes glycolipids in the context of CD1d. Of note, the source of the glycolipids presented by CD1d may be either endogenous (self)[104,105] or exogenous (i.e. microbial or synthetic)[105-109].

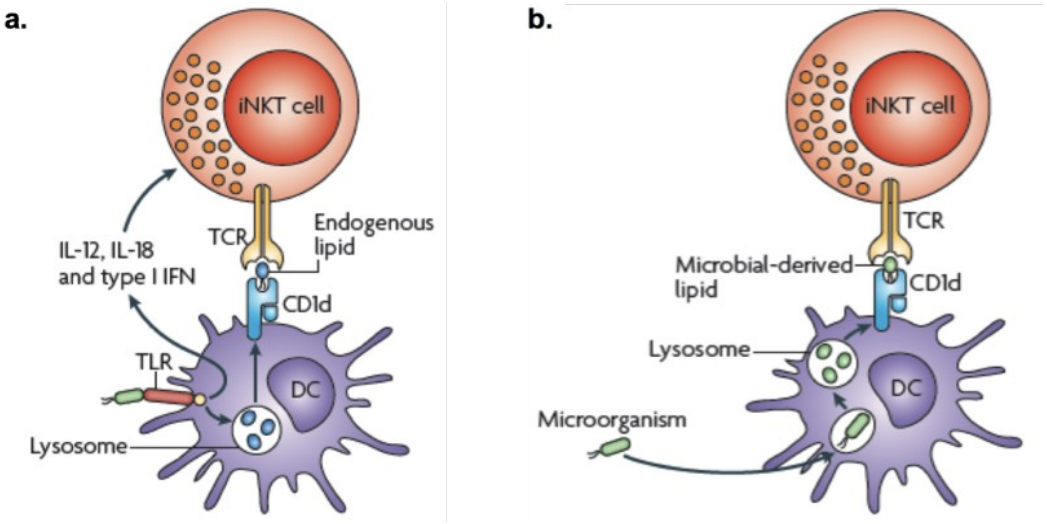


Figure 5: iNKT cell activation by endogenous and exogenous lipids; (a) Signalling through Toll-like receptors (TLRs) leads to the loading of endogenous (self) lipids onto CD1d molecules. Presentation of the self-lipid, along with TLR-induced IL-12, IL-18 and type I interferon (IFN) secretion by the antigen presenting cell (APC), activates the iNKT cell[110-113]. The TLR-induced cytokines amplify the activating signals generated by the self-glycolipids, however may activate the iNKT cells irrespective of TCR-lipid-CD1d engagement[114]; **(b)** APCs process and present microbial lipids on CD1d molecules that can activate iNKT cells. DC, dendritic cell *(Adopted from Cerundolo et al Nat Rev Immunol 9 (1), 28-38 (2009)[115].*

The scope of lipid reactive T cell biology is extensive and the nature and range of self and microbial lipids that activate iNKT cells is unfolding. The first (and most potent) lipid antigen to be identified was α-

galactosylceramide (αGalCer), derived from the glycosphingolipid extract of the marine sponge *Agelas mauritianus*[116]. Subsequently, the α-linked glycan in αGalCer was shown to be an α-anomeric linked structural motif common to most of the identified self and microbial antigens that activate iNKT cells[117]. Following this, several analogues have been synthesized that are capable of activating iNKT cells both in vitro and in vivo[108,118,119]. The use of these pharmacological compounds, in particular αGalCer, in experimental models that investigate iNKT cell function has become commonplace as they activate iNKT cells more rapidly and efficiently than TLR agonists and microbes[105-107,120]. Moreover, the use of αGalCer-loaded CD1d tetramers has enabled the definitive analysis of iNKT cells in such experiments[117].

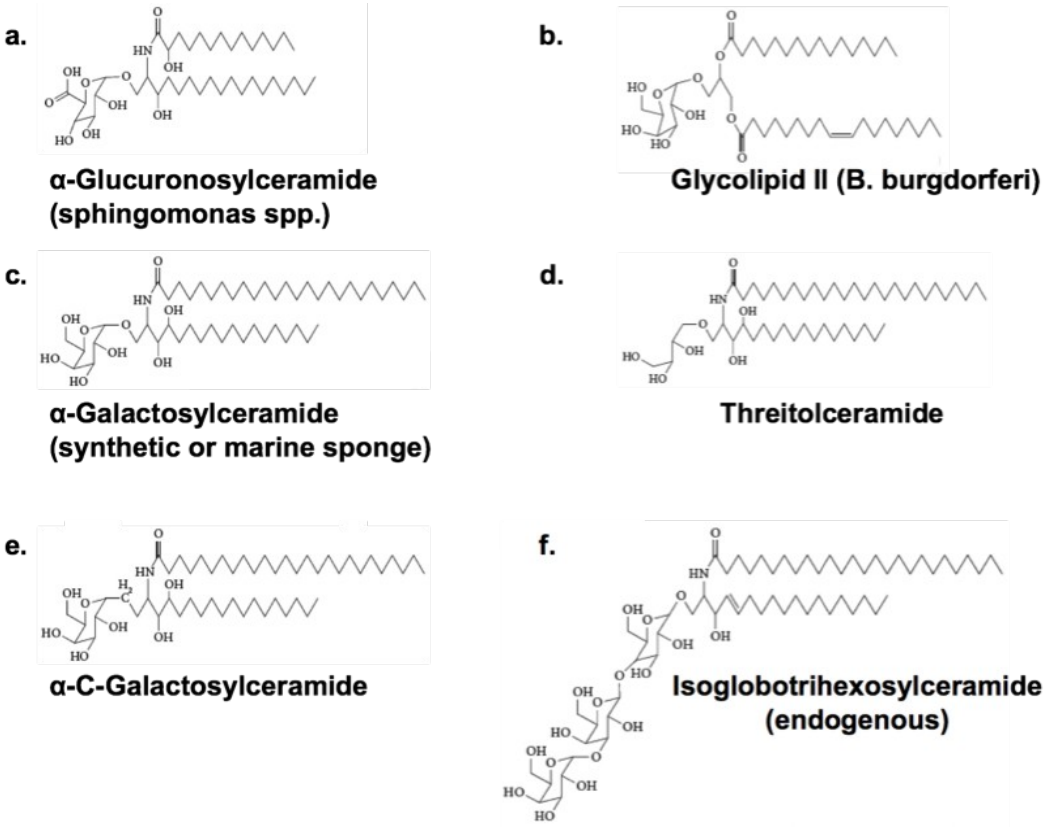


Figure 6: Invariant-Natural-Killer-T cell agonists; Microbial ligands that activate iNKT cells have been characterized from both **(a)** Sphingomonas spp. (α-Glucuronosylceramide)[107] and **(b)** Borrelia burgdorferi (α-galactosyldiacylglycerol or Glycolipid II)[106]; Identified synthetic ligands include **(c)** α-Galactosylceramide[118], **(d)** Threitolceramide[108] and **(e)** α-C-

galactosylceramide[119]; an example of an endogenous ligand thus far identified is **(f)** Isoglobotrihexosylceramide[104] (Figures adopted from *Cerundolo et al Nat Rev Immunol 9 (1), 28-38 (2009).*

1.5.2 iNKT cells Regulate the Immune Response

iNKT cells modulate the function of many other cell types, both directly through cell-cell contact and indirectly through the rapid production of an array of cytokines. As such, they play an important role in selective recruitment, activation and polarization of the innate and adaptive immune response[117].

During an infection, iNKT cells are classically elicited earlier than cells of the adaptive immune system but later than the innate effectors[121]. This can largely be attributed to iNKT cell development, from which the iNKT cells emerge with a distinct memory-activated phenotype[117]. Stetson et al provided deeper understanding of the capacity of iNKT cells to secrete cytokines within hours of stimulation by also demonstrating the constitutive presence of preformed cytokine-encoding mRNA transcripts in the cytoplasm of iNKT cells[122].

Phenotypically iNKT cells have high surface levels of the activation markers CD44 and CD69 and low expression of CD62L[123,124], thereby resembling effector memory T cells. The soluble factors secreted by iNKT cells following TCR stimulation include T helper 1 (T_H1) cytokines, such as IFNγ, IL-2, and TNFα, often simultaneously with T_H2 cytokines including IL-4, IL-5, IL10 and IL-13[124-126]. More recently, IL-17 (a pro-inflammatory cytokine

associated with autoimmune disease) and IL-21 (a cytokine with pleiotropic effects) have been added to the list of factors produced by iNKT cells upon activation[127-129].

It is important to note that different circumstances/pathologies elicit separate subsets of iNKT cells with distinct functions. In mice iNKT cells have been identified as either $CD4^+$ or double negative (DN, $CD4^-CD8^-$), while in humans a third subset of $CD8^+$ iNKT cells also exists. Human $CD8^+$ and DN iNKT cells produce T_H1-type cytokines and are highly cytolytic, whereas $CD4^+$ iNKT cells produce both T_H1 and T_H2 cytokines[125,130]. CD1d-restricted T cells, in particular the $CD4^+$ subset in both humans and mice, have been reported to drive DC maturation following recognition of antigenic glycolipids. Specifically, stimulation of iNKT cells through recognition of αGalCer-CD1d complexes results in rapid IFNγ and IL-4 production and an increased expression of CD40 ligand (CD40L); signalling via CD40 has been shown to up-regulate surface MHC Class II and co-stimulatory molecules and push the DCs to produce IL-12, a cytokine which promotes T_H1 polarization[131-133]. The effect of iNKT cells on DC maturation is so potent that it has prompted the use of αGalCer as an adjuvant to co-administered protein antigens for the enhancement of immune responses[134,135]. It has become clear that timely provision of iNKT-cell-derived signals is important for optimizing DC and consequently T-cell responses.

The effects of iNKT cells on the immune response however go beyond enhancing DC maturation and cytokine-induced polarization of the innate

and adaptive arms of the immune system. For instance, the up-regulation of CD70[136] and OX40 ligand[137] by mature DCs has recently been shown to be important for co-stimulating iNKT cells which in turn promote an antigen-specific $CD8^+$ T-cell response. Additionally, iNKT cells have been found to provide cognate help for B cells, in a CD40-dependent manner, after recognition of a lipid antigen presented by the B cells in the context of CD1d; the cognate help specifically results in the production of antigen-specific, class-switched antibody responses in vivo[138,139]. Coupled with influencing T and B cell responses, iNKT cells also contribute to NK cell activation through IFNγ and IL-2 secretion by either the iNKT cells themselves or iNKT-stimulated APCs[140-142]. Further, both murine and human iNKT cells can acquire potent cytotoxic function following activation. Through the expression of FasL and TRAIL as well as the up-regulation of granzyme B and perforin, iNKT cell killing of tumour cells has been observed[143,144].

1.5.3 iNKT and Myeloid-derived Suppressor cells

De Santo et al recently showed that iNKT cells can increase antigen-specific immune responses by regulating the suppressive activity of $CD11b^+Gr\text{-}1^+$ MDSCs[145]. In De Santo's study, they observed greater numbers of MDSCs in iNKT-cell-deficient mice and CD1d-deficient mice than wild-type mice during infection with influenza A virus (flu), suggesting a role for iNKT cells in regulating the frequency and activity of MDSCs during infection with this virus[145]. Further, adoptive transfer of iNKT cells into flu-infected iNKT-cell-deficient mice reduced the ARG1 and iNOS activity of the MDSCs thereby abolishing their suppressive activity and rescuing the ability of these mice to

clear the flu infection. These results were extended to humans by showing the presence of MDSCs in the peripheral blood of individuals infected with flu and the ability of iNKT cells to abolish the activity of the suppressor cells ex vivo[145].

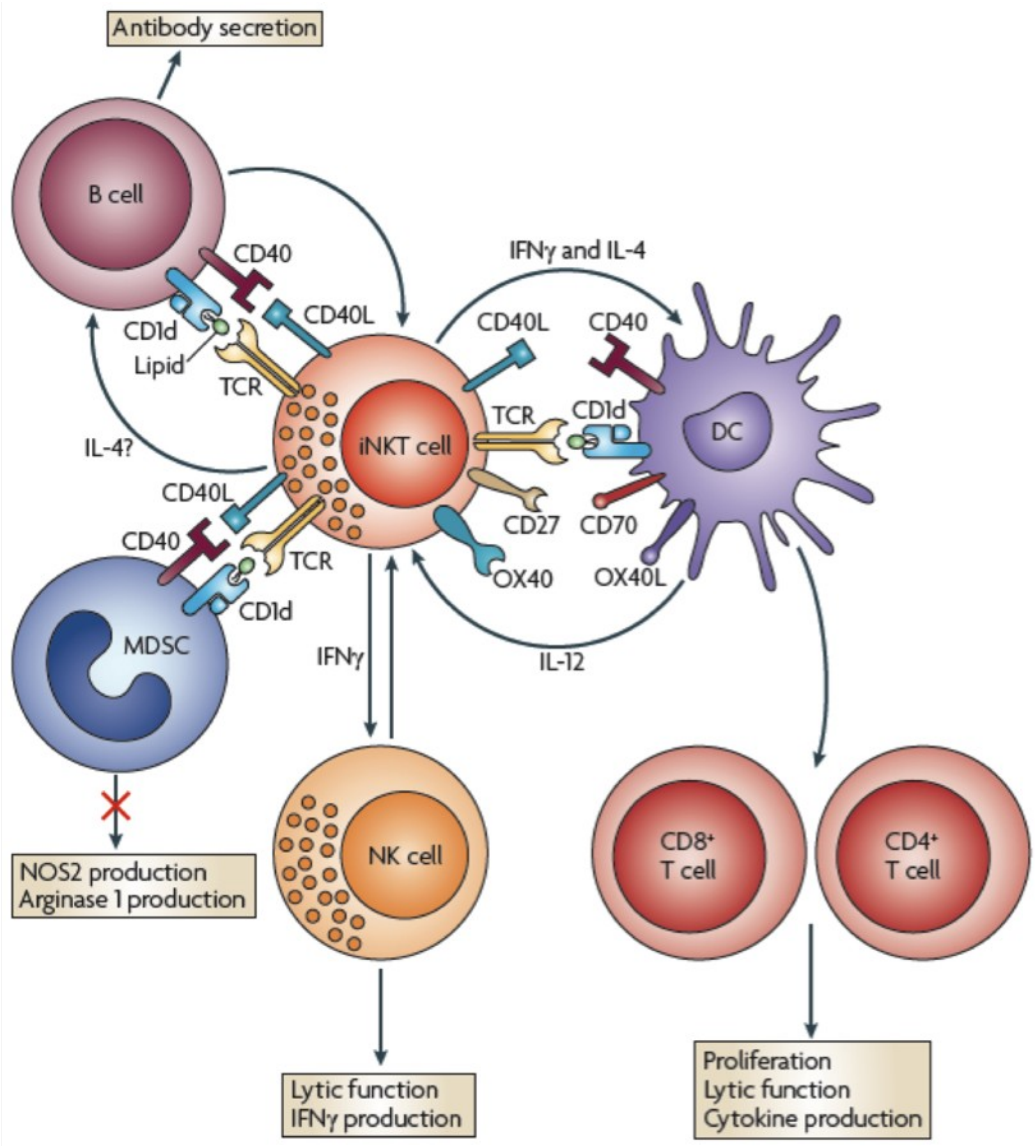


Figure 7: **iNKT cells modulate the function of many other cell types.** During the immune response invariant natural killer T (iNKT) cells directly and indirectly interact with other cell types in a bidirectional manner. Signals are received through T cell receptors (TCRs) recognizing glycolipid-CD1d complexes, through co-stimulatory receptors, such as CD40, CD27 and OX40 recognizing their ligands CD40L, CD70 and OX40L respectively, as well as through cytokine mediators. iNKT cells are seen to modulate the lytic

function of NK cells, the suppressive activity of MDSCs, the maturation of antigen-presenting cells and the responses of B and T cells in various pathologies *(Figure adopted from Cerundolo et al Nat Rev Immunol 9 (1), 28-38 (2009).*

Indeed, the above results highlighting the ability of iNKT cells to modulate the presence and activity of MDSCs during acute inflammatory processes has been mirrored in tumour models as higher numbers of MDSCs have been found in the spleen and ascites fluid of tumour-bearing iNKT-cell-deficient mice than in that of wild-type mice[146]. However, from all this data on iNKT cells and MDSCs, a larger question arises: specifically, is influencing the differentiation and function of myeloid cells a role iNKT cells have during an immune response? Acquiring a deeper understanding of iNKT cells' ability to modulate myelopoiesis is an overarching theme of this thesis.

1.5.4 iNKT cells and myelopoiesis

Several lines of evidence have pointed to an important role for iNKT cells in regulating haematopoiesis. Firstly, activated iNKT cells secrete haematopoietic growth factors such as GM-CSF and IL-3[125,130]. In fact, mice treated with a single intravenous injection of αGalCer develop increased committed haematopoietic progenitor activity (Colony forming units) in the bone marrow, spleen and peripheral blood, as well as an efflux of granulocytes from the bone marrow (mostly neutrophils), all shown to be associated with the production of the NKT-cell derived GM-CSF and IL-3[147].

Expounding on the above findings, Kotsianidis et al showed that haematopoiesis is impaired in iNKT-cell deficient mice. Specifically,

Kotsianidis found that there was reduced short and long-term progenitor activity in the absence of NKT cells, coupled with peripheral blood cytopenias, reduced marrow cellularity and a lower frequency of hematopoietic stem cells (HSCs). However, it is worth noting that Kotsianidis highlighted that it was the myeloid clonogenic potential of human haematopoietic progenitors that increased in the presence of glycolipids-activated GM-CSF-secreting iNKT[97], thereby concluding that iNKT cells served to predominantly enhance myelopoiesis[97].

It is widely accepted that polymorphonuclear leukocytes (PMNs or neutrophils) provide the first line of defence against invading pathogens by producing a wide range of effector molecules essential for pathogen killing[148]. As such, the appropriate differentiation of myeloid progenitors for the expansion of PMNs as effectors during an infection is an integral part of the immune response. While the above reports on the ability of αGalCer-stimulated iNKT cells to increase myelomonocytic activity[97,147] may allude to a role for iNKT cells in augmenting myelopoiesis, mechanisms through which iNKT cells play a physiological role in regulating myelopoiesis in response to infections or tumour-bearing states remain to be demonstrated.

We believe that the greater expansion of suppressive $CD11b^{+}Gr1^{+}$ cells in flu-infected iNKT-cell deficient mice (as compared to wild type mice)[145] may be providing an intriguing window of insight into the influence iNKT cells may have on the differentiation of myeloid progenitors in response to infection. Following this observation we hypothesize that during an

influenza A virus infection iNKT cells may be modulating myeloid progenitor differentiation towards effector cells, like PMNs, thereby counterbalancing a possible influenza-derived cytokine-driven lineage commitment of progenitors towards MDSCs that would occur if iNKT cells were absent or anergic.

Consistent with our hypothesis, recent reports suggest that PMNs can be differentially polarized into effector neutrophils (N1) and immunoregulatory neutrophils (N2)[149,150]. Firstly, Fridlender et al described the ability of tumour-derived transforming growth factor-b (TGF-b) to induce the expansion of a regulatory $CD11b^{+}Ly6G^{+}$ hyper-segmented population capable of suppressing CD8 T-cell activation[149]. Subsequently, Zhang et al showed that in bacterial and fungal infections, the co-triggering of TLR-MyD88 and C-type lectin receptor (CLR)-Syk-dependent pathways in myeloid cells results in the differentiation of a $CD11b^{+}Ly6G^{+}$ hyper-segmented population that secrets the anti-inflammatory cytokine IL-10 rather than any pro-inflammatory cytokines (such as IL-12p40, IL-1b, IL-6 or CXCL1)[150].

It is important to note at this juncture that while MDSCs have been characterized as $CD11b^{+}Gr1^{+}$, Gr1 antibodies (clone RB6-8C5) recognize both Ly6G and Ly6C antigens; Ly6G is known to be a neutrophil marker in mice while Ly6C is expressed by monocytes and dendritic cells[151]. Therefore, we now question whether the $CD11b^{+}Gr1^{+}$ cells described by De Santo that expand in flu-infected iNKT-deficient mice are in fact IL-10 secreting regulatory neutrophils as described in Zhang's bacterial infection model,

with the ability to suppress CD8 T cell proliferation as highlighted by Fridlender's tumour model.

1.6 Thesis Aims

We believe that investigating the skew observed in myeloid differentiation towards MDSCs in flu-infected iNKT-deficient mice (as compared to wild type mice) may shed light on whether iNKT cells have a physiological role in modulating myelopoiesis during an immune response. With this concept as a starting point, this thesis is structured around addressing the following:

(i) To determine if the myeloid-derived suppressor cells that expand in flu-infected iNKT-deficient mice are suppressive IL-10 secreting neutrophils.

(ii) To determine if differences in response to flu infection can be detected at the level of the granulocyte-macrophage progenitor (GMP) population in the bone marrow of iNKT-deficient mice versus wild type mice.

(iii) To determine if iNKT cells can directly crosstalk with the GMPs and thereby modulate their differentiation and/or function.

And if objective (iii) is met, then:

(iv) To determine what soluble factors present during flu infection modulate myelopoiesis by facilitating myeloid progeny's interaction with iNKT cells.

CHAPTER 2

IL-10-secreting immunosuppressive granulocyte expansion regulated by invariant NKT cells

2.1 Introduction

Several investigators have set about characterizing subsets within the heterogeneous myeloid-derived suppressor cell population. Dolcetti et al rightly pointed out that MDSC subset definition is not a barren exercise since it can address several important issues: firstly, it has the potential to reduce MDSC heterogeneity and eliminate elements that do no directly contribute to the main functional properties of MDSCs, and secondly the process could place MDSCs more directly into the haematopoietic phylogenetic tree[152].

The focus of this chapter adds a third reason to Dolcetti's list, in that we aimed to better define the MDSCs that expand in flu-infected iNKT-deficient mice[145] so as to gain deeper insight into how iNKT cells are modulating the differentiation of myeloid cells. Specifically, we were curious to determine if the IL-10-secreting $CD11b^{+}Ly6G^{+}$ cells described by Zhang et al are akin to

the $CD11b^{+}Gr1^{+}$ suppressive cells described by De Santo in flu-infected iNKT-deficient mice. We attempted to repeat De Santo's conditions and screen for IL-10 levels and then go on to investigate whether the expansion of the MDSCs identified was indeed influenced by CD1d expression and therefore the ability of myeloid cells to crosstalk with iNKT cells.

2.2 MDSC expansion in flu-infected iNKT-deficient mice is accompanied by higher IL-10 levels in the serum

With a view to compare IL-10 levels between flu-infected iNKT-deficient and wild type (WT) mice we first sought to reproduce De Santo's model of flu-infection. Mice were injected with 160 Hemagglutinin Units (HIU) of the Influenza A/Puerto Rico/8/34 virus (flu) intra-nasally and culled 7 days thereafter to analyse the percentage of $CD11b^{+}Ly6G^{+}$ cells in the lungs and the ability of these cells to suppress an OT-1 proliferation assay.

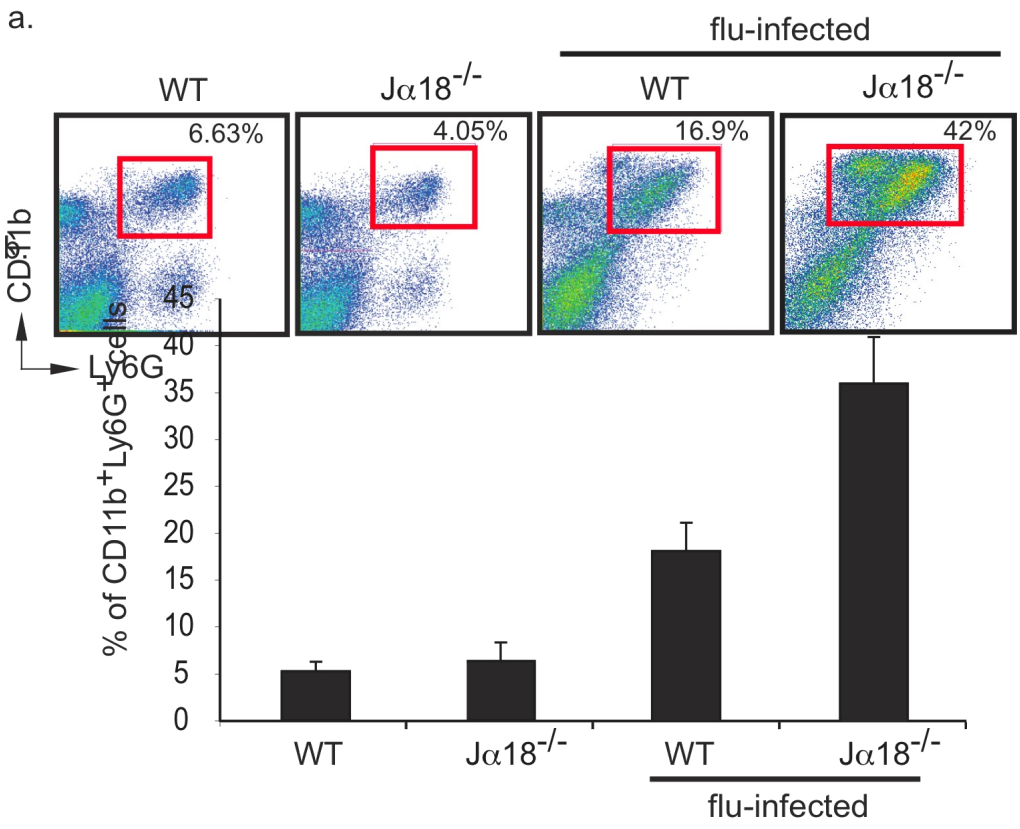


Figure 8: CD11b$^+$Ly6G$^+$ cells expand in the lungs of flu-infected iNKT-deficient mice; (a) FACS analysis comparing the percentage of CD11b$^+$Ly6G$^+$ cells in the lungs of naïve versus flu-infected WT and Jα18$^{-/-}$ mice; (**b)** Bar graph showing a greater expansion of CD11b$^+$Ly6G$^+$ cells in the lungs of flu-infected Jα18$^{-/-}$ mice versus infected WT mice or the naïve counterparts; (n=6 for all groups).

Indeed there was a greater expansion of CD11b$^+$Ly6G$^+$ cells in the lungs of flu-infected Jα18$^{-/-}$ mice as compared to flu-infected WT mice (**Figure 8**). Further, only those CD11b$^+$Ly6G$^+$ cells isolated from the lungs of infected Jα18$^{-/-}$ mice (and not from either the WT or naïve mice) had the ability to

exquisitely suppress T cell proliferation as determined with an OT-1 proliferation assay (**Figure 9**).

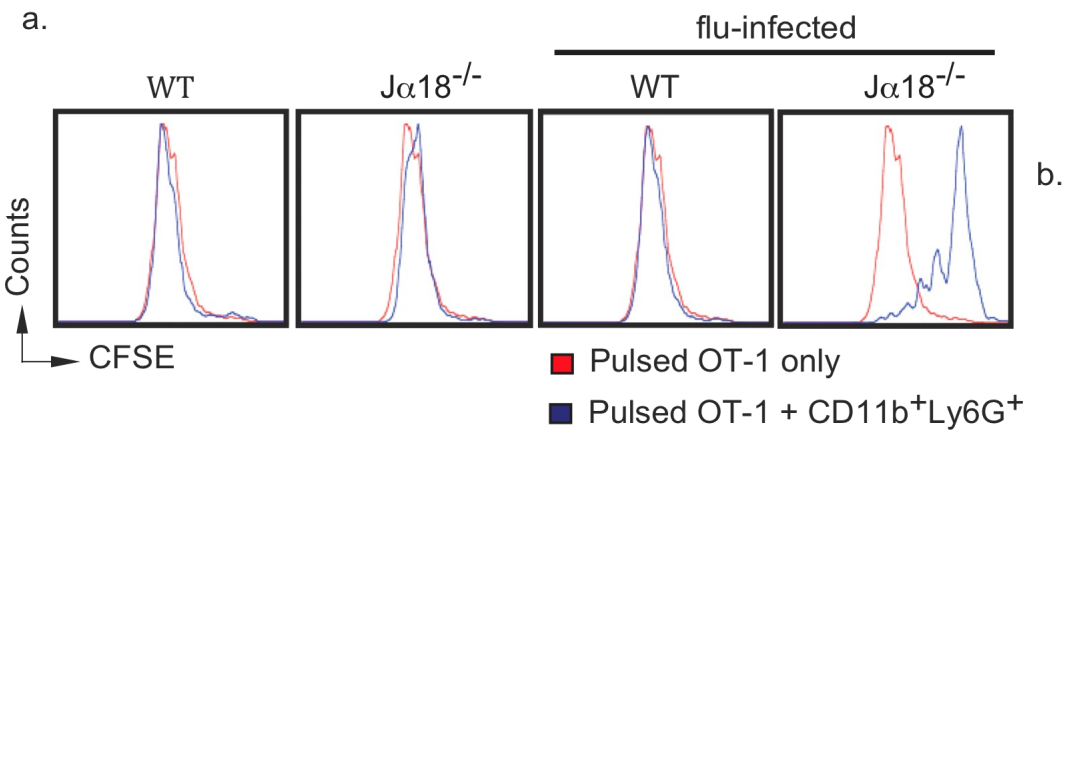


Figure 9: CD11b$^+$Ly6G$^+$ cells expand in the lungs of flu-infected iNKT-deficient mice and suppress T cell proliferation; (a) OT-1 Proliferation assay graph showing the ex-vivo suppression of the proliferation of CFSE-labelled OT-1 splenocytes by CD11b$^+$Ly6G$^+$ cells isolated from the lungs of flu-infected Jα18$^{-/-}$ mice; **(b)** Histogram showing the ex-vivo suppression of CFSE-labelled OT-1 splenocyte proliferation by CD11b$^+$Ly6G$^+$ cells isolated from the lungs of flu-infected Jα18$^{-/-}$ mice (n=6 for all groups).

Importantly, on day 7 post flu infection, before culling we also bled the WT and Jα18$^{-/-}$ mice, and showed for the first time that coupled with the above findings there was a significantly higher level of IL-10 in the serum of flu-infected Jα18$^{-/-}$ mice as compared to flu-infected WT mice or the naïve counterparts (**Figure 10**).

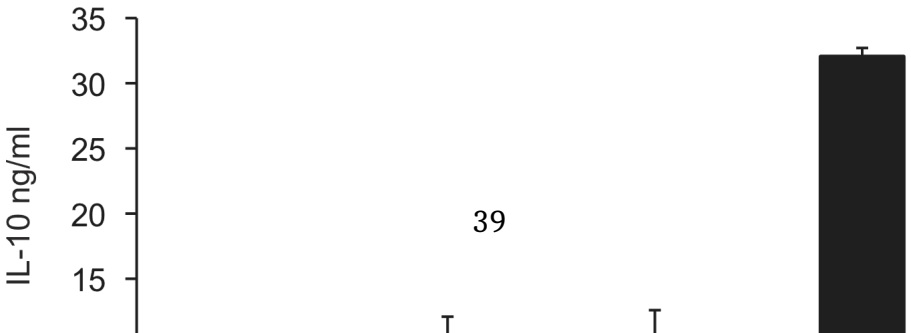


Figure 10: Bar graph showing higher levels of IL-10 in the serum of flu-infected Jα18$^{-/-}$ as compared to flu-infected WT mice or the naïve counterparts (n=6 for all groups).

Two questions arose from the above findings. Firstly, whether secretion of IL-10 by immunosuppressive CD11b^{+}Ly6G^{+} cells contributed to the increased levels of IL-10 in the serum of the iNKT–deficient mice, and secondly whether the ability to crosstalk with iNKT cells influences the expansion of such a population of suppressive myeloid cells.

2.3 Generation of IL-10-secreting CD11b^{+}Ly6G^{int} suppressive cells in flu infection is CD1d-dependent

In an attempt to address both questions posed above using one experimental model we engineered 50:50 mixed bone marrow chimeras in lethally irradiated mice, in which half of the hematopoietic cells were CD1d and CD45.1 positive (from B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ, herein referred to as B6-SJL, donors), while the other 50% of hematopoietic cells were CD1d negative and CD45.2 positive (obtained from CD1d-deficient donors on a B6 background), and therefore unable to interact with iNKT cells (**Figure 11**).

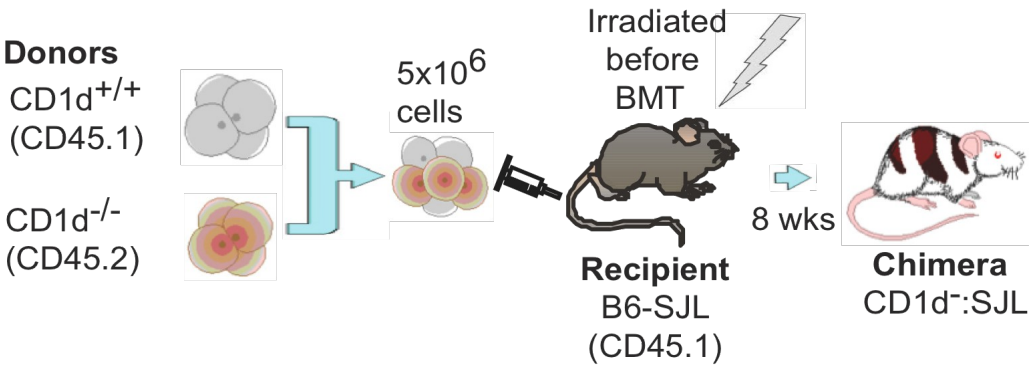


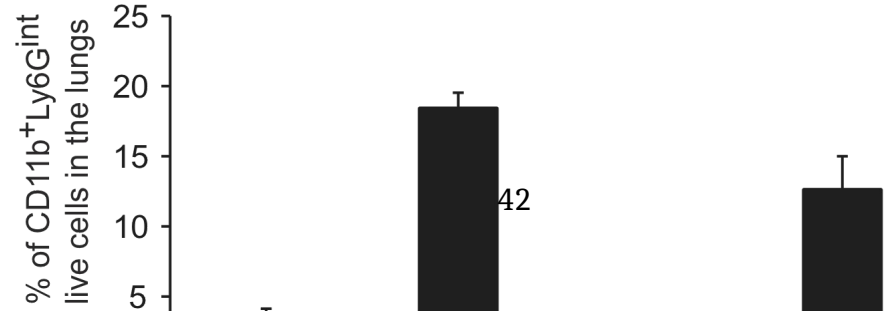
Figure 11: Experimental design for engineering CD1d$^{-/-}$:SJL mixed bone marrow chimeras. B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (B6-SJL) mice were irradiated with two doses of 450rads, 3 hours apart at the Biomedical Services Unit, John Radcliffe Hospital, University of Oxford. Mice were then immediately reconstituted with bone marrow cells from donor mice by intravenous injection of 5 x 10^6 bone marrow cells in an inoculum of 100ml using a 30 G needle. Sample mice were reconstituted to have a 50:50 ratio of CD1d^{-} CD45.2: CD1d^{+}CD45.1 hematopoietic cells (as shown in the figure) while control mice were reconstituted to have a 50:50 ratio of CD1d^{+}CD45.2: CD1d^{+}CD45.1 hematopoietic cells. The mice were kept in micro-isolator cages with sterile sawdust, food and water while they were allowed to reconstitute for eight weeks. The blood of all animals was thereafter examined for reconstitution by using antibodies and FACS.

We hypothesized that if the differentiation and expansion of IL-10-secreting immunosuppressive CD11b^{+}Ly6G^{+} cells was influenced by contact with iNKT cells, then flu infection may preferentially induce such an expansion in cells derived from the CD1d^{-} (CD45.2) population and not the CD1d^{+} (CD45.1) population. To ensure that any effect seen was due to the expression of CD1d on the hematopoietic cells, control 50:50 (B6:SJL) mixed bone marrow chimera mice were engineered from C57BL/6 (CD45.2) and B6-SJL (CD45.1) and infected in parallel with the CD1d^{-}:SJL mice.

Before flu infection, both sample and control animals were bled and examined for a 50:50 reconstitution of CD45.1 and CD45.2 cells. Seven days post intranasal flu infection, both CD1d^{-}:SJL and B6:SJL bone marrow

chimera mice were culled, lungs harvested, homogenized and then analysed by FACS for $CD11b^{+}Ly6G^{+}$ cell infiltration. Interestingly, there was an expansion of a $CD11b^{+}$ population that had an intermediate level of expression of Ly6G ($CD11b^{+}Ly6G^{int}$) in the lungs of all flu-infected mice (**Figure 12b**). Of note, the expansion of $CD11b^{+}Ly6G^{int}$ cells in the lungs of the flu-infected $CD1d^{-}$:SJL chimeras was only marginally greater than the expansion in the lungs of the control B6:SJL bone marrow chimeras, although the difference proved to be statistically significant (**Figure 12a**). However, on analyzing the composition of the expanded $CD11b^{+}Ly6G^{int}$ population, the lungs of the flu-infected $CD1d^{-}$:SJL chimera mice had a considerably greater expansion of the CD45.2 ($CD1d^{-}$ derived) $CD11b^{+}Ly6G^{int}$ cells as compared to the CD45.1 (SJL $CD1d^{+}$ derived) positive $CD11b^{+}Ly6G^{int}$ cells, the former making up almost 80% of the expanded $CD11b^{+}Ly6G^{int}$ population (**Figure 12c**). By contrast, the $CD11b^{+}Ly6G^{int}$ cells in the lungs of the naïve as well as the flu-infected control B6:SJL bone marrow chimeras had an equal (50:50) representation of CD45.2 and CD45.1 cells (**Figure 12c**).

a.



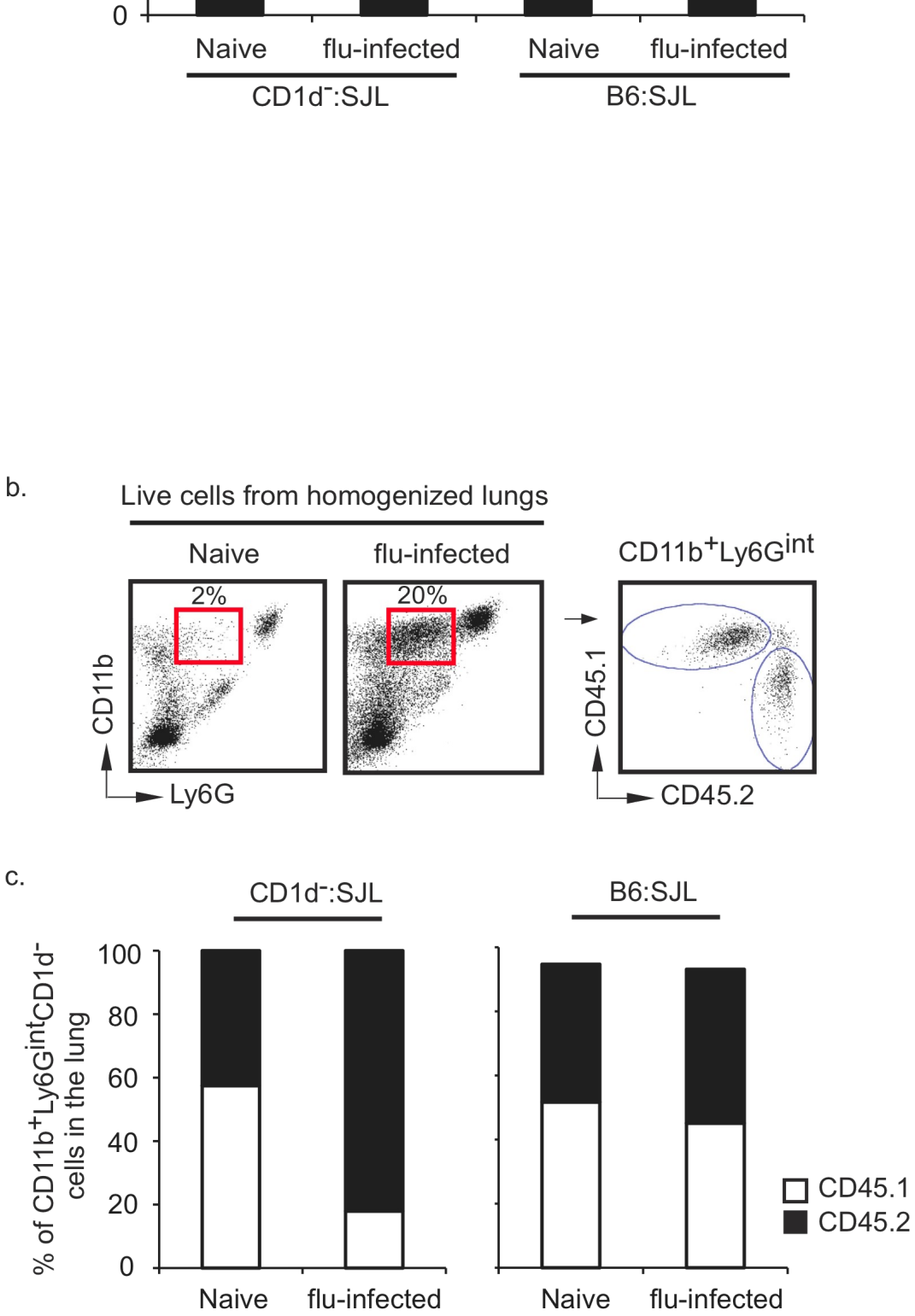
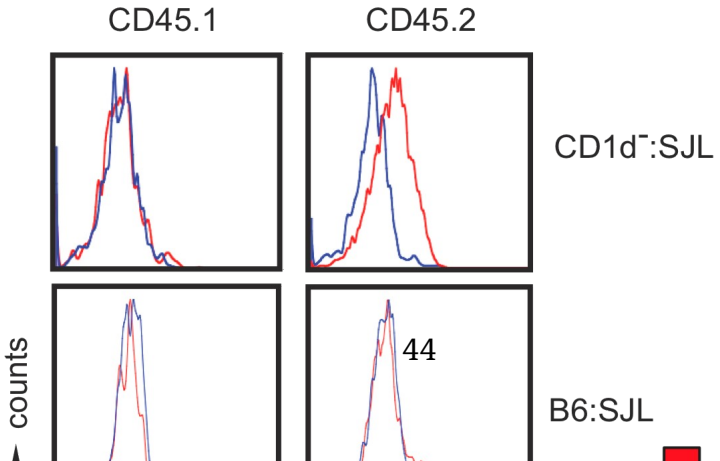


Figure 12: Expansion of CD11b^{+}Ly6G^{int} cells in the lungs of flu-infected mice; (a) Bar graph showing the expansion of CD11b^{+}Ly6G^{int} cells in the lungs of flu-infected mice. The increase in CD11b^{+}Ly6G^{int} cells in the flu-infected CD1d^{-}:SJL mice was marginally but statistically significantly higher than the expansion of the CD11b^{+}Ly6G^{int} cells in flu-infected control B6:SJL bone marrow chimera mice; **(b)** FACS analysis showing the expansion of CD11b^{+}Ly6G^{int} cells in flu-infected mice, and the relative expression of CD45.1 (SJL CD1d^{+} derived) versus CD45.2 (B6 CD1d^{-} derived) cells within the CD11b^{+}Gr1^{int} population: **(c)** Graph showing the relative expansion of the CD45.2 (B6 CD1d^{-} derived) population of CD11b^{+}Ly6G^{int} cells within the lungs of flu-infected CD1d^{-}:SJL mice as compared to the CD45.1 (SJL CD1d^{+} derived) CD11b^{+}Ly6G^{int} population. Both naïve and flu-infected B6:SJL bone

marrow chimera mice had a 50:50 ratio of CD45.1:CD45.2 cells within the $CD11b^{+}Ly6G^{int}$ population within the lungs.

We used FACS to gate on the $CD11b^{+}Ly6G^{int}$ population from the homogenized lungs of the control and sample bone marrow chimera mice and separately sorted the CD45.1 and CD45.2 cells. Intracellular staining with anti-IL10 antibody revealed that only the $CD11b^{+}Ly6G^{int}CD45.2^{+}$ cells (which therefore lack CD1d expression) isolated from the lungs of the flu-infected $CD1d^{-}$:SJL bone marrow chimeras produced IL-10 (**Figure 13**). Further it was this population of $CD11b^{+}Ly6G^{int}CD45.2^{+}$ cells from the lungs of the flu-infected $CD1d^{-}$:SJL chimeras that was able to suppress OT-1 proliferation *ex-vivo*. Neither $CD11b^{+}Ly6G^{int}CD45.1^{+}$ cells (which are SJL.$CD1d^{+}$ derived) from the lungs of flu-infected $CD1d^{-}$:SJL chimeras nor any of the $CD11b^{+}Ly6G^{int}$ cells isolated from the flu-infected B6-SJL control chimeras demonstrated the ability to suppress T cell proliferation as demonstrated by an OT-1 proliferation assay (**Figure 13**).

a.



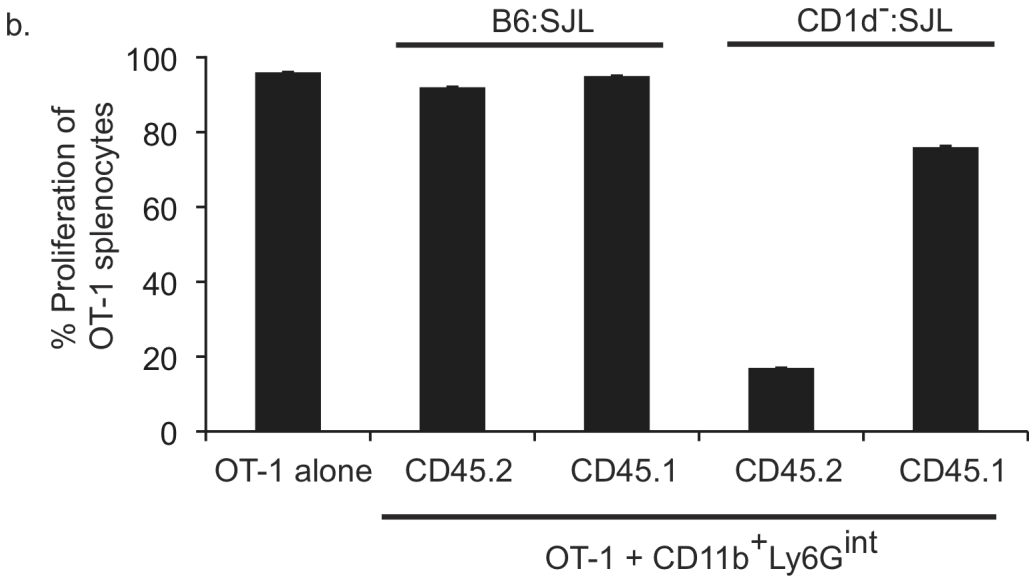


Figure 13: Generation of IL-10-secreting CD11b^{+}Ly6G^{int} suppressive cells in flu infection is CD1d-dependent; (a) Histogram of intracellular staining with anti-IL-10 antibody (**Red**) showing that only the CD11b^{+}Ly6G^{int}CD45.2^{+} cells (which lack CD1d expression) isolated from the lungs of flu-infected CD1d^{-}:SJL chimeras produced IL-10; **(b)** Bar graph showing OT-1 proliferation in the presence of CD11b^{+}Ly6G^{int} cells that are either CD45.2 or CD45.1 derived, and isolated from the lungs of flu-infected bone marrow chimera mice. Only CD11b^{+}Ly6G^{int}CD45.2^{+} cells (which lack CD1d expression) isolated from the lungs of flu-infected CD1d^{-}:SJL chimeras suppressed OT-1 proliferation *ex-vivo*; neither the CD11b^{+}Ly6G^{int}CD45.1^{+} from the lungs of flu-infected CD1d^{-}:SJL chimeras nor any lung CD11b^{+}Ly6G^{int} cells from flu-infected B6-SJL control chimeras demonstrated the ability to suppress T cell proliferation in the OT-1 assay (n=4 per group).

2.4. Discussion

There are four main findings worth highlighting from experiments described in this chapter: (a) There is significantly greater IL-10 in the

serum of Influenza A virus (flu) infected iNKT-deficient mice than in both flu-infected WT mice and the naïve counterparts (b) Flu infection induces the expansion of a $CD11b^{+}Ly6G^{int}$ population of cells in the lungs of the mice; (c) $CD11b^{+}Ly6G^{int}$ cells derived from CD1d negative cells (which are unable to interact with iNKT cells) gain a flu-induced ability to produce IL-10, while $CD1d^{+}CD11b^{+}Ly6G^{int}$ cells do not; and (d) The flu-induced IL-10 producing $CD11b^{+}Ly6G^{int}$ ($CD1d^{-}$) cells are capable of suppressing T cell proliferation.

IL-10 is widely accepted as an anti-inflammatory cytokine[153]. Among its regulatory functions is the ability to enhance the differentiation of regulatory T (T_{Reg}) cells[154-156] and inhibit the innate effector functions of macrophages and DCs, which in turn inhibit subsequent T_H1 and T_H2 responses. Many cells of both arms of the immune system express IL-10. Mast cells, DCs, macrophages and natural killer cells[154] are among the innate effectors listed as IL-10 secreting, while T_H1, T_H2, T_H17, B and $CD8^{+}$ T cells[154,156-160] have been cited as IL-10 producing cells in the adaptive arm of the immune system. More relevant to this project however is Zhang et al's finding of IL-10-secreting $CD11b^{+}Ly6G^{+}$ neutrophils in bacterial infections[150], as mentioned earlier in the background literature review.

Many publications highlight the crucial role of IL-10 in preventing inflammatory and autoimmune pathologies[154,161,162]. Interestingly, although Zhang's findings demonstrate a delicate balance between the direct bacterial elimination effects and the IL-10 (anti-inflammatory) effects in an acute mycobacterial infection, depletion of the neutrophils in the chronic

phase of the infection (when mycobacteria numbers are high and stable) proved to be beneficial for the host, as it enabled more rigorous T_H1 and T_H17 responses to control the infection[150]. As such it is evident that there are conditions in which the regulatory effect of IL-10 on the immune response may be exacerbating the pathology.

Indeed, the phenotype and regulatory effect of the neutrophils described in Zhang's mycobacterial model naturally raise the question as to whether they are any different from the granulocytic subset of MDSCs, previously described by Youn et al as $CD11b^+Ly6G^+Ly6C^-$ [163]. Using several tumour types, Youn identified the expansion of a $CD11b^+Ly6G^+Ly6C^-$ (granulocytic) MDSC subset separate from a $CD11b^+Ly6G^-Ly6C^{high}$ (monocytic) MDSC subset, highlighting arginase-dependent reactive oxygen species (ROS) production as the primary mechanism of the granulocytic MDSCs' ability to suppress T cell responses, as compared to the iNOS-dependent mechanism of the monocytic MDSCs[163]. Our findings in influenza A virus infected mice have indeed bridged Zhang and Youn's reports, through the identification of the expansion of an IL-10-producing population of $CD11b^+Ly6G^{int}$ cells capable of suppressing T cell proliferation.

The significance of the intermediate brightness of the Ly6G marker on the MDSCs expanded in our studies may not just be anecdotal. In January of 2010 Luigi Dolcetti et al published on a hierarchy of immunosuppressive strength among myeloid-derived suppressor cells based on the brightness of Gr-1 expression[152]. Using three different mouse tumour models known to induce MDSC expansion (namely CT26 - a carcinogen-induced

undifferentiated colon carcinoma, 4T1 – a mouse mammary carcinoma cell line and MCA203 – a 3-methylchalanthrene-induced Fibrosarcoma) Dolcetti showed that within the $CD11b^+$ population the $Gr\text{-}1^{high}$ subset exerted only a marginal suppressive influence at high cell numbers, while the $Gr\text{-}1^{int}$ subset in all three tumour models had stronger suppressive activity. In short, *in vitro* mixed leukocyte peptide cultures and alloantigen stimulated lymphocyte assays (MLR) showed that $CD11b^+Gr\text{-}1^{int}$ cells from all three models of tumour-bearing mice were capable of suppressing $CD8^+$ T cell activation. However $CD11b^+Gr\text{-}1^{high}$ cells from the CT26 and MCA203 tumour-bearing mice (and not the mammary-carcinoma) exerted weak suppressive activity, observed only at high MDSCs numbers[152]. Additionally, only the $CD11b^+Gr\text{-}1^{int}$ and not the $CD11b^+Gr\text{-}1^{high}$ cells were immunosuppressive *in vivo* following adoptive-transfer[152]. Therefore the fact that the immunosuppressive cells expanding in the flu-infected mice in our studies are the $CD11b^+Gr\text{-}1^{int}$ and not the $CD11b^+Gr\text{-}1^{high}$ population correlates beautifully with Dolcetti's hierarchy of immunosuppressive MDSCs. Moreover, our use of the anti-Gr-1 antibody from the 1A8 clone (which recognizes Ly6G) allowed us to better define the flu-induced immunosuppressive $CD11b^+Gr\text{-}1^+$ population originally described by De Santo et al.

Perhaps the most intriguing finding in this chapter however is the influence CD1d expression (and therefore the ability to crosstalk with iNKT cells) has on the generation of IL-10-producing suppressive $CD11b^+Ly6G^{int}$ in flu-infected mice. By using the $CD1d^-$:SJL bone marrow chimeras, in which half

of the hematopoietic stem cells in the bone marrow express CD1d and the other half are CD1d-deficient, we observed that only $CD11b^{+}Ly6G^{int}$ cells that were CD1d negative acquired an flu-induced ability to produce IL-10 and suppress T cell proliferation. These findings definitely confirm and expound upon De Santo et al's initial report that iNKT cells influence the expansion of immunosuppressive $CD11b^{+}Gr\text{-}1^{+}$ in flu infection[145].

Following the above findings, a question naturally arises as to what stage is the modulation of myelopoeisis by iNKT cells occurring so as to counterbalance any flu-induced differentiation of immunosuppressive IL-10-secreting regulatory $CD11b^{+}Ly6G^{int}$ cells. Specifically, are iNKT cells (which are a small population of T cells) influencing the differentiation of myeloid cells at the site of an infection in the periphery or are they acting in the bone marrow before myeloid cells are mobilized in response to an infection? We attempt to address this question in the following chapter.

CHAPTER 3

iNKT cells influence Granulocyte-macrophage progenitor differentiation and function during influenza A virus infection

3.1 Introduction

It is likely that the frequency and tissue distribution of iNKT cells allude to where this relatively small subset of regulatory T cells may be best modulating myeloid differentiation and function. In the mouse, iNKT cells have been shown to represent approximately 0.5% of the T cell population in the blood and peripheral lymph nodes, about 2.5% of T cells in the spleen, mesenteric and pancreatic lymph nodes, but up to 30% of T cells in the liver, where they appear to patrol the liver sinusoids[164]. Although De Santo et al have shown that the immunosuppressive activity of the $CD11b^{+}Gr\text{-}1^{+}$ cells isolated from the lungs of flu-infected iNKT-deficient mice can be abrogated by a CD1d- and CD40-dependent crosstalk with iNKT cells[145], we believe that it is improbable that iNKT cells are acting in the periphery to curtail the differentiation of these bone marrow derived MDSCs because of the relative paucity of iNKT cells in the periphery compared to the MDSCs' frequency measured in a flu infection. We therefore hypothesized that iNKT cells may be acting further upstream, on the myeloid progenitors in the bone marrow so as to influence myeloid differentiation before cells have matured and expanded in response to an infection. The comparable frequency of myeloid progenitors and iNKT cells in the bone marrow niche supports this notion and exploring this hypothesis is therefore the focus of this chapter.

Sorting granulocyte-macrophage progenitors (GMPs) from the bone marrow of both naïve and flu-infected WT and $J\alpha18^{-/-}$ mice to analyse the progenitors themselves for phenotypic and functional differences was the first step taken to investigate whether the presence or absence of iNKT cells

during a flu infection has any impact on the myeloid progenitors, and by extension on myelopoiesis.

As done for the MDSC analysis in flu-infected mice in the previous chapter, both WT and Jα18$^{-/-}$ mice were injected with 160 HIU of the IAV/PR8 virus intra-nasally and culled 7 days thereafter; once the bone marrow was harvested from each of the mouse samples, the surface markers and staining protocol published by Akashi et al[11] was used for the GMP isolation (**Figure 14**).

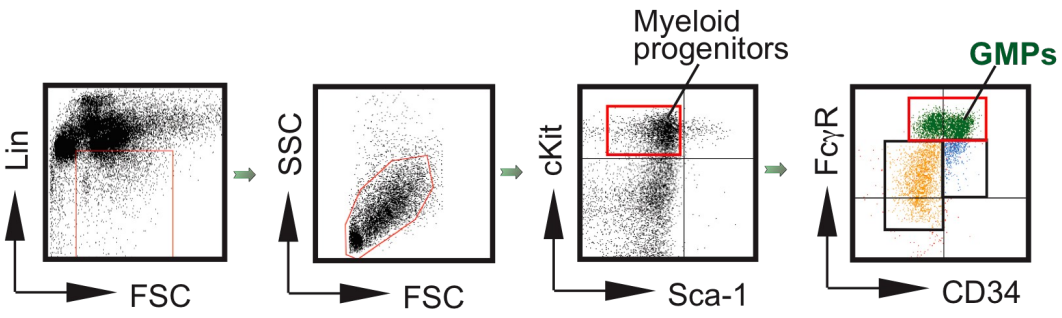


Figure 14: Dot plots showing the markers and gating used to analyse and sort granulocyte-macrophage progenitors (GMPs) from the bone marrow of naïve and flu-infected WT and Jα18$^{-/-}$ mice (the figure shows the analysis of one mouse but represents the protocol used for all mice in the experiment, the populations sequentially gated on being those in the red boxes); by definition the myeloid progenitors are a Lin^{-}cKit^{+}Sca-1^{-} population of cells (the lineage cocktail comprising of anti-CD4, CD5, CD8α, CD11b, Gr-1, TER119, B220 and the lymphoid marker CD127); GMPs (**green**) were identified as the FcgR^{hi}CD34^{+} fraction of cells within the population of myeloid progenitors. The protocol used and shown above is as described by Akashi et al[11]. Of note the Common Myeloid progenitors, CMPs (**blue**), considered by Akashi in this protocol to be the FcgR^{lo}CD34^{+} cells within the population of myeloid progenitors were later shown by Pronk et al to not be a pure population of cells as the gate included subsets of intermediate myelo-erythroid precursors as well[12]. As such we decided to focus on the better-accepted pure GMP population in our studies. Megakaryocyte-erythrocyte progenitors, MEPs (**orange**), are the FcgR^{lo}CD34^{-} fraction of cells within the myeloid progenitor population.

3.2 CD1d is expressed on GMPs, but CD40 expression is up-regulated only on the GMPs of flu infected WT mice and not infected Jα18$^{-/-}$ mice

For our hypotheses to be on firm footing we first had to determine if GMPs even had the ability to crosstalk with iNKT cells. For crosstalk to occur between an iNKT cell and any other cell, the latter must be able to present lipid antigen in the context of a CD1d molecule to thereby activate the TCR of the iNKT cell. Additionally, direct activation of iNKT cells by antigen presenting cells also requires the interaction of CD40 (a co-stimulatory molecule) with CD40L on the surface of iNKT cells. As such GMPs from the bone marrow of WT and Jα18$^{-/-}$ mice were analyzed with fluorescent antibody cell sorting (FACS) for CD1d as well as CD40 expression.

Interestingly, GMPs from both WT and Jα18$^{-/-}$ mice expressed CD1d inferring an ability to present glycolipids to iNKT cells. Even more intriguing however was the evidence of CD40 up-regulation on the GMPs of flu-infected WT mice but not on those from infected Jα18$^{-/-}$ mice (**Figure 15**).

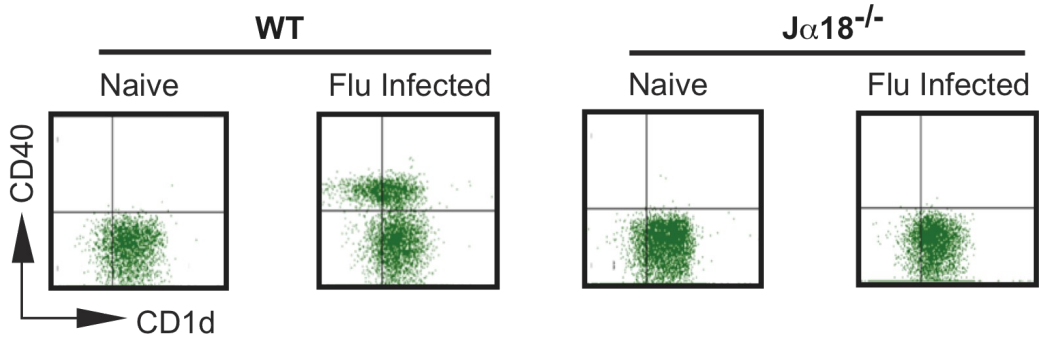


Figure 15: CD1d expression and CD40 up-regulation on GMPs; FACS analysis showing CD1d expression on the GMPs of both naïve and flu-infected WT and Jα18$^{-/-}$; of note however, only flu-infected WT mice up-regulate CD40 expression. The results represent plots confirmed with three separate experiments.

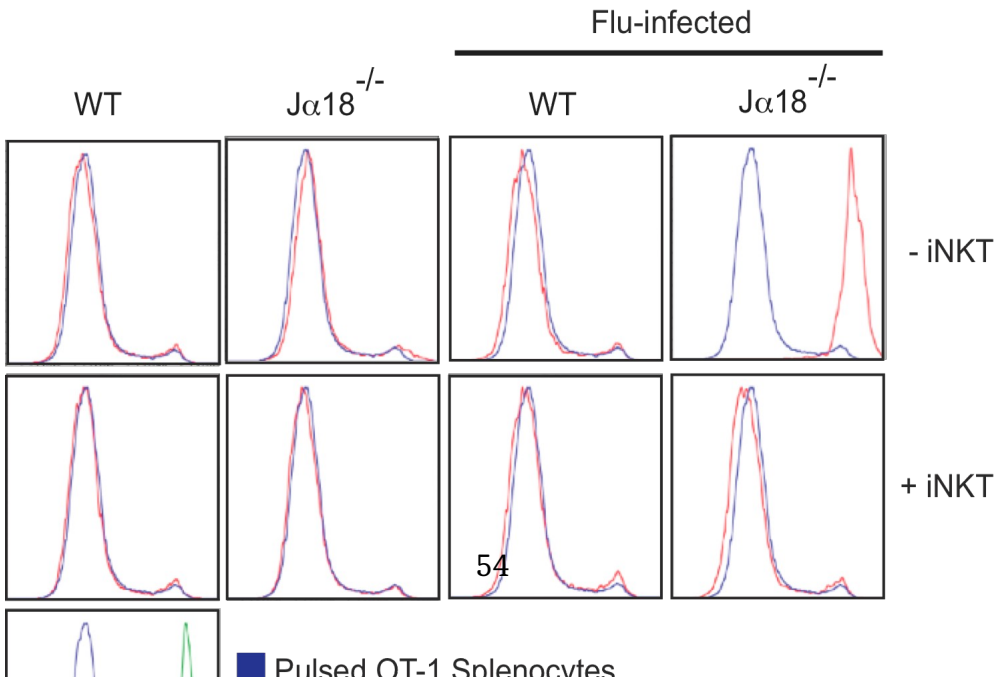
This novel finding led us to follow up with *ex vivo* experiments to determine if a functional difference accompanied this phenotypic difference. Specifically, we sought to determine if immunosuppressive ability was indeed acquired as early as GMPs in the myeloid lineage if iNKT cells were absent during a flu infection.

3.3 T cell proliferation is suppressed by GMPs from the bone marrow of flu-infected Jα18$^{-/-}$ mice but not by that from infected WT mice

CFSE-labelled OT-1 splenocyte proliferation was induced by pulsing CFSE labelled OT-1 splenocytes with their cognate antigen (SIINFEKL) and then culturing for 3 days in the presence or absence of GMPs acquired from the

bone marrow of either naïve or flu-infected WT or Jα18$^{-/-}$ mice. We observed that OT-1 proliferation was indeed inhibited when co-cultured with the GMPs from flu-infected Jα18$^{-/-}$ mice, but not when co-cultured with GMPs from infected WT mice or with that from naïve mice (**Figure 16**).

To investigate whether the presence of activated iNKT cells could abrogate the suppressive potential of the GMPs from infected Jα18$^{-/-}$ mice, we pulsed GMPs *ex vivo* with 100ng/ml of the iNKT agonists αGalCer first, and then co-cultured with iNKT cells for 24 hours. Following this, peptide pulsed CFSE-labelled OT-1 splenocytes were added to the GMP-iNKT cell mix. (Of note: iNKT cells were sorted to greater than 98% purity from the spleen of Vα14TCR transgenic mice). We observed that co-culturing activated iNKT cells with the GMPs from infected Jα18$^{-/-}$ mice relieved the suppressive activity of the GMPs, thereby rescuing OT-1 proliferation (**Figure 16**).



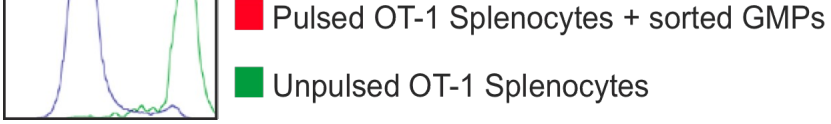


Figure 16: iNKT cells relieve the suppressive activity of GMPs from flu-infected mice; OT-1 Proliferation Assay showing the suppression of CFSE-labelled OT-1 splenocytes by the GMPs from the bone marrow (BM) of IAV/ PR8 infected Jα18$^{-/-}$ mice but not by GMPs from infected WT mice or from the naïve WT or Jα18$^{-/-}$ mice *(first row)*; Co-culturing activated iNKT cells with the GMPs from the infected Jα18$^{-/-}$ abrogated the suppressive activity and rescued OT-1 proliferation *(second row).* Unpulsed (and therefore non-proliferating) OT-1 cells are depicted as a control *(third row).*

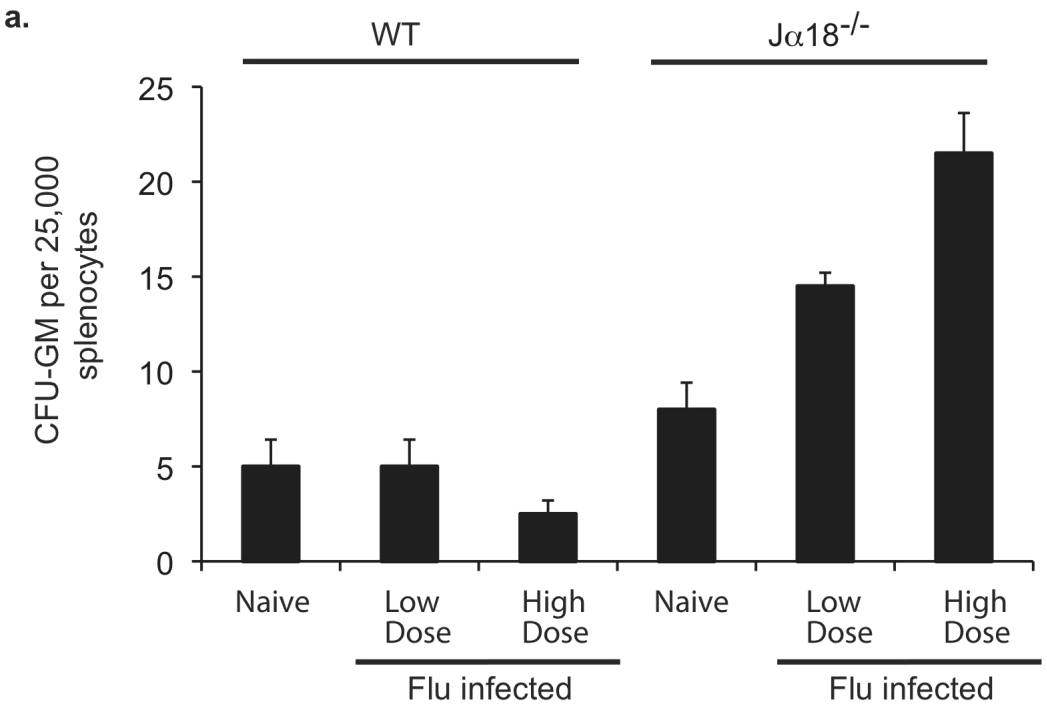
These findings suggest that in the absence of iNKT cells during a flu infection, GMPs not only lack CD40 up-regulation but also acquire immunosuppressive properties, thereby possibly contributing to the T cell suppression seen in flu infection[145]. Furthermore, activated iNKT cells have the ability to abrogate the suppressive activity of the GMPs. This prompted us to look in the periphery of infected WT and Jα18$^{-/-}$ mice to see if the development of suppressive GMPs was accompanied by mobilization of the myeloid progenitors.

3.4 CFU-GM colonies expand in the periphery of flu-infected Jα18$^{-/-}$ mice but not in that of infected C57BL/6 mice.

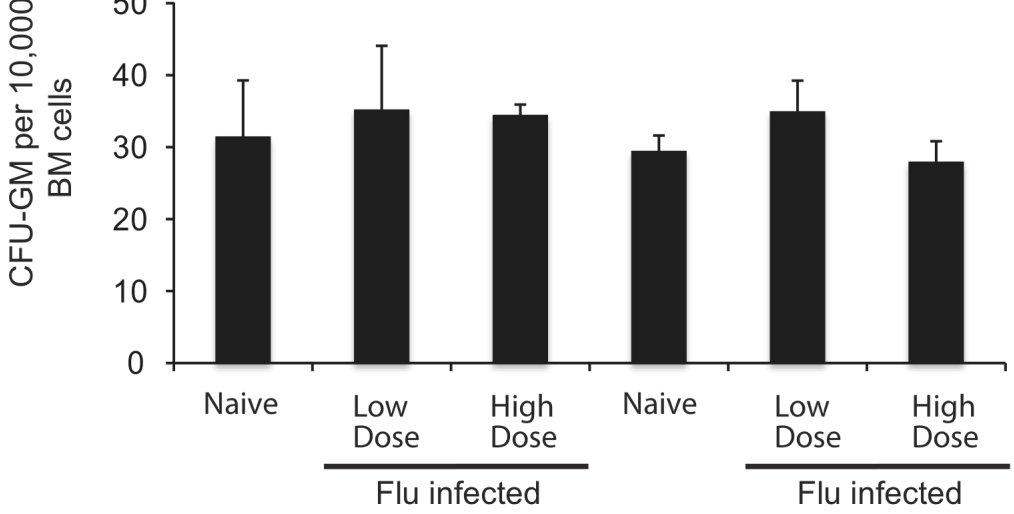
Cytokine-replete Colony forming unit (CFU) assays were plated to compare the number of cells with granulocyte-macrophage (GM) clonogenic potential in the periphery and bone marrow (BM) of naïve versus flu-infected mice for both WT and Jα18$^{-/-}$ mice. Mice were culled on day 7 post flu infection,

and plates for splenocytes (representing the periphery) were seeded with 25,000 cells while those for BM were seeded with 10,000. Interestingly, the number of CFU-GM colonies in the periphery of the infected Jα18$^{-/-}$ mice was significantly higher than in the naïve Jα18$^{-/-}$ mice, while those in the periphery of the WT remained at baseline levels. Additionally, the number of CFU-GM colonies in the bone marrow of Jα18$^{-/-}$ mice remained the same when comparing the naïve to the flu-infected mice.

Notably, when the experiment was performed using both a lower dose of IAV/PR8 (40 HIU, considered as the *'low dose'*) and the original dose of IAV/PR8 (160 HIU, considered as the *'high dose'*), the same pattern of increased CFU-GMs in the periphery of infected Jα18$^{-/-}$ mice emerged in a dose dependent manner (**Figure 17a**).



b.
WT
Jα18-/-



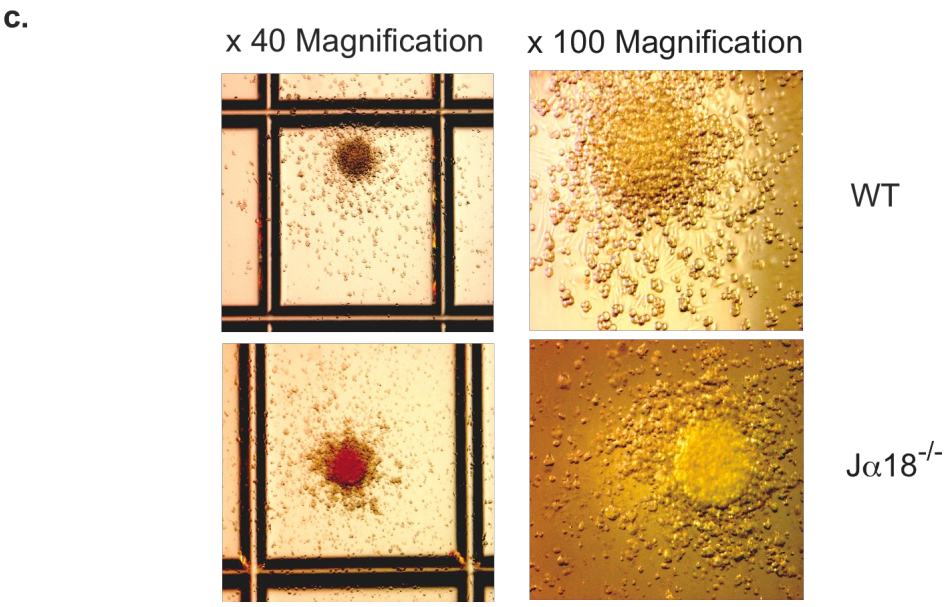


Figure 17: Increased numbers of Granulocyte-macrophage colony-forming units (CFU-GMs) in the periphery of flu-infected Jα18$^{-/-}$ mice; (a) Histogram showing the number of granulocyte-macrophage colony

forming units cultured from 25,000 splenocytes harvested from both naïve and flu-infected WT and Jα18$^{-/-}$ mice; **(b)** Histogram of the number of CFU-GM colonies cultured from 10,000 bone marrow (BM) cells from naïve and flu-infected WT and Jα18$^{-/-}$ mice; **(c)** Images of granulocyte-macrophage colonies counted with microscopy (any cluster of greater than 50 cells was considered to be one colony); colonies at x40 and x100 magnification from spleens of WT and Jα18$^{-/-}$ mice are depicted (Colony assays were done in Methylcellulose replete with mGM-CSF 5ng /ml, hFlt3L 10ng/ml, mIL-3 2ng/ml, hG-CSF 10ng/ml).

A possible explanation for the above findings is that mice with a suboptimal immune response, due to MDSC expansion in response to the infection, may have signals driving the BM to mobilize more myeloid cells to combat the infection, unwittingly some of which may be contributing to the impaired immune response through suppression of CD8 T cells. This is merely a speculative argument and would have to be delved into further to be a credible hypothesis. As such, possible causes of these findings and the details of iNKT involvement were explored further and discussed below.

3.5 GMPs crosstalk with iNKT cells in a CD1d-dependent manner

Having shown GMPs to be CD1d^{+}, we sought to investigate whether GMPs could crosstalk with the iNKT cells in a CD1d-dependent manner. For this purpose sorted GMPs (98% of purity) from the bone marrow of WT mice were pulsed with 100ng/ml of αGalCer and co-culture with sorted iNKT cells in the presence or absence of CD1d-blocking antibody (30μg/ml) for 24hours. We then analyzed IFNγ and IL-12p40 levels in the supernatant of these co-cultures by ELISA. Indeed we demonstrated that GMPs and iNKT cells could interact as evidenced by IFNγ and IL-12p40 production, suggesting there is bidirectional activation. Furthermore, we showed that

this crosstalk was CD1d-dependent, as defined by the lack of IFNγ and IL-12p40 secretion in those wells in which iNKT cells and αGalCer-pulsed GMPs were co-cultured in the presence of a CD1d-blocking antibody (**Figure 18**).

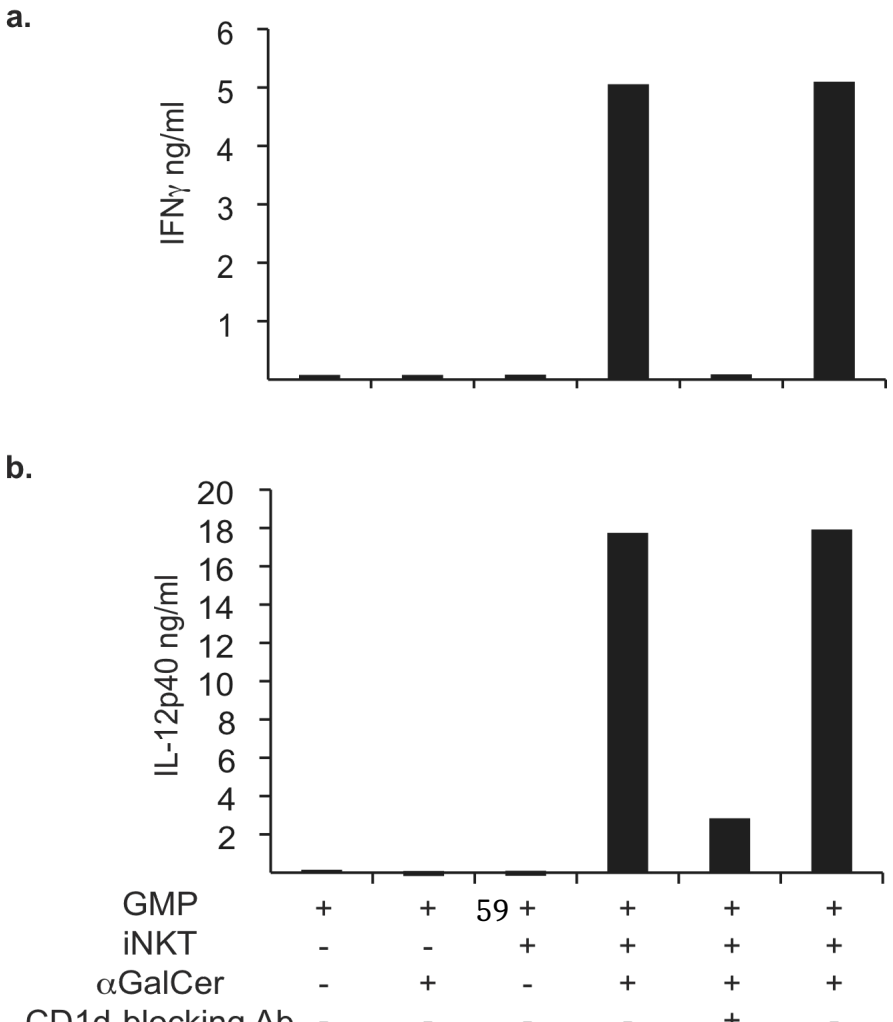


Figure 18: Crosstalk between GMPs and iNKT cells is CD1d-dependent; (a) Histogram showing IFNγ production when GMPs are pulsed with αGalCer and co-cultured with iNKT cells, abrogated by adding CD1d-blocking antibody, but not blocked by adding the isotype control of the CD1d-blocking Ab; **(b)** Histogram showing IL-12p40 production when GMPs are pulsed with αGalCer and co-cultured with iNKT cells, abrogated by adding CD1d-blocking antibody, but not blocked by adding the isotype control of the CD1d-blocking Ab.

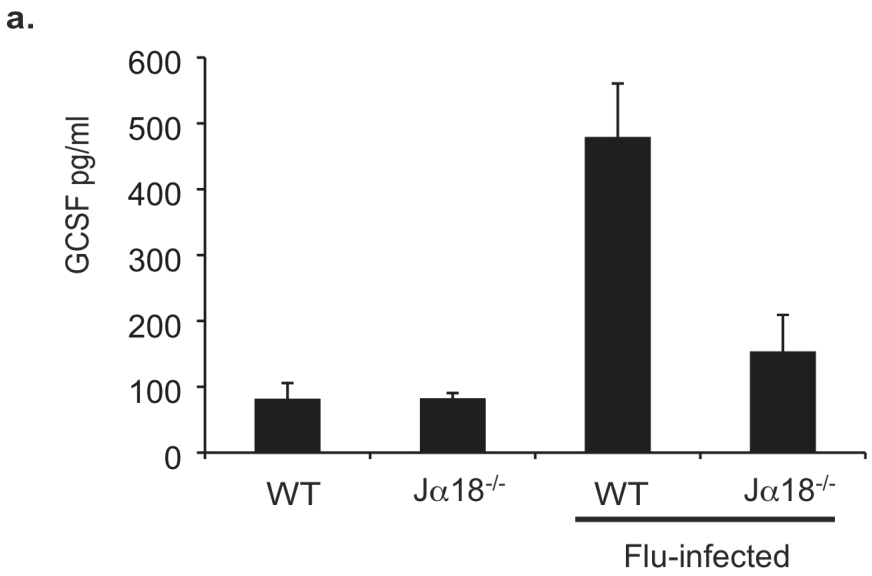
The results above led us to ask the question: what factor(s) during an Influenza A virus infection facilitate the crosstalk between iNKT cells and GMPs and/or MDSCs?

It has previously been shown that a wide array of pro-inflammatory cytokines are secreted in significant amounts by type II pneumocytes and alveolar macrophages during an IAV/PR8 (flu) infection, (including IL-1β, IL-6, IL-1Ra, IL-29, IP-10, MIP-1α, MIP-1β, RANTES, TNF-α, and MCP-1)[165], but differences in the levels of myelopoietic factors, such as GM-CSF, G-CSF, Flt3L or IL-3 weren't reported on. As such, with the goal of determining factors that influence myelopoiesis during a flu infection we sought to investigate the serum of flu-infected mice versus naïve mice for any changes in the levels of the myelopoietic growth factors as well. Notably, to allow any finding to be correlated with the expansion of MDSCs seen in infected mice that lack iNKT cells, the serum levels of any myelopoietic

growth factor investigated were compared in the context of flu-infected WT versus Jα18$^{-/-}$ mice.

3.6 Flu infection induces a significant increase in GCSF production in the serum of WT mice but not in that of iNKT-deficient mice

On the serum collected from naïve and flu infected WT and Jα18$^{-/-}$ mice 7 days post intranasal inoculation, ELISAs were first done for GCSF and Flt3L. Notably, the levels of GCSF in the serum of flu infected WT mice were strikingly higher than that of naïve WT mice while flu-infected Jα18$^{-/-}$ mice showed no significant change in GCSF levels above the baseline level seen in the naïve Jα18$^{-/-}$ mice. However, there was no difference in the pattern of Flt3L levels between WT and Jα18$^{-/-}$ mice as both showed a similar decrease in Flt3L levels when infected with flu compared to their naïve counterparts (**Figure 19**).



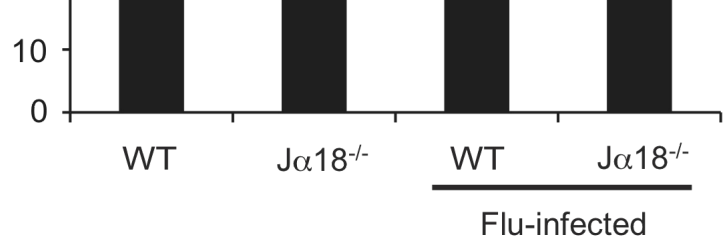


Figure 19: Increased levels of G-CSF in the serum of flu-infected WT mice; a. Histogram showing increased levels of G-CSF in the serum of flu-infected WT mice as compared to naïve WT mice; no significant increase of GCSF is seen for infected Jα18$^{-/-}$ mice in comparison to naïve Jα18$^{-/-}$ mice; **b.** Histogram showing a decrease in the levels of Flt3L in the serum of both infected WT and Jα18$^{-/-}$ mice as compared to the naïve mice.

With GCSF being known for the ability to mobilize myeloid progenitors, we initially found this to be a counter intuitive result as we had shown previously that more CFU-GM colonies were in the periphery of infected Jα18$^{-/-}$ mice as compared to infected WT mice. However, by taking two of the main differences demonstrated above between flu-infected mice into account, specifically, that infected mice with iNKT cells had (i) an increase in GCSF levels and (ii) no MDSCs expansion, we then sought to investigate firstly whether an iNKT interaction with GMPs and/or MDSCs could be facilitated by GCSF and secondly, if iNKT cells had a role in GCSF production.

3.7 GCSF can induce a CD1d-dependent crosstalk between GMPs and iNKT cells

Having shown that GMPs can cross-talk with iNKT cells in a CD1d-dependent manner through the presentation of the exogenous lipid αGalCer, we replicated this *in vitro* assay by using GCSF in place of αGalCer. Test wells were those with GMPs that were pulsed with 100ng/ml of GCSF alone and then co-cultured with sorted iNKT cells, in the presence or absence of CD1d-blocking antibody; after 24 hours IFNγ and IL-12p40 levels in the supernatant were measured by ELISA.

Excitingly, we demonstrated that GCSF facilitated a crosstalk between GMPs and iNKT cells. Of note, this crosstalk was CD1d-dependent, evident by the lack of IFNγ and IL-12p40 in the presence of CD1d-blocking antibody (**Figure 20**).

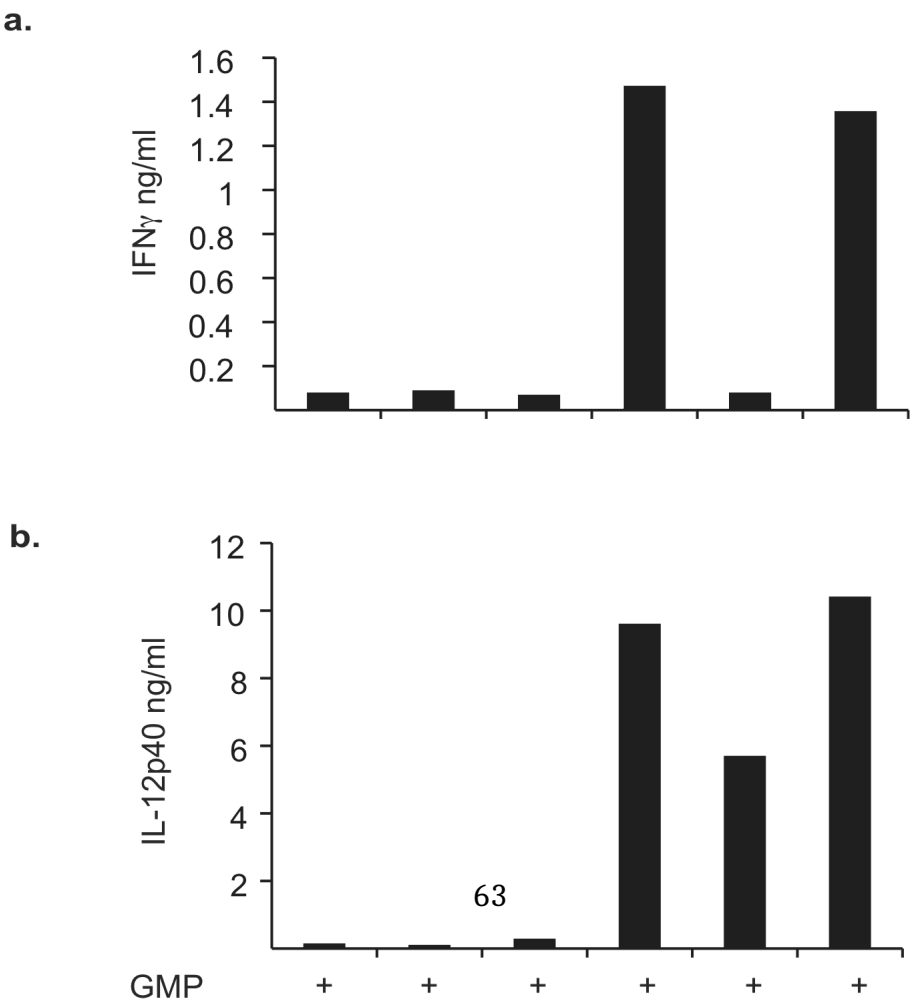


Figure 20: GCSF facilitates the crosstalk between iNKT cells and GMPs; (a) Histogram showing IFNɣ production when GMPs are pulsed with 100ng/ml of GCSF and co-cultured with iNKT cells, abrogated by adding CD1d-blocking antibody, but not blocked by adding the isotype control of the CD1d-blocking Ab; **(b)** Histogram showing IL-12p40 production when GMPs are pulsed with 100ng/ml of GCSF and co-cultured with iNKT cells, abrogated by adding CD1d-blocking antibody, but not blocked by adding the isotype control of the CD1d-blocking Ab.

Importantly, these results suggest that in the presence of GCSF, GMPs may up-regulate a stimulatory endogenous lipid in the context of CD1d that facilitates iNKT cell activation.

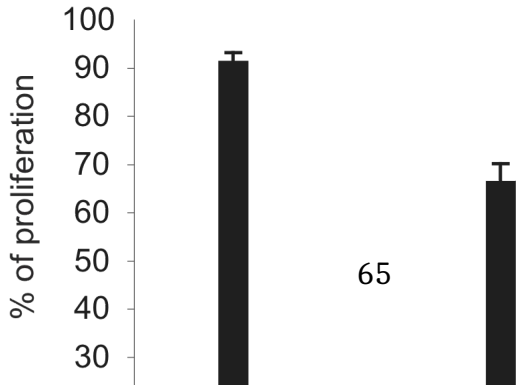
We sought to extend these findings to investigate the MDSC-iNKT cell interaction. De Santo et al showed that MDSCs through the presentation of αGalCer could activate iNKT cells. Moreover, they demonstrated that the crosstalk would in turn cause down regulation of iNOS and ARG1 in the MDSCs and abrogate their ability to suppress T cells[145]. We therefore set about to determine whether we could replicate these findings using GCSF in place of αGalCer to facilitate the crosstalk between MDSCs and iNKT cells.

3.8 GCSF facilitates the ability of iNKT cells to abrogate the suppressive activity of MDSC on T cell proliferation

To study the crosstalk between MDSCs and iNKT cells, MDSCs were first generated by culturing the BM of naïve WT mice with 1ng/ml of GM-CSF for 5 days in R-10 medium at 37˚C, and then selected for by using CD11b-MACS beads (protocol adopted from De Santo et al[145]), while iNKT cells were sorted from the spleen of Vα14TCR transgenic mice.

For the purposes of controls, the CFSE-labelled OT-1 proliferation assay described in chapter 2 was used to show that the GM-CSF-differentiated BM-derived MDSCs, when co-cultured with SIINFEKL stimulated OT-I splenocytes, suppress the proliferation of T cells. Additionally, we confirmed that by adding iNKT cells to the culture and pulsing the MDSCs with 100ng of αGalCer we relieved this suppressive activity, thereby rescuing T cell proliferation.

All test samples were set up to mirror the controls but using GCSF instead of αGalCer to pulse the MDSCs. Remarkably, co-culturing iNKT cells with MDSCs pulsed with 100ng/ml of GCSF did abrogate the suppressive activity of the MDSCs and allow T cell proliferation. Furthermore, we showed that this was a CD1d-dependent effect as confirmed by the suppression of T cell proliferation in those wells with CD1d-blocking antibody, as well as in those wells in which GM-CSF derived MDSCs were generated from the BM of $CD1d^{-/-}$ mice (**Figure 21**).



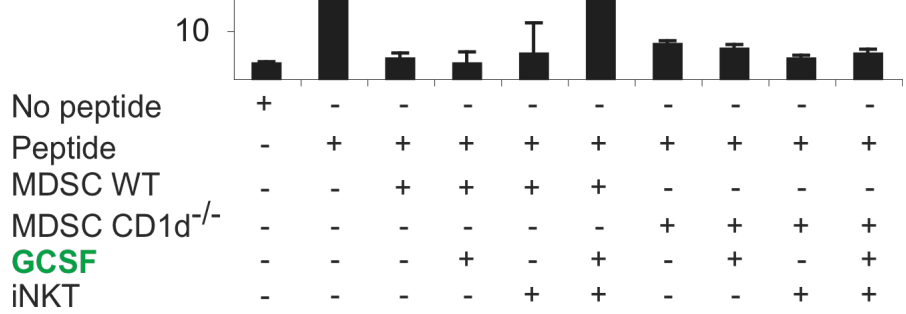


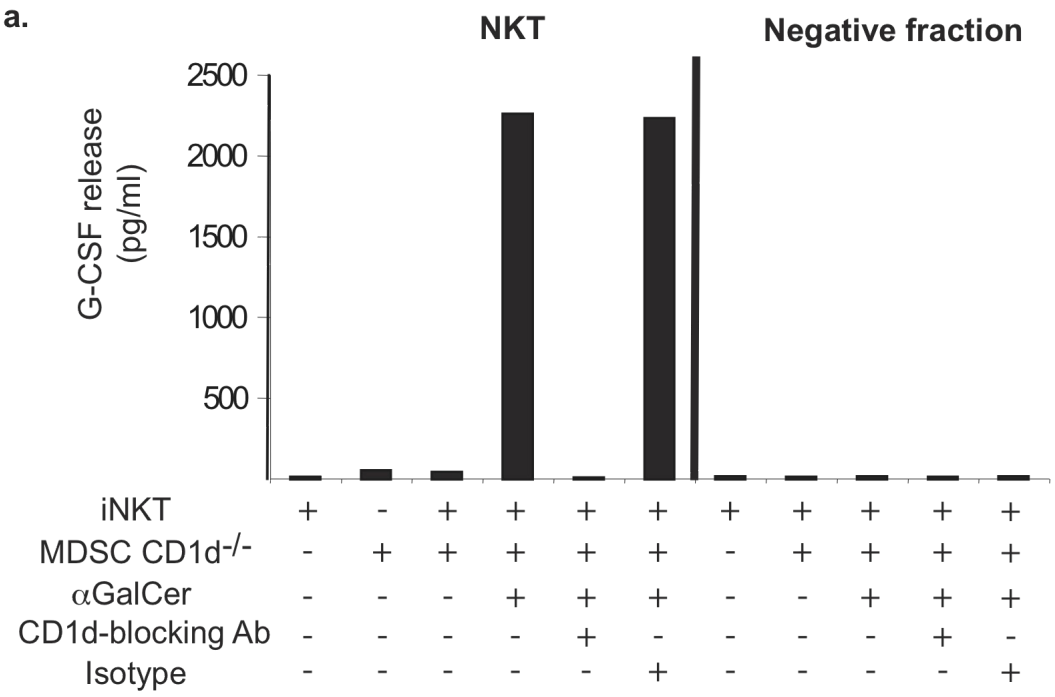
Figure 21: GCSF facilitates iNKT-mediated relief of MDSCs' suppressive action on proliferating T cells; Histogram showing an OT-1 proliferation assay; SIINFEKL (peptide)-pulsed OT-1 cells co-cultured with BM-derived MDSCs were suppressed from proliferating; OT-1 proliferation was rescued in those wells in which the BM-derived MDSCs were pulsed with GCSF and co-cultured with iNKT cells; pulsing BM-derived MDSCs with GCSF alone did not relieve the suppressive activity of the MDSCs nor could iNKT cells alone relieve the suppressive activity of the MDSCs. The interaction between MDSCs and iNKT cells was CD1d-dependent as evidenced by the inability to relieve the suppressive activity of MDSCs with GCSF and iNKT cells in the presence of a CD1d-blocking antibody (not shown) or when MDSCs were derived from the BM of a $CD1d^{-/-}$ mouse.

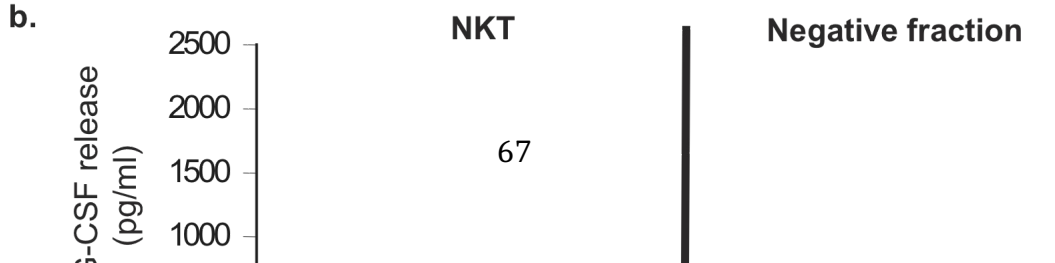
These CD1d-dependent results suggest that a GCSF-induced up-regulation of an endogenous lipid may be a mechanism by which iNKT cells can relieve the suppressive activity of MDSCs.

However, though these findings may support the hypothesis that GCSF may facilitate the crosstalk between iNKT cells and MDSCs and/or GMPs *in vivo*, they don't explain why there is a much higher level of GCSF in the serum of flu-infected WT mice than in that of infected $J\alpha18^{-/-}$ mice. This led us to hypothesize that not only did GCSF possibly have a role in facilitating iNKT activation, but also that iNKT cells may have a role in GCSF production.

3.9 iNKT cell crosstalk with MDSCs or GMPs results in GCSF production

To investigate whether iNKT cells have a role in the production of GCSF we co-cultured GM-CSF-differentiated BM-derived MDSCs pulsed with αGalCer with sorted iNKT cells and then after 24 hours measured the level of GCSF in the supernatant by ELISA. This interaction resulted in the secretion of a significant amount of GCSF in the supernatant, not seen if either MDSC or iNKT cells alone were pulsed with αGalCer or if a CD1d-blocking antibody were added to the culture (**Figure 22**).





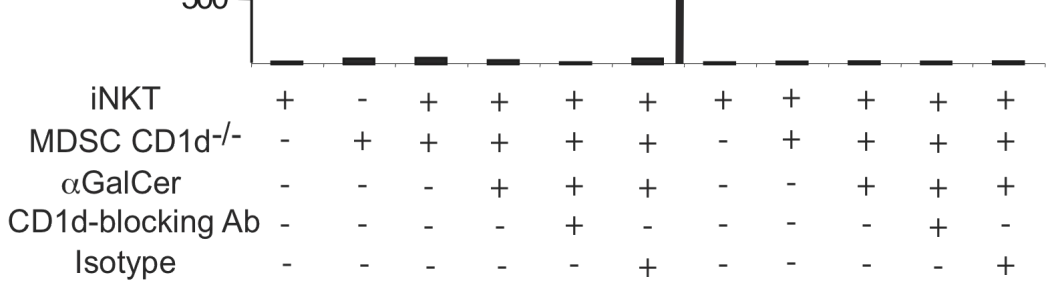
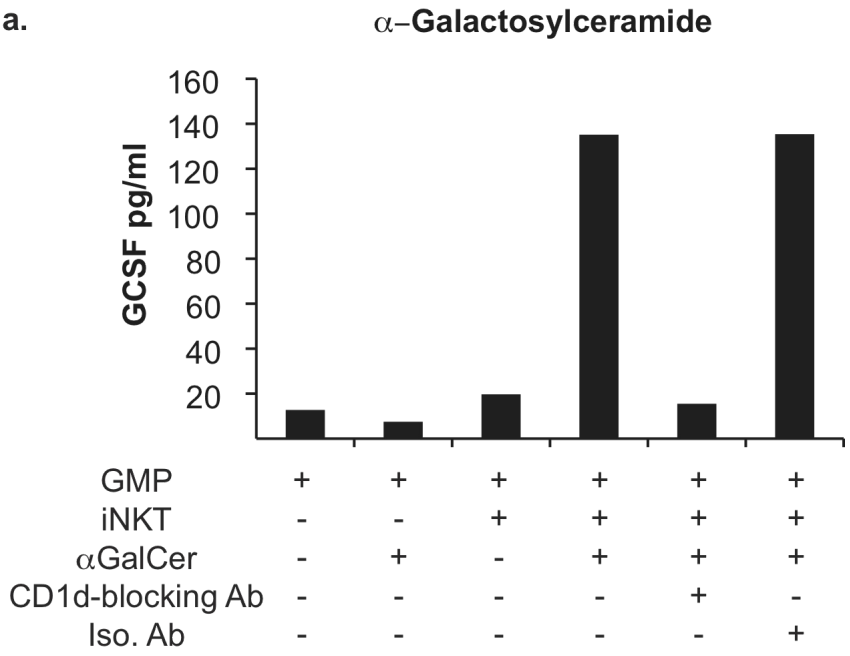


Figure 22: GCSF production from an iNKT cell-MDSCs interaction; (**a**) Histogram showing an increased level of GCSF in the supernatant of wells containing αGalCer-pulsed BM-derived MDSCs and iNKT cells. iNKT cells were responsible for this phenomenon as co-culturing the BM-derived MDSCs with the negative fraction from the spleen of the Vα14 transgenic mice from which the iNKT cells were sorted failed to cause GCSF production. Further the GCSF producing interaction between MDSCs and iNKT cells was CD1d-dependent as evidenced by no GCSF increase in the wells containing CD1d-blocking antibody; (**b)** Histogram showing the same experiment using αGalCer-pulsed MDSCs derived from $CD1d^{-/-}$ mice showing no increase in GCSF when co-cultured with iNKT, thereby confirming that the GCSF production from the interaction is CD1d-dependent.

We replicated the above *in vitro* assay using GMPs sorted from the bone marrow of WT mice (with the Akashi staining protocol described above) in place of MDSC, and indeed a CD1d-dependent iNKT-GMP crosstalk resulted in GCSF production (**Figure 23**); Furthermore, the results were reproducible using the exogenous glycolipid agonist Threitolceramide in place of α-Galactosylceramide (**Figure 23b**).

These results suggest that the direct activation of iNKT cells via the presentation of a stimulatory lipid in the context of CD1d can result in GCSF production and may contribute to the findings above showing flu-infected WT mice having significantly more GCSF in their serum than those flu-infected mice lacking iNKT cells.

a.


α–Galactosylceramide
GCSF pg/ml
160
140
120
100
80
60
40
20
GMP + + + + + +
iNKT - - + + + +
αGalCer - + - + + +
CD1d-blocking Ab - - - - + -
Iso. Ab - - - - - +

b.

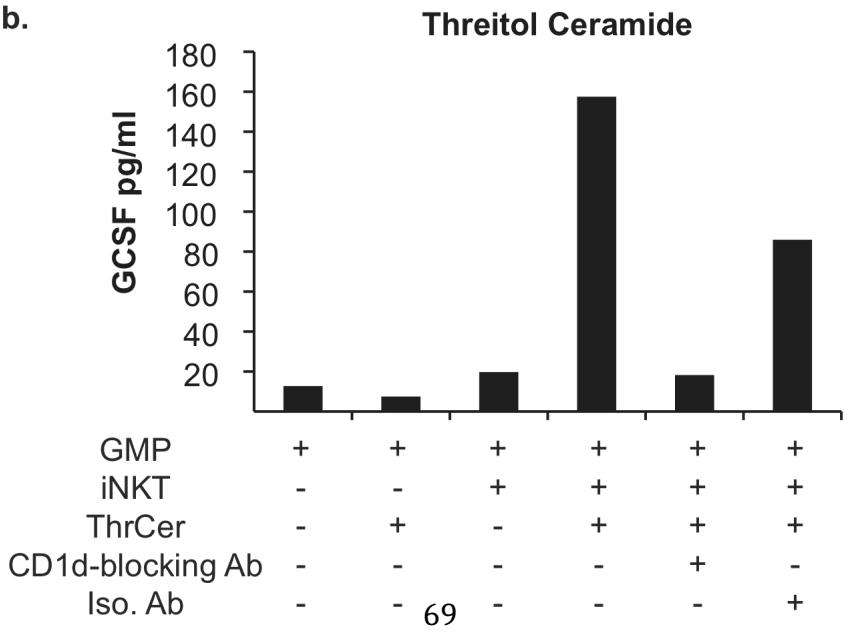
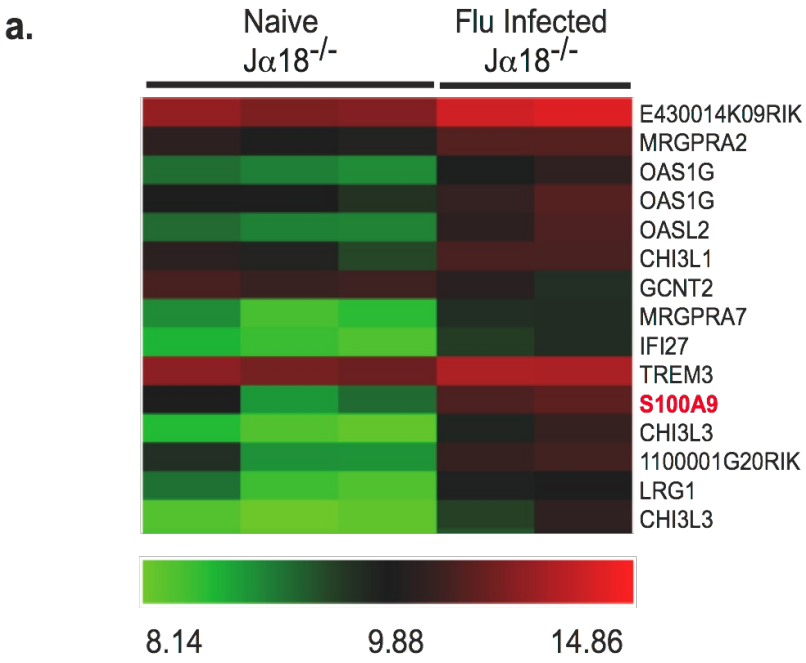

Threitol Ceramide
GCSF pg/ml
180
160
140
120
100
80
60
40
20
GMP + + + + + +
iNKT - - + + + +
ThrCer - + - + + +
CD1d-blocking Ab - - - - + -
Iso. Ab - - - - - +

Figure 23: GCSF production from iNKT-GMP crosstalk; (**a)** Histogram showing an increased level of GCSF in the supernatant of wells containing iNKT cells and αGalCer-pulsed GMPs from the BM of WT mice. This GCSF producing interaction between GMPs and iNKT cells was CD1d-dependent as evidenced by no GCSF increase in the wells containing CD1d-blocking antibody; (**b)** Histogram showing the same experiment using threitolceramide-pulsed GMPs (instead of αGalCer-pulsed GMPs) yielding the same CD1d-depedent GCSF production when crosstalk occured with iNKT cells.

3.10 S100A9 up-regulation in the Granulocyte-macrophage progenitors of flu-infected Jα18$^{-/-}$ mice

Having demonstrated a framework that suggests it is indeed possible for iNKT cells to be playing a role in modulating myelopoiesis, as evidenced by phenotypic and functional (immunosuppressive) differences seen in the GMPs of flu-infected iNKT-deficient versus wild type mice, we then sought to investigate whether differences in gene expression at the level of the GMPs during flu infection could garner further insight on how iNKT cells influence GMPs. To this end GMPs were sorted from the bone marrow of naïve and flu-infected WT and Jα18$^{-/-}$ mice and analyzed by microarray to see if any differences in gene expression existed. The most striking finding from this analysis was a 4.4-fold up-regulation of the myeloid-related protein S100A9 in flu-infected Jα18$^{-/-}$ mice as compared to the naïve Jα18$^{-/-}$ mice. The up-regulation of S100A9 was the largest fold-change of the 15 genes that had any statistically significant change in expression when comparing naïve to flu-infected Jα18$^{-/-}$ mice (Figure 24a). QPCR was used to

confirm the increase in S100A9 expression seen in flu-infected Jα18$^{-/-}$ mice in a separate experiment (done in triplicate using the same conditions described above). Of note, each sample analyzed with QPCR represented the pooling of GMPs from six mice (Figure 24b).



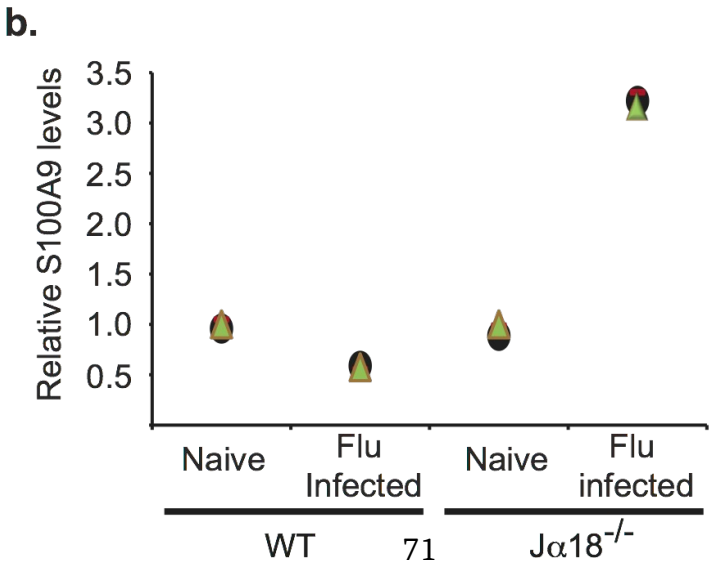


Figure 24: Increased S100A9 expression by GMPs in the bone marrow of flu-infected Jα18$^{-/-}$ mice. (a) Heat map of the genes that had a statistically significant change in expression when comparing the GMPs from naïve and flu-infected Jα18$^{-/-}$ mice. A statistically significant change was determined to be any up- or down regulation of a gene achieving a B value greater than 0 (each sample/column in the heat map represents the pooling of GMPs from six mice). S100A9 had the largest change in expression (4.4-fold increase) of all the genes shown; (**b)** PCR showing the relative increase in S100A9 expression by GMPs of flu-infected Jα18$^{-/-}$ mice as compared to those of naïve Jα18$^{-/-}$ mice; no change in expression was observed when comparing the GMPs of the WT counterparts; the QPCR was run in triplicate, each sample representing the pooling of 6 mice).

Importantly, naïve WT mice showed no change in S100A9 expression as compared to flu-infected WT mice on microarray, although at baseline naïve WT mice did have a higher level of expression than the naïve Jα18$^{-/-}$ mice. Comparatively, no statistically significant change in any gene expression was observed in flu-infected versus naive WT mice, although when comparing naïve WT to naïve Jα18$^{-/-}$ mice, at baseline there were 60 genes with differing levels of expression.

From the results of the microarray, we decided to focus on S100A9, and did so for three main reasons: firstly, as already mentioned, S100A9 showed the largest change in expression by GMPs when comparing the naïve and flu-infected iNKT-deficient mice; secondly, investigating every gene showing any difference in expression would have been beyond the temporal scope of this thesis; and thirdly (but most importantly) a paper recently published by Cheng et al showed that over-expressing S100A9 in HPCs caused differentiation of the HPCs into CD11b^{+}Gr1^{+} MDSCs, while S100A9 down-regulation was associated with differentiation of HPCs into DCs [38].

Therefore, on the background of Cheng et al's results, our findings that MDSCs accumulate in flu-infected iNKT-deficient mice, and that S100A9 is up-regulated in the GMPs of flu-infected iNKT-deficient mice (and not flu-infected WT mice), we hypothesized that iNKT cells may be influencing myelopoiesis by modulating GMP differentiation away from a myeloid-derived suppressor cell phenotype, and doing so in part by affecting S100A9 expression levels. To explore this hypothesis further we sought to replicate the $CD1d^{+}$: $CD1d^{-}$ chimera model described in Chapter 2 so as to determine if CD1d expression (and therefore the ability to crosstalk with iNKT cells) had any effect on S100A9 expression during flu infection.

3.11 CD1d expression influences S100A9 and Arginase-1 up-regulation by $CD11b^{+}Ly6G^{+}$ cells during flu infection

Once more, B6.SJL-$Ptprc^{a}Pep3^{b}$/BoyJ (B6-SJL) mice were irradiated with two doses of 450rads 3 hours apart. Mice were then immediately reconstituted with bone marrow cells from donor mice by intravenous injection of 5 x 10^{6} bone marrow cells in an inoculum of 100μl using a 30 G needle. Sample mice were reconstituted to have a 50:50 ratio of $CD1d^{-}$ CD45.2: $CD1d^{+}$CD45.1 hematopoietic cells (as shown in the **Figure 11**). After confirming the equal engraftment of CD45.1 and CD45.2 cells, we inoculated half of the mice with 160 HIU of the Influenza A/Puerto Rico/8/34 virus (flu) intra-nasally. Five days post flu infection infected and naïve mice were culled, lungs homogenised separately and FACS used to sort for $CD1d^{-}CD11b^{+}Ly6G^{+}$ cells versus $CD1d^{+}CD11b^{+}Ly6G^{+}$ cells. Protein

lysates were then made from the sorted cells and Western blots done for S100A9.

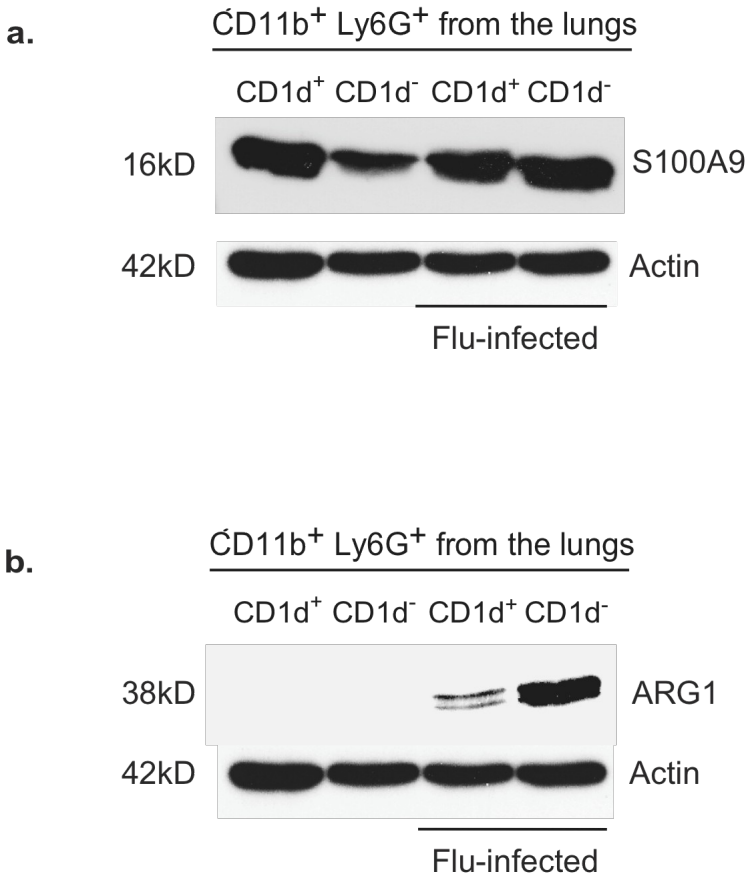


Figure 25: CD1d-dependent S100A9 and ARG1 up-regulation by CD11b$^+$Ly6G$^+$ cells in the lungs of flu-infected chimera mice. Naïve bone marrow chimera mice with a 50:50 ratio of CD1d$^-$CD45.2: CD1d$^+$CD45.1 hematopoietic cells were compared to those infected for 5 days with influenza A virus (flu); CD11b$^+$Ly6G$^+$CD1d$^+$ and CD11b$^+$Ly6G$^+$CD1d$^-$ cells sorted separately from the lungs of the naïve and flu-infected mice were analysed by Western blot for S100A9 and Arginase-1 (ARG1) expression. **(a)** Western blot showing the up-regulation of S100A9 by the CD1d$^-$ population of CD11b$^+$Ly6G$^+$ cells in the lungs of the flu-infected mice in comparison to the CD1d$^-$CD11b$^+$Ly6G$^+$ cells in the lungs of the naïve mice.

Notably, the $CD1d^+CD11b^+Ly6G^+$ cells had a higher baseline level of S100A9 expression in the naïve mice than that of the $CD1d^-$ population but had no increase in S100A9 expression when mice were infected with flu; (**b)** Western blot showing a significant up-regulation of the granulocytic-MDSC associated enzyme ARG1 by the $CD1d^-CD11b^+Ly6G^+$ cells in the lungs of flu-infected mice in comparison to those in the lungs of naïve mice; $CD1d^+CD11b^+Ly6G^+$ cells showed a marginal increase in ARG1 expression in the flu-infected mice suggesting a CD1d-independent mechanism for modulating ARG1 expression on $CD11b^+Ly6G^+$ cells during flu infection may exist. Actin was the housekeeping gene blotted for to ensure equal loading was achieved for each sample in the Western blot.

As anticipated, there was a flu-induced up-regulation of S100A9 expression by the $CD1d^-CD11b^+Ly6G^+$ cells (which lacked the ability to crosstalk with iNKT cells) but not by the $CD1d^+CD11b^+Ly6G^+$ cells in the lungs of the flu-infected mice (Figure 25a); the $CD1d^-$ population is notably the same granulocytic-MDSC population in flu-infected chimera mice shown in Chapter 2 to have the immunosuppressive capability of secreting IL-10 and inhibiting T-cell proliferation. Although flu infection did not induce an increase in S100A9 expression on $CD1d^+CD11b^+Ly6G^+$ cells, the $CD1d^+CD11b^+Ly6G^+$ cells did have a higher baseline S100A9 expression level than that of the $CD1d^-CD11b^+Ly6G^+$ cells in the lungs of naïve mice (correlating perfectly with the results of the microarray that compared $CD1d^+$ and $CD1d^-$ GMPs from naïve versus flu infected WT and $J\alpha18^{-/-}$ mice).

The fact that a flu-induced increase in S100A9 expression is seen only on $CD11b^+Ly6G^+$ cells that lack the ability to crosstalk with iNKT cells suggests that iNKT cells may indeed be playing role in regulating S100A9 expression. However, although Cheng et al definitely correlated S100A9 expression with MDSC differentiation[38], the baseline expression of S100A9 by WT GMPs and by $CD1d^+CD11b^+Ly6G^+$ in chimera mice (neither of which show

immunosuppressive ability) in our results suggests that S100A9 may not be reliable as a sole marker of MDSCs. As such, we probed the Western blot of the $CD1d^+$ versus $CD1d^-$ $CD11b^+Ly6G^+$ cells from the naïve and flu-infected chimera for Arginase 1 (ARG1), the enzyme previously shown by Youn et al to be the primarily responsible for the ROS production and immunosuppressive capability by the granulocytic subset of MDSCs[163]. Once again, flu-induced a significant up-regulation in ARG1 expression by $CD11b^+Ly6G^+$ cells derived from the $CD1d^-$ population of cells but only a slight change in expression by the $CD11b^+Ly6G^+$ cells derived from the $CD1d^+$ population of cells (**Figure 25b**). The results taken together therefore highlight the integral role iNKT cells play in regulating the markers of MDSC differentiation and function and, by extension, myelopoiesis on a whole during flu infection.

3.12 Discussion

The results of the experiments described in this chapter highlight 7 main findings: (a) iNKT cells can crosstalk with granulocyte-macrophage progenitors (GMPs); (b) if iNKT cells are absent, flu infection induces up-regulation of S100A9 and immunosuppressive ability as early as the level GMPs; (c) myeloid progenitors in iNKT-deficient mice mobilize in response to a flu infection; (d) Mice with iNKT cells respond to a flu infection with an increased level of GCSF secretion, as well as lack MDSC expansion in the periphery; (e) GCSF can induce a CD1d-dependent crosstalk between iNKT cells and GMPs as well as between iNKT cells and MDSCs, thereby facilitating the abrogation of the immunosuppressive ability of these myeloid cells; (f) iNKT cells may amplify the response outlined in 'e' as activation of iNKT cells by antigen presenting cells results in GCSF production; and finally (g) iNKT cells via CD1d interaction influence MDSC differentiation by regulating S100A9 and ARG1 expression.

3.12.1 S100A9 and immunosuppression

S100A9, also known as myeloid-related protein 14 (MRP14) or Calgranulin A, is a member of the large S100 family of proteins. It was initially described as a molecule that when secreted by neutrophils and monocytes as a heterodimer with S100A8 functioned to recruit leukocytes to sites of inflammation[166]. However, Cheng et al subsequently showed S100A9 expression during myeloid differentiation to be also associated with MDSC proliferation and accumulation[38].

The experiments outlined in this chapter not only support Cheng's findings but have taken them further with the novel assertion that the presence or absence of iNKT cells plays a role in influencing S100A9 expression (and therefore MDSC differentiation) during a flu infection. For example, by comparing wild type to S100A9 KO mice, Cheng demonstrated that the expansion of $CD11b^{+}Gr\text{-}1^{+}$ MDSCs in response to EL-4 lymphoma inoculation *in vivo* is dependent on S100A9 expression by the myeloid cells. Then, in separate experiments, Cheng showed that the over-expression of S100A9 in embryonic stem cells resulted in hyper-production of these MDSCs and blockade of DC differentiation[38]. These results were consistent with Hashimoto et al's findings that to progress toward macrophage and dendritic cell differentiation, myeloid cells must down regulate S100A9 expression[167]. We've extended these findings to now show that the presence or absence of iNKT cells influences S100A9 expression, and therefore MDSC differentiation, during a flu infection.

Additionally, it has also previously been shown that transgenic mice over-expressing S100A9 in myeloid cells accumulated IMCs (immature myeloid

cells), which expressed c-kit and demonstrated the ability to form colonies *in vitro*. Moreover, these IMCs over-expressing S100A9 had strong immunosuppressive effects on T-cell proliferation, much like that seen with the $CD11b^{+}Gr\text{-}1^{+}$ cells above[38]. Similarly, in the experiments discussed in this chapter we found an accumulation of GM-CFUs in the periphery of flu-infected iNKT-deficient mice (as compared to WT mice) and that the GMPs in these mice up-regulated S100A9 and gained the ability to suppress T-cell proliferation. Additionally, we went even further to show that iNKT cells could cross-talk with the GMPs and abrogate the flu-induced immunosuppressive ability, proving that iNKT cells indeed can act at the level of the progenitors to influence myelopoiesis in a pathological setting.

Interestingly, although Cheng et al showed MDSC differentiation to be dependent upon S100A9 expression, they also used tumour models to demonstrate that $Gr\text{-}1^{+}$ cells from both naïve and tumour-bearing mice expressed S100A9, despite the fact that only those from the later had immunosuppressive capability[38]. Similarly, we found that although S100A9 up-regulation was only observed on GMPs from flu-infected iNKT-deficient mice, or on $CD11b^{+}Ly6G^{+}$ cells derived from the $CD1d^{-}$ population in flu-infected chimera mice, we also noted that WT mice and $CD1d^{+}$ $CD11b^{+}Ly6G^{+}$ cells from the naïve chimera mice expressed a baseline level of S100A9 as well. Therefore, by amalgamating Cheng's previously published findings with those in this manuscript we asserted that while MDSC differentiation may depend on S100A9 expression, S100A9 expression ought not be used as a sole marker of MDSCs. As such, by using the chimera model comparing

the $CD1d^+$ to $CD1d^-$ $CD11b^+Ly6G^+$ cells in flu infected mice, we extended our findings even further and revealed that iNKT cells were modulating granulocytic-MDSC differentiation not only by influencing S100A9 expression, but by also modulating ARG1 expression.

3.12.2 GCSF-mediated iNKT influence on Myelopoiesis

Granulocyte colony-stimulating factor is said to be the major regulator of neutrophils[168]. First noted for its ability to stimulate the growth of neutrophilic granulocytic colonies, GCSF has been shown to increase proliferation and survival at every level of the granulocytic lineage as well as shorten the passage of the progenitors through the cell cycle[169,170]. Additionally, GCSF is known to stimulate stem cell mobilization to the periphery, in part through neutrophil mediated proteolytic degradation of CXCR4 on the HPCs and of its ligand stromal cell-derived factor-1 (SDF-1) expressed in the bone marrow niche[171-173].

In 2009 Morris et al in a letter to Nature Medicine highlighted that the administration of GCSF to recipients of a bone marrow transplantation increased the expansion of $CD8^+$ T cells and accelerated graft versus host disease (GVHD) mortality[174]. The associated effect of GCSF on GVHD was however not seen in iNKT-deficient grafts and was shown to be GCSFR, CD1d, CD40 and IFN-γ-dependent, as knock-out mice for either of these molecules failed to show the increased cytotoxicity and GVHD seen in WT

mice. Morris thereby put forth a conceptual model in which total body irradiation had up-regulated the GCSFR on APCs of the recipient enabling GCSF treatment to cause a CD1d-dependent iNKT activation through crosstalk with the APCs; IFN-γ secretion from this interaction subsequently enhanced the CD40-dependent priming of the cytotoxic T lymphocyte arm of GVHD[174].

In this chapter we have confirmed and expanded upon the concept that GCSF can facilitate the crosstalk between iNKT cells and APCs by showing for the first time a GCSF-mediated CD1d-dependent interaction between GMPs and iNKT cells. Moreover, the GCSF-mediated iNKT-MDSC interaction results in abrogation of the flu-induced immunosuppressive activity of the MDSCs thereby revealing yet another role for both GCSF and iNKT cells during demand driven myelopoiesis. Of note, we also found GCSF production from the iNKT-myeloid cell crosstalk itself, which can potentially explain why we observed a higher level of GCSF in the serum of flu-infected WT mice as compared to iNKT-deficient mice. Furthermore, it is possible that this amplification of GCSF production could be a means to propagate this mechanism of modulating GMP differentiation away from a flu-induced suppressive phenotype, concomitantly decreasing the number of MDSCs in response to a flu infection.

Together these results provide the framework of a model for a role of iNKT cells in modulating appropriate myeloid cell differentiation and function in response to flu infection. However, the question of what is the initial trigger from the periphery during a flu infection that precipitates the snowball

effect of the iNKT-GMP interaction ensuring an appropriate myelopoietic response during an infection remains to be answered. Addressing this question is the focus of the next chapter.

CHAPTER 4

The Reciprocal Role of Serum Amyloid A – inducing and relieving MDSC activity

4.1 Introduction

The results in the previous chapter not only point out GCSF's capability of inducing iNKT activation but also demonstrate that GCSF production can itself result from iNKT activation during flu infection. Therefore questions arose as to what factors during flu infection are triggering the iNKT activation in the first place to initiate this positive feedback loop.

It is well accepted that changes in the self-lipid repertoire in antigen-presenting cells (APC) upon microbial exposure have been found to skew towards more stimulatory self-lipids and iNKT cell activation[112,175]. However, we sought to explore the relatively recently published concept that Serum Amyloid A (SAA) induces GCSF production and neutrophilia *in vivo*[176]. SAA is a generic term for a family of acute-phase proteins with a high degree of homology between species, the dominant isotype being SAA1[177]; it is one of

the first major acute-phase proteins produced by many cells including hepatocytes, tissue macrophages and synoviocytes in response to bacterial products and inflammatory cytokines[178-180]. As such, although many aspects of SAA's role remain largely unknown, it is commonly used as a marker of inflammation.

He et al demonstrated that SAA, through Toll-like receptor 2 (TLR2), induced GCSF production by macrophages[176]. In order to investigate whether SAA was indeed a candidate molecule for being the systemic signal triggering iNKT involvement we first sought to confirm He et al's finding that SAA induces GCSF production.

4.2 SAA induces TLR2-dependent GCSF secretion by Macrophages

Similar to He's protocol[176], murine bone marrow derived macrophages (BMDMs) were stimulated *in vitro* with 1μM of recombinant SAA in R10, in the presence or absence of TLR2 blocking antibody, and GCSF production determined by ELISA of the supernatant after 16 hours of culture. GCSF was detected in significant amounts in the supernatant of macrophages pulsed with SAA as compared to untreated macrophages and indeed production was abolished in those wells in which macrophages were pulsed with TLR2 blocking antibody prior to treatment with SAA. To ensure the specificity of the blocking antibody, the TLR2 ligand PAM3CyS was used as a control in place of SAA in wells mirroring the test samples (**Figure 26**).

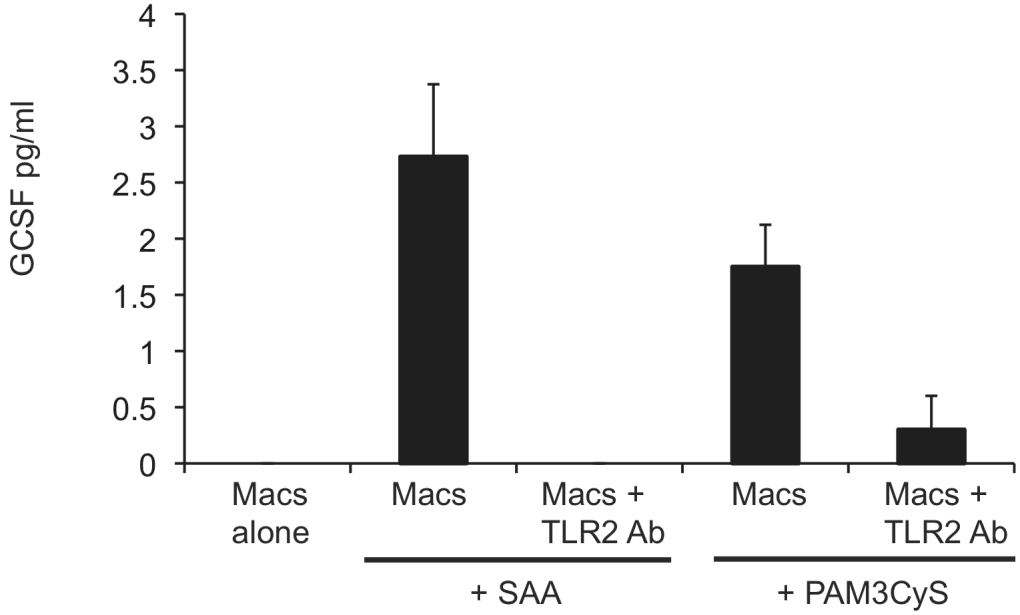


Figure 26: SAA-induced TLR2-dependent GCSF secretion by macrophages. Histogram showing GCSF secretion in wells in which macrophages were stimulated with SAA (1μM) or PAM3CyS (1μg/ml) for 16 hours; GCSF production was abolished in those wells in which macrophages were pulsed with a TLR2 blocking antibody prior to SAA or PAM3Cys treatment, suggesting that SAA stimulated GCSF production in a TLR2 dependent manner. All samples and controls were done in triplicate.

Having confirmed a relationship between SAA and GCSF production *in vitro* we next sought to determine whether SAA was increased *in vivo* during a flu infection.

4.3 SAA is increased in both flu infected WT and Jα18$^{-/-}$ mice

Using the same experimental model described in chapters 2 and 3, WT and Jα18$^{-/-}$ mice were inoculated with 160 Hemagglutinin Units (HIU) of the

Influenza A/Puerto Rico/8/34 virus (flu) intra-nasally and 7 days thereafter the serum collected for ELISA. In both WT and iNKT-deficient mice the concentration of SAA in the serum increased significantly to similar levels when either was infected with flu as compared to their naïve counterparts. This result suggested that SAA is a systemic signal of inflammation found in flu infection and that its production was not affected by the presence or absence of iNKT cells (**Figure 27**).

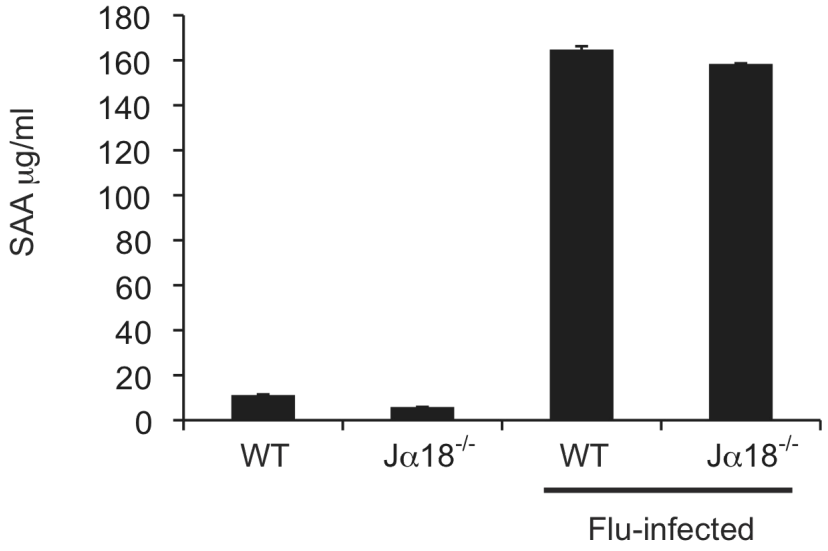


Figure 27: Increased levels of SAA in the serum of flu-infected mice. Histogram showing an equivalent increase in SAA in the periphery of both WT and iNKT-deficient mice infected with flu as compared to their naïve counterparts (n = 3 for each bar in the graph).

Demonstrating a flu-induced increase in SAA levels *in vivo* makes this acute-phase protein a feasible candidate for triggering the cascade of GCSF mediated iNKT modulation of myelopoiesis. Additionally, showing that the significantly greater increase in GCSF concentration in the periphery of flu-infected WT mice compared to iNKT-deficient mice is despite there being comparable increased levels of SAA in the flu infected mice lends support to

the hypothesis that iNKT cells play a role in amplifying GCSF production during the immune response to flu. With the above evidence we therefore deemed it appropriate to then investigate in *vitro* whether SAA could trigger a GCSF-mediated MDSC-iNKT crosstalk.

4.4 SAA-induced MDSC-iNKT crosstalk is GCSF-mediated

As explained previously, to study the crosstalk between MDSCs and iNKT cells, MDSCs were first generated by culturing the BM of naïve WT mice with 1ng/ml of GM-CSF for 5 days in R10 medium at 37˚C, and then selected for by using CD11b-MACS beads (protocol adopted from De Santo et al[145]), while iNKT cells were sorted from the spleen of Vα14TCR transgenic mice.

Recombinant SAA (1μM) was used to pulse a 4:1 ratio co-culture of MDSCs and iNKT cells and after 16 hours IFNγ levels in the supernatant of these co-cultures were analyzed using ELISA. Controls were set up to mirror test sample conditions by using GCSF (100ng/ml) in place of SAA. As anticipated, SAA induced iNKT-MDSC crosstalk as evidenced by IFNγ production (controls demonstrating that IFNγ is not produced if either MDSCs or iNKT cells are pulsed with SAA separately). More importantly, culturing the SAA-pulsed iNKT-MDSC mix with a CD1d-blocking antibody or a GCSF neutralizing antibody prevented IFNγ production, suggesting that the SAA-induced MDSC-iNKT cell crosstalk was not only CD1d-dependent but also GCSF-mediated (**Figure 28**).

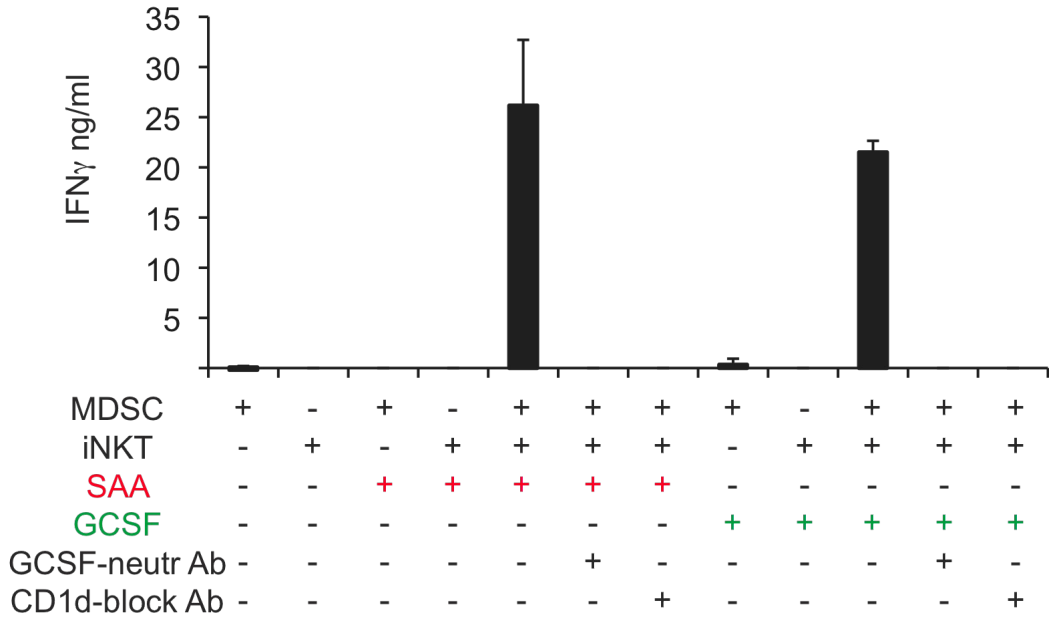


Figure 28: SAA-induces iNKT-MDSC crosstalk via GCSF production; Histogram showing IFNγ production when MDSC are pulsed with recombinant SAA (1μM) and then co-cultured with iNKT cells; IFNγ release is inhibited by adding either GCSF-neutralizing antibody or CD1d-blocking antibody suggesting that the MDSC-iNKT crosstalk was both CD1d- and GCSF- dependent. Control wells set up using 100ng/ml of GCSF in place of SAA under the same conditions yielded the same results. All test and control samples were set up in triplicate.

Conceivably the findings shown in Figure 28 suggest that SAA may be able to facilitate a GCSF-mediated iNKT influence on demand-driven myelopoiesis ensuring the immune response isn't hampered by an expansion of suppressor cells during an infection. However Sander et al in a recent publication purported SAA to be a promoter rather than an inhibitor of MDSC proliferation[181]. Specifically, Sander showed that transgenic mice with the inability to secrete SAA (because of a hepatic gp130-STAT3

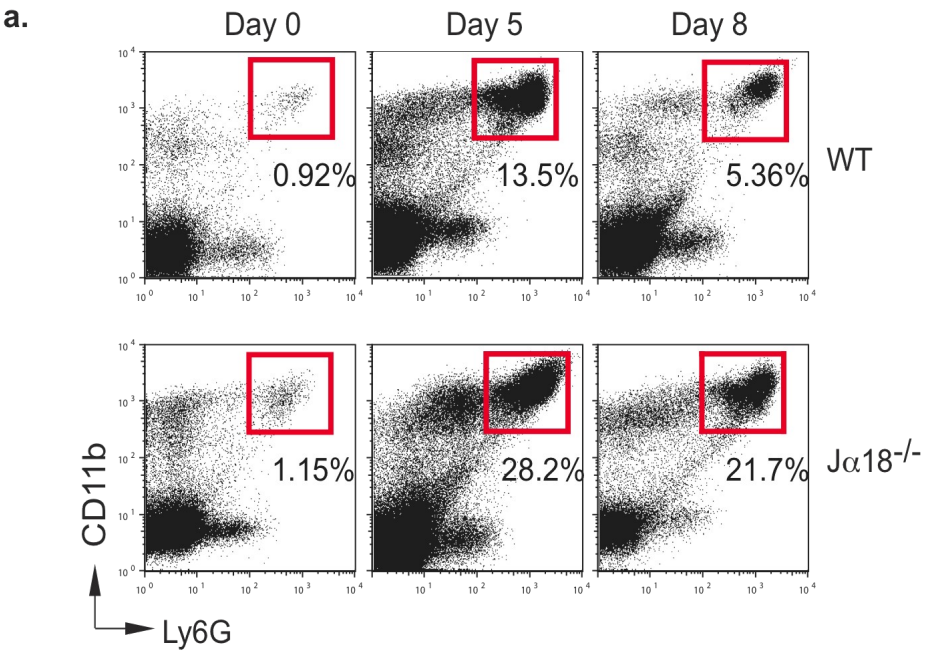
deletion) had much higher morbidity and mortality during polymicrobial sepsis which the authors attributed to diminished sepsis-induced MDSC accumulation, mobilization and survival; MDSCs in the polymicrobial setting were seen as regulatory cells without which much of the systemic damage was suggested to be due to an unchecked immune response[181].

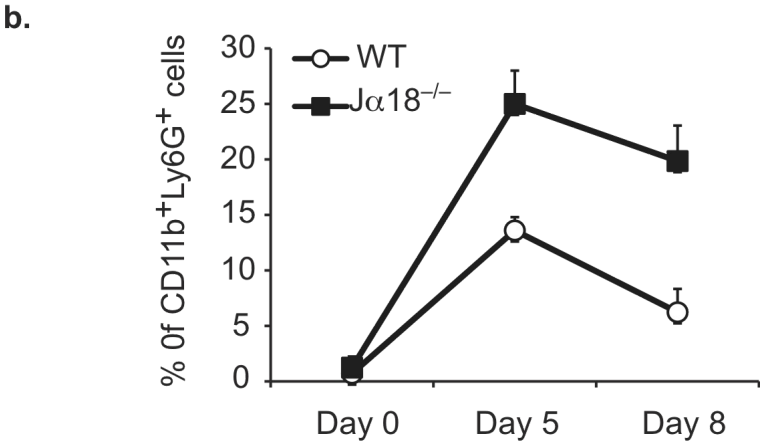
Taking Sander's findings into account, we then sought to determine whether SAA indeed contributes to the expansion of MDSCs, in particular the IL-10 secreting granulocytic subset of MDSCs that expand in flu infection; specifically, our overarching question was whether SAA could be playing a dual reciprocal role by both promoting MDSC proliferation while concomitantly facilitating iNKT activation to provide a mechanism to abrogate the MDSC generation and function.

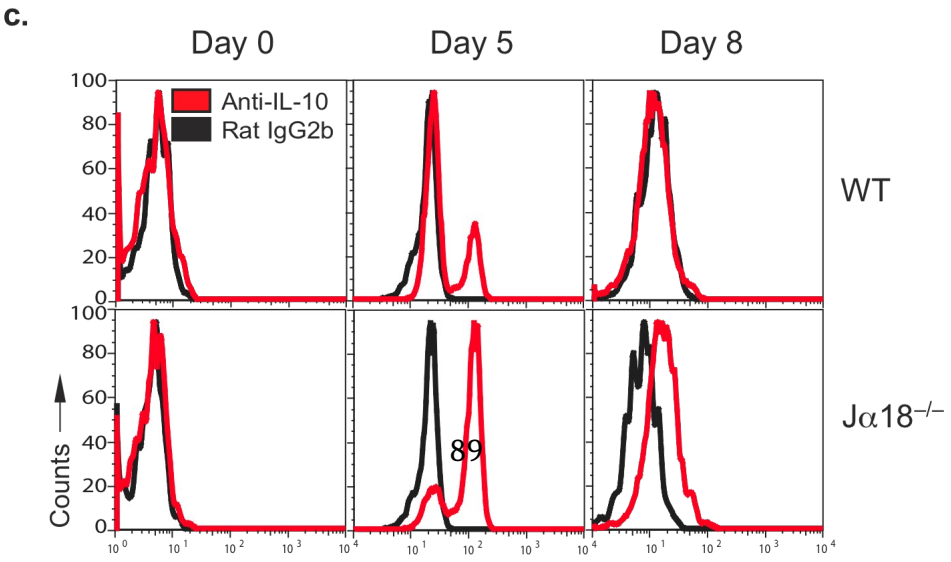
4.5 SAA induces the proliferation of IL-10-secreting granulocytic-MDSCs in iNKT-deficient mice

To determine if a relationship indeed exists between SAA, IL-10-secreting granulocytic-MDSCs and iNKT cells we injected WT and iNKT-deficient ($J\alpha18^{-/-}$) mice with 120μg of SAA/kg subcutaneously daily for 5 days. Consistent with previously published data by He et al that SAA can induce neutrophila[176], we demonstrated that SAA induced an expansion of $CD11b^{+}Ly6G^{+}$ cells in the periphery of WT mice. However, expanding on He's findings, we found firstly that SAA induced a greater expansion of $CD11b^{+}Ly6G^{+}$ cells in $J\alpha18^{-/-}$ mice (28.2%) than in WT mice (13.5%) and

secondly that the population of $CD11b^{+}Ly6G^{+}$ cells decreased at a slower rate in the $J\alpha18^{-/-}$ mice when the SAA injections were stopped (**Figure 29a and b**).







d.

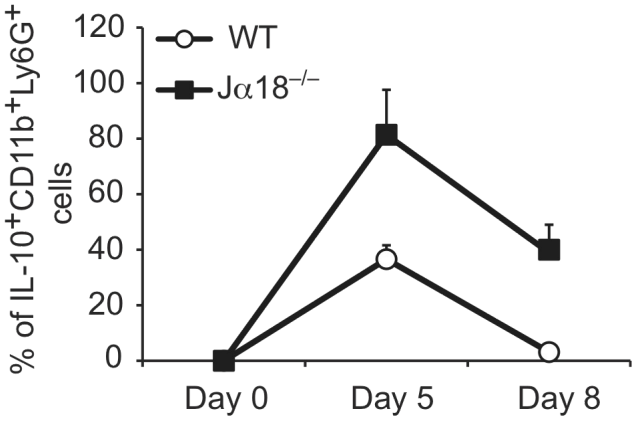


Figure 29: The expansion of IL-10-secreting CD11b$^+$Ly6G$^+$ cells in iNKT-deficient mice injected with SAA; (a) FACS plots showing the expansion of CD11b$^+$Ly6G$^+$ cells in the periphery of wild type (WT) and Jα18$^{-/-}$ mice injected daily with 120μg/kg of recombinant SAA subcutaneously for 5 days. Blood samples were collected from the tail vein of each mouse on Day 0, day 5 and three days after the final SAA injection; the percentages represent the CD11b$^+$Ly6G$^+$ cells in the outlined red boxes of each plot; **(b)** Graph showing the frequency of CD11b$^+$Ly6G$^+$ cells in the periphery of wild type and Jα18$^{-/-}$ mice (n = 6 for each phenotype) injected with SAA as described in **a**; **(c)** Flow cytometry of circulating CD11b$^+$Ly6G$^+$ cells (from WT and Jα18$^{-/-}$ mice injected with SAA subcutaneously for 5 days) stained *ex-vivo* for IL-10 or isotype-matched control antibody (rat immunoglobulin G2b (IgG2b); **(d)** Graph showing the frequency of IL-10$^+$CD11b$^+$Ly6G$^+$ cells from both Jα18$^{-/-}$ and WT mice that were injected with SAA as described in **c**.

Notably, by using intracellular staining, we further revealed that in contrast to the CD11b$^+$Ly6G$^+$ cells in the SAA-treated wild type mice, a large percentage of the CD11b$^+$Ly6G$^+$ cells expanding in the periphery of the SAA-treated Jα18$^{-/-}$ mice produced IL-10 (**Figure 29 c and d).** Moreover, OT-1 proliferation assays revealed that the CD11b$^+$Ly6G$^+$ cells from the SAA-treated Jα18$^{-/-}$ mice had a much stronger ability to suppress the proliferation of stimulated OT-1 lymphocytes than either cells from naïve or WT SAA-treated mice (**Figure 30**).

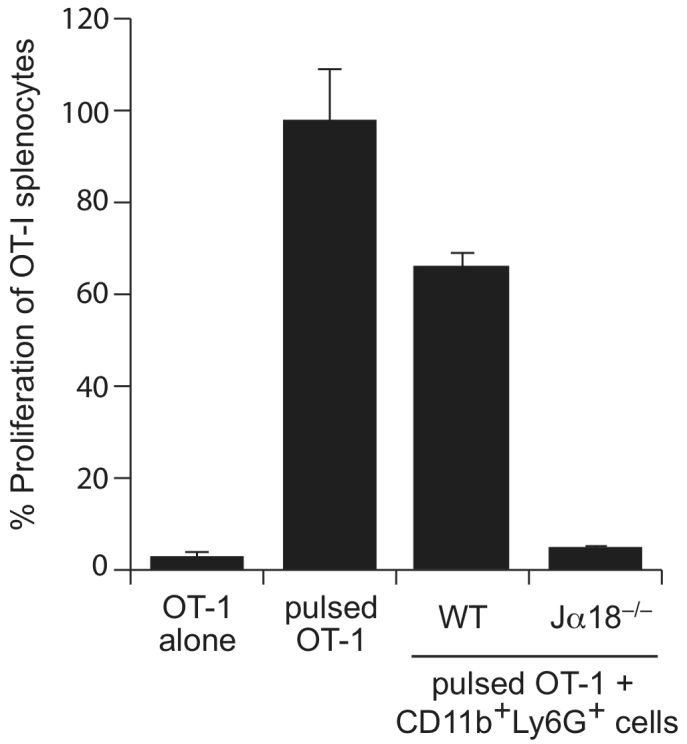


Figure 30: Histogram showing the *ex-vivo* suppression of CFSE-labelled OT-1 splenocyte proliferation by CD11b^{+}Ly6G^{+} cells isolated from the peripheral blood of SAA-injected Jα18$^{-/-}$ mice. CFSE-labelled OT-1 splenocytes were pulsed with their cognate antigen (ovalbumin peptide, SIINFEKL) and co-cultured in a 10:1 ratio with CD11b^{+}Ly6G^{+} cells sorted from SAA-treated WT and Jα18$^{-/-}$ mice; proliferation of the OT-1 cells was determined by flow cytometry 3 days after the co-cultures were set-up. (n = 3 for each bar on the graph)

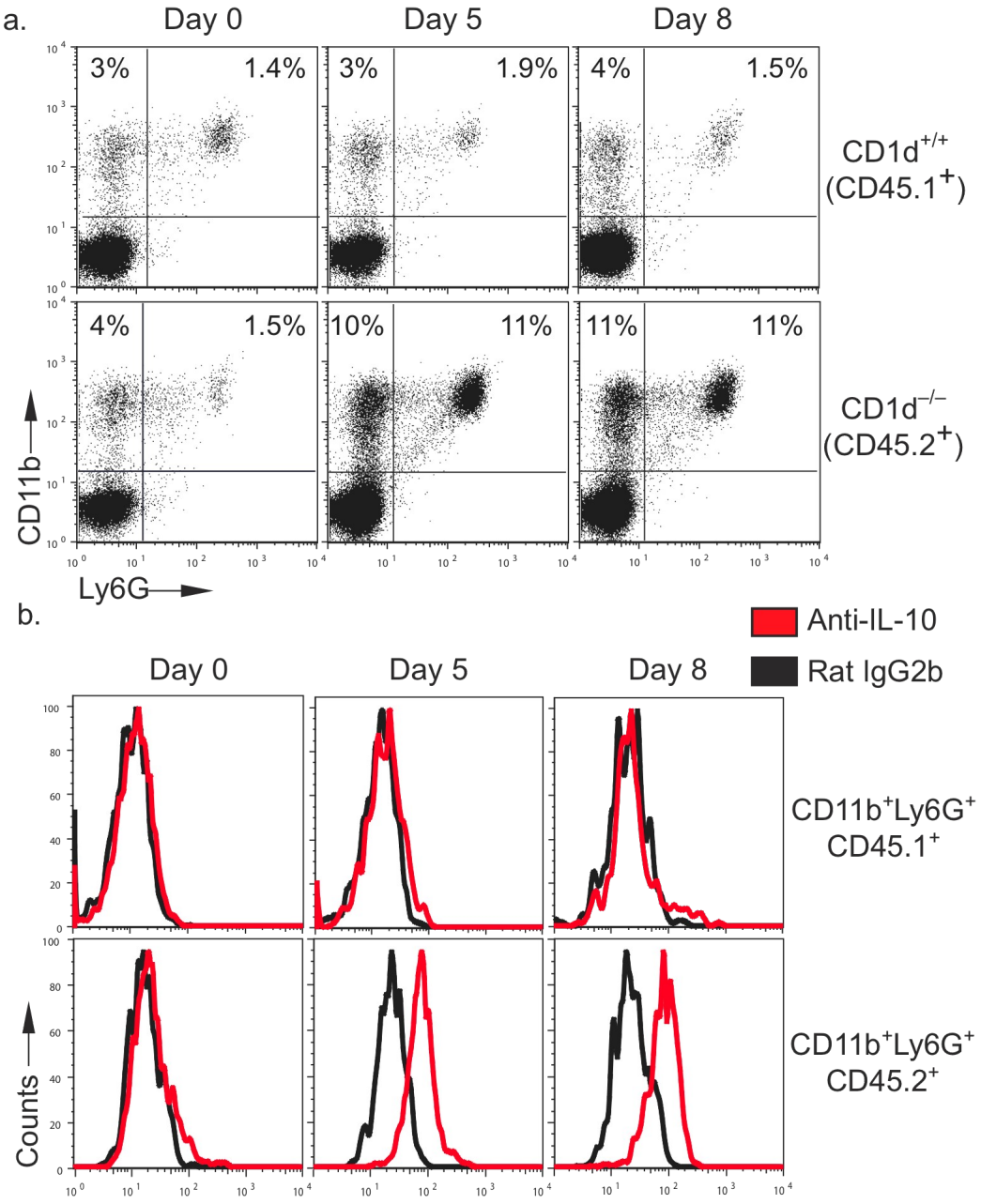
Together the results shown in figures 29 and 30 suggest that SAA indeed has the ability to induce the proliferation of suppressive IL-10-secreting CD11b^{+}Ly6G^{+} cells *in vivo*, and that the presence of iNKT cells can abrogate the SAA-induced proliferation of these IL-10 producing immunosuppressive granulocytes.

4.6 Abrogation of SAA-induced IL-10-secreting $CD11b^{+}Ly6G^{+}$ cell development *in vivo* is CD1d-dependent

We demonstrated in chapter 2 with the chimera mouse model that the generation of IL-10-secreting $CD11b^{+}Ly6G^{+}$ cells during flu infection was CD1d-dependent. We believe that the results above suggest SAA to be the candidate soluble factor in flu infection inducing the IL-10-secreting $CD11b^{+}Ly6G^{+}$ cell proliferation when iNKT cells are absent. However for this hypothesis to stand, we sought to ensure that the mechanism via which iNKT cells influence IL-10-secreting $CD11b^{+}Ly6G^{+}$ cell differentiation is the same in SAA-injected mice as it is in flu-infected mice. In short, iNKT cells could be influencing IL-10-secreting $CD11b^{+}Ly6G^{+}$ cell proliferation by either direct CD1d-dependent cell-cell crosstalk or indirectly through soluble factors; having shown the former to be the case in flu infection we sought to replicate these findings in SAA-treated mice using the chimera mouse model.

Once again we reconstituted lethally irradiated B6.SJL-$Ptprc^{a}Pep3^{b}$/BoyJ (B6-SJL) mice with a 50:50 ratio of $CD1d^{-}$CD45.2: $CD1d^{+}$CD45.1 hematopoietic bone marrow cells (as described in the previous chapters and shown in **Figure 11**). After confirming by FACS the equal engraftment of CD45.1 and CD45.2 cells, we injected the chimeras with 120μg of SAA/kg subcutaneously daily for 5 days. On analysis of peripheral blood samples on Day 5, we observed a greater expansion of $CD11b^{+}Ly6G^{+}$ cells derived from $CD1d^{-/-}$ (CD45.2) bone marrow than from $CD1d^{+/+}$ (CD45.1) bone marrow

(**Figure 31a**), the former persisting in the periphery even three days after SAA injections were ceased.



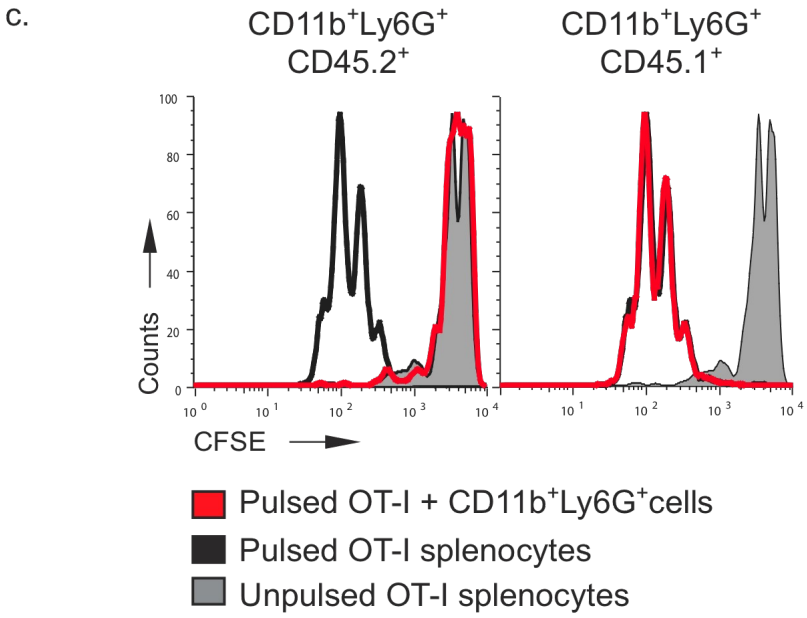


Figure 31: SAA-induced CD1d-dependent expansion of IL-10-secreting granulocytic-MDSCs in chimera mice; (a) FACS plots showing the percentages of $CD1d^-$ (CD45.2) versus $CD1d^+$ (CD45.1) $CD11b^+Ly6G^+$ cells in the periphery of bone marrow chimeras injected with 120μg of SAA/kg subcutaneously daily for 5 days; blood samples were analysed on days 0, and 5 and three days after the cessation of SAA injections. Numbers in quadrants indicate the percent of $CD11b^+Ly6G^-$ (top left) and $CD11b^+Ly6G^+$ (top right) cells. (**b**) Flow cytometry of $CD1d^-CD11b^+Ly6G^+$ and $CD1d^+CD11b^+Ly6G^+$ cells stained intra-cellularly for IL-10, analysed from the peripheral blood of SAA-treated chimera mice at the same time points as described in **a**. (**c**) Flow cytometry showing the inhibition of the proliferation of SIINFEKL-pulsed OT-1 splenocytes by $CD1d^-CD11b^+Ly6G^+$ (and not by $CD1d^+CD11b^+Ly6G^+$) sorted from the periphery of SAA-treated chimera mice.

Further, as hypothesized, the $CD1d^-CD11b^+Ly6G^+$ cells (and not the $CD1d^+CD11b^+Ly6G^+$ cells) when sorted by FACS were found by intracellular

staining to produce IL-10 (**Figure31b**) and to have the ability to suppress OT-1 proliferation in an OT-1 proliferation assay (**Figure 31c**). These findings provide conclusive evidence that an *in vivo* CD1d-dependent crosstalk between iNKT cells and myeloid cells in SAA-treated mice results in less IL-10-secreting immunosuppressive granulocytic MDSCs.

4.7 SAA up-regulates markers of MDSC differentiation and function

Having shown SAA to be a key soluble factor contributing to the differentiation of IL-10-producing granulocytic-MDSCs *in vivo*, we then ventured to gain further mechanistic insight into how SAA may be influencing known markers of MDSCs function. Specifically, by using $CD11b^+$ MACS-beads and magnetic columns, $CD11b^+$ cells were purified from the peripheral blood of naïve WT mice and treated *in vitro* with 1μM of recombinant SAA for 24 and 48 hours. Thereafter, building upon the associations made in the previous chapter, Western blots for ARG1 and S100A9 were done on cell lysates of untreated $CD11b^+$ and those treated with SAA for 24 and 48 hours respectively (**Figure 32**).

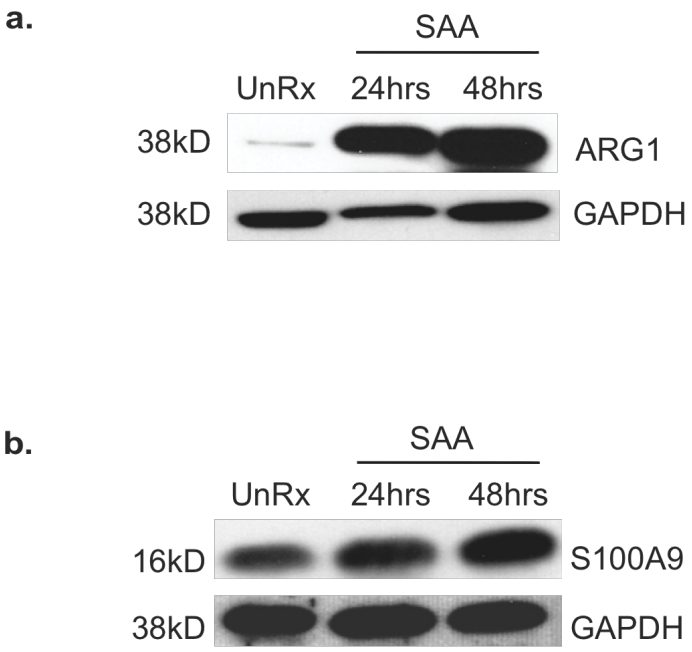


Figure 32: **The expression of Arginase-1 and S100A9 on SAA-treated CD11b cells.** $CD11b^+$ cells (purified from the peripheral blood of WT mice using CD11b-beads and MACS magnetic columns) were treated with 1μM of SAA for 24 and 48 hours *in vitro.* Western blotting was used to compare the level of expression of (**a**) Arginase-1 (ARG1) and (**b**) S100A9 of untreated CD11b cells, CD11b cells treated with SAA for 24 hours and CD11b cells treated for 48 hours. GAPDH was used as the housekeeping gene ensuring equal loading of protein.

Importantly, for the first time we demonstrated that SAA can (i) up-regulate ARG1, thereby conferring the $CD11b^+$ cells with the ability to suppress T cells via ROS production and arginine depletion and (ii) up-regulate S100A9, a marker known to be associated with differentiation of myeloid cells into a MDSC phenotype.

4.8 SAA can differentiate human IL-10-secreting granulocytic MDSCs and promote crosstalk between the human iNKT cells and MDSCs

In De Santo et al's seminal publication on MDSCs in influenza A virus infections, $CD11b^+$ cells isolated from the peripheral blood of influenza A virus (flu) infected humans were shown to have the ability to suppress T cell proliferation, and iNKT cells were shown to be able to abrogate the suppressive activity of these flu-induced MDSCs in a CD1d- and CD40-dependent manner[145]. Many years before De Santo's findings, Whicher et al published that SAA rapidly increased in all the subjects of a series of flu infected individuals[182]. On the background of these two publications on flu-infected humans, we sought to investigate whether our mouse model findings of SAA playing a role in inducing the differentiation of IL-10-secreting granulocytic-MDSCs during infection could be extended to humans.

Firstly, by using a Ficoll gradient to separate the peripheral blood of healthy human donors', neutrophils were collected as a layer above the pelleted red cells; a purity of 95% $CD11b^+CD15^+$ was evident on staining a sample of the cells collected (data not shown), $CD15^+$ being a marker of granulocytes; however to ensure complete homogeneity, the cells collected were then also sorted for $CD11b^+$ by using $CD11b^+$ MACS-beads and magnetic columns. In a

similar fashion to the *in vitro* experiments of murine cells, we pulsed the human $CD11b^{+}CD15^{+}$ cells with increasing amounts of SAA for 24 hours and then performed ELISAs of each well for IL-10. Of note, controls were pulsed with C-reactive protein (CRP), an acute phase protein conserved in humans but not in mice. Excitingly, consistent with our results in mice, SAA (and not CRP) indeed induced IL-10 production by the $CD11b^{+}CD15^{+}$ cells in a dose-dependent manner (**Figure 33**).

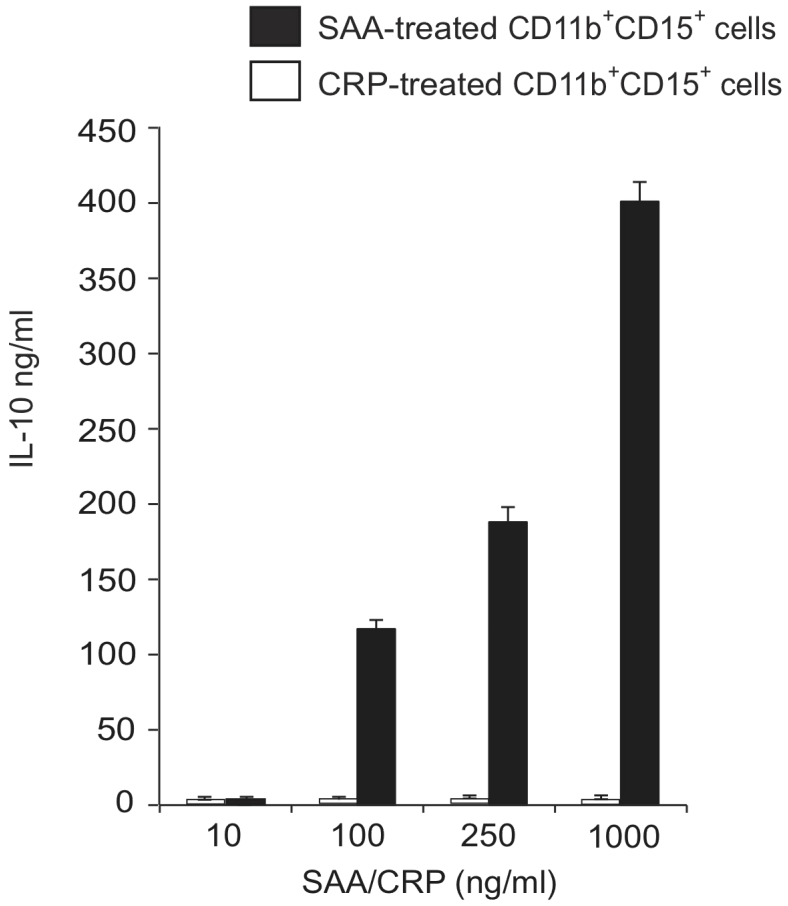


Figure 33: SAA stimulates IL-10 production by human granulocytes. Graph showing dose-dependent IL-10 production by $CD11b^{+}CD15^{+}$ cells purified from the blood of healthy human donors and stimulated with SAA in increasing concentrations. C-reactive protein (CRP) did not however induce IL-10 production by the $CD11b^{+}CD15^{+}$ cells. The data represent five independent experiments.

Secondly, by using a mixed leukocyte reaction (MLR) as the read out, we investigated whether the SAA-treated $CD11b^+CD15^+$ cells also demonstrated functional suppressive ability. Once again, in line with our findings in mice, the SAA-treated $CD11b^+CD15^+$ cells when cultured in the presence of alloreactive T cells and allogenic DCs exhibited the ability to suppress T cell proliferation as evidenced by their capacity to inhibit the MLR (**Figure 34**).

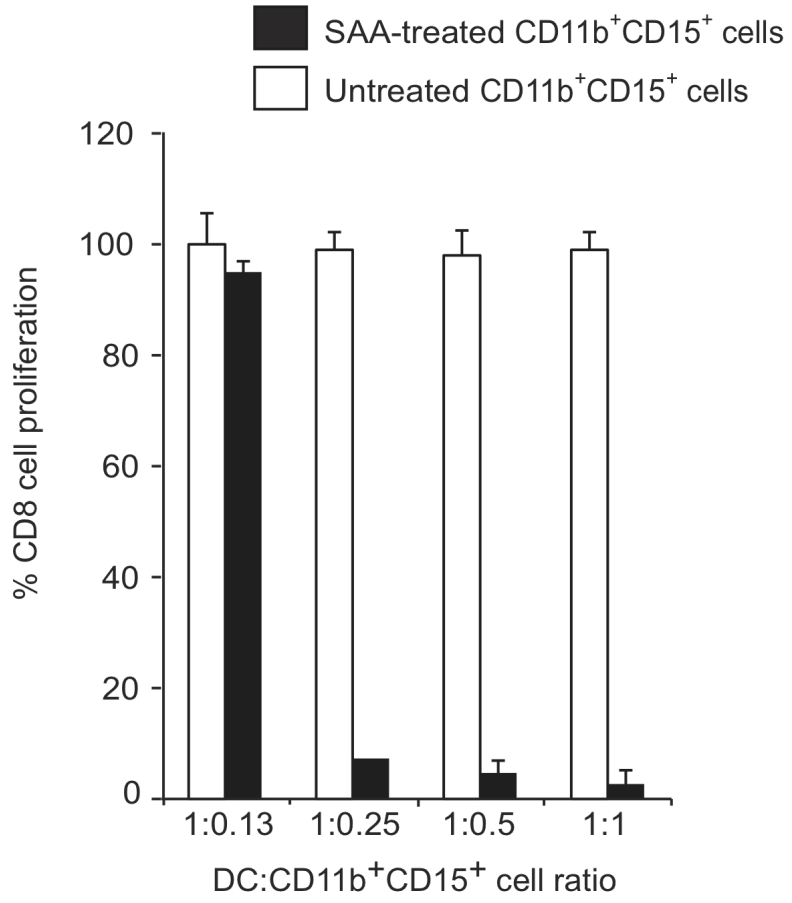


Figure 34: SAA differentiates human granulocytic MDSCs. Graph showing the abrogation of proliferation of alloreactive T cells that are in the presence of allogenic DCs and graded numbers of SAA-pretreated $CD11b^+CD15^+$ cells from a third party healthy donor (100% proliferation corresponds to 7 x 10^4 c.p.m.). The data represents five independent experiments.

Thirdly we sought to determine whether a SAA-facilitated crosstalk between human MDSCs and iNKT cells could also be demonstrated. For this

purpose $CD11b^{+}CD15^{+}$ cells, sorted from Ficoll-purified PBL of healthy donors (as explained previously), were treated with 1μM of SAA for 24 hours in the presence or absence of human iNKT cells in a 4:1 ratio. As anticipated, intra-cellular staining of the iNKT cells after the 24 hours of culture revealed IFNγ production; further, the SAA-induced human MDSC-iNKT cell crosstalk proved to be CD1d-dependent as culturing in the presence of a CD1d-blocking antibody prevented IFNγ production (**Figure 35a**).

Finally, to complete the comparison of our human and mouse data, ELISAs were performed on the supernatant of the SAA-pulsed human MDSC-iNKT cultures described in the previous paragraph and indeed the results revealed that the SAA-induced MDSC-iNKT interaction abrogated the IL-10 production that was otherwise seen in the wells of SAA-pulsed $CD11b^{+}CD15^{+}$ cells alone; the abrogation of IL-10 production was once again shown to be a CD1d-dependent interaction between the MDSC-iNKT cells as increased IL-10 levels were restored in those wells in which SAA-pulsed $CD11b^{+}CD15^{+}$ cells were in the presence of iNKT cells and a CD1d-blocking antibody (**Figure 35b**).

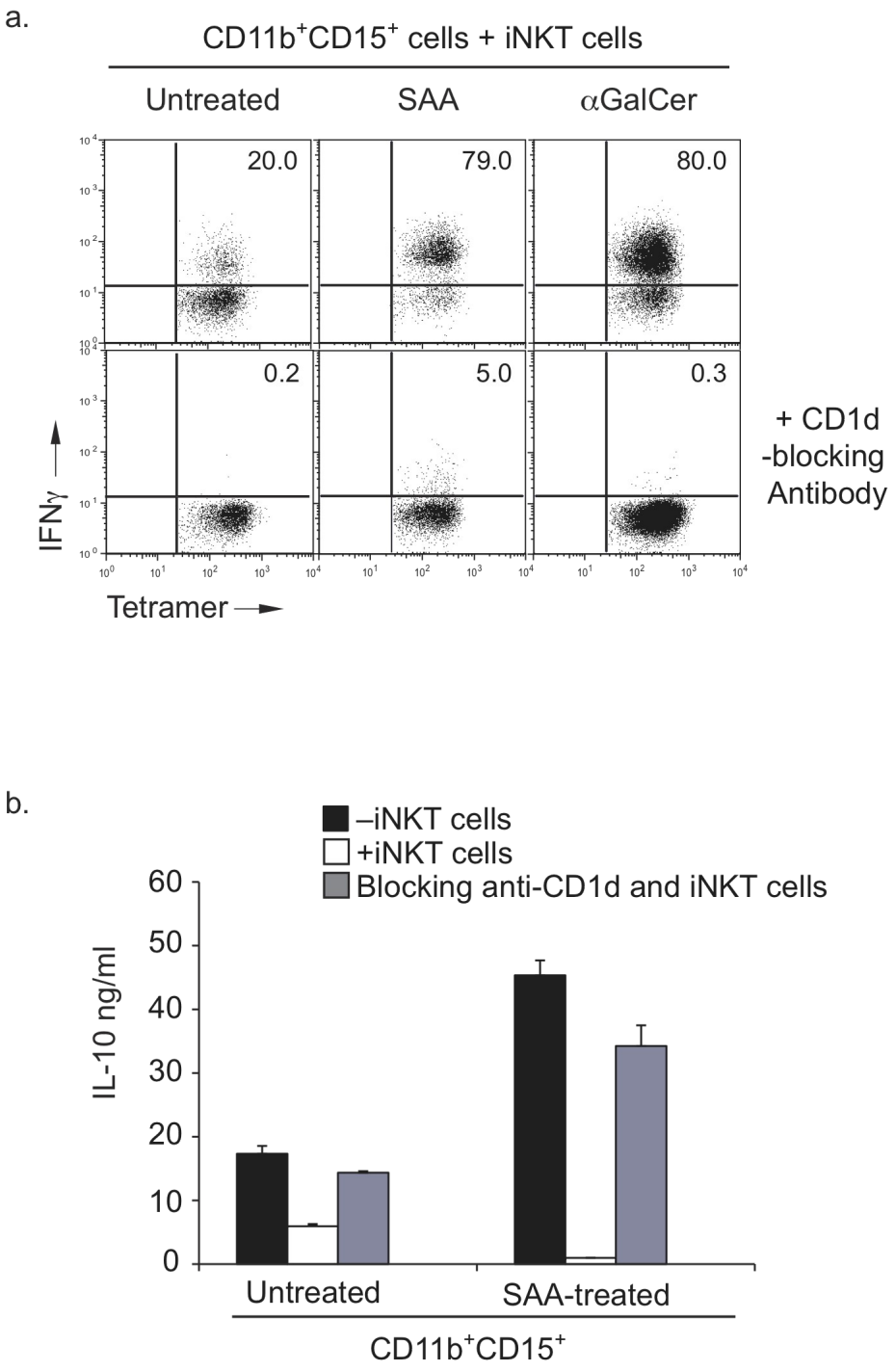


Figure 35: SAA-facilitated human MDSC-iNKT crosstalk abrogates IL-10 production by the MDSCs. (**a**) Intra-cellular anti-IFNγ staining of αGalCer

tetramer-positive human iNKT cells cultured together with untreated (Left), SAA-treated (middle) or αGalCer-treated (right) $CD11b^+CD15^+$ cells, in the presence or absence of CD1d-blocking antibody. (**b**) Bar graph showing the release of IL-10 by SAA-treated $CD11b^+CD15^+$ cells being abrogated by a CD1d-dependent iNKT-$CD11b^+CD15^+$ cell crosstalk

4.9 Discussion

In this final chapter of results we demonstrate a reciprocal dual role of the acute phase protein Serum Amyloid A (SAA). We show that SAA can induce the differentiation of IL-10-secreting granulocytic MDSCs, but can also concomitantly counterbalance this effect by facilitating the abrogation of the suppressive activity of the MDSCs through a SAA-induced GCSF-mediated CD1d-dependent interaction of the MDSCs with iNKT cells. These results suggest that SAA is involved in inducing both pro- and anti-inflammatory responses to flu infection and indeed reflect the complexity of its role in the acute phase response.

4.9.1 Immunomodulation by Serum Amyloid A

The acute phase response primarily functions to isolate and destroy pathogens and promote repair processes in restoring host homeostasis[183,184]. SAA has long been used as a marker of the acute phase response and can increase up to 1000-fold that in the non-inflammatory state[177]. Although C-reactive protein is widely used clinically, SAA is frequently a more sensitive marker of inflammation. Further it has the experimental advantage of being highly conserved and involved in the acute phase response in vertebrates other than humans, such as mice[185].

Investigators have previously shown the immunomodulatory effects of SAA to include: an ability to act as a chemoattractant for monocytes, neutrophils and T-cells[186,187], rescue neutrophils from apoptosis by preventing mitochondrial dysfunction[188], and promote the secretion of pro- and anti-inflammatory cytokines including IL-1β, IL-1 receptor agonist, IL-8, IL-10, IL-12, IL-23 and TNFα from granulocytes[189,190], lymphocytes[177], and monocytes/macrophages[191-193]. However, demonstrating the ability of SAA to differentiate IL-10-secreting granulocytic MDSCs is a novel finding and sheds further light on the influence SAA has on the anti-inflammatory arm of the immune response.

4.9.2 SAA-induced Myeloid-derived Suppressor Cells

Only recently has SAA been associated with MDSCs. It was Sander et al who first observed that SAA secretion during polymicrobial sepsis enhanced $CD11b^{+}Gr1^{+}$ MDSC survival by demonstrating a greater number of annexin V^{+} apopototic $CD11b^{+}Gr1^{+}$ cells in the periphery of septic gp130-deficient mice (which lack the ability to secrete SAA) as compared to in septic wild type mice. From those results Sander proposed that SAA functioned to prevent apoptosis of the sepsis-induced MDSCs without which there was increased morbidity and mortality, as the MDSCs were hypothesized to be mitigating any untoward host "collateral" damage that would otherwise occur if the MDSCs were absent and the immune response went unchecked[181]. The results in this manuscript have not only again made an association between SAA and MDSCs, but have also gone further in

identifying a more integral role for SAA in the differentiation of a more defined subset of MDSCs.

In contrast to the $CD11b^+Ly6^+$ cells (PMN- or granulocytic- MDSCs) we have described which are themselves IL-10-secreting, Sander et al described a heterogeneous population of $CD11b^+Gr1^+$ MDSCs in the polymicrobial sepsis model that functioned to stimulate primary macrophages to secrete IL-10[181]. Interestingly however, the MDSCs defined in this thesis do coincide perfectly with the relatively recently described "suppressive neutrophils"; akin to the classification of macrophages into M1 and M2, evidence of PMNs undergoing "N2 polarization" toward a suppressive phenotype (as opposed to the canonical pro-inflammatory neutrophils which are "N1 polarized) has recently been suggested[149,194]. As mentioned in Chapters 1 and 2, hyper-segmented $CD11b^+Ly6^+$ cells were shown by Zhang et al to secrete IL-10 and by Fridlender et al to suppress $CD8^+$ T cell proliferation[149,150]; like the MDSCs described in our flu-model, these suppressive N2 neutrophils were shown to be detrimental to the host as they were recruited as an immune-escape mechanism by tumours and mycobacteria infections alike[149,150]. The association we have identified in flu infection between SAA and suppressive $CD11b^+Ly6^+$ cells now provides possible mechanistic insight into the differentiation of these N2 neutrophils, firstly by demonstrating SAA's ability to induce IL-10 secretion by the $CD11b^+Ly6^+$ cells, and then by highlighting the SAA-mediated up-regulation of S100A9 and ARG1, the latter being the primary mechanism through which these granulocytic MDSCs suppress CD8 T cells[163].

Extending our findings to humans by demonstrating a SAA-induced ability of $CD11b^{+}CD15^{+}$ cells to secrete IL-10 and suppress T cell proliferation gave further relevance to the association identified between SAA and granulocytic MDSCs. Significantly, in parallel to our findings in flu infection, we demonstrated the presence of SAA-induced IL-10-secreting suppressive $CD11b^{+}CD15^{+}$ cells in melanoma patients (data not shown in this manuscript)[195]. Indeed, similar to the IL-10-secreting $CD11b^{+}Ly6^{+}$ cells in the flu-infected mice, the $CD11b^{+}CD15^{+}$ cells sorted from the melanoma patients also expressed ARG1 and suppressed antigen-specific T cell responses, although the differentiation of the suppressive neutrophils by SAA was found to be via the FPR2 receptor in humans as opposed to being TLR2-dependent in mice (data not shown in this manuscript)[195]. Demonstrating the ability of these suppressive neutrophils to impair the immune response to certain infections and tumours make them possible viable targets in treatment strategies.

4.9.3 iNKT cell influence on myeloid function and differentiation

Even more exciting is our finding that SAA also promotes the crosstalk between MDSCs (suppressive neutrophils) and iNKT cells, which results in abrogation of the suppressive activity of the MDSCs and underscores once again the immunomodulatory capacity of the iNKT cells. The results are consistent with the hypothesis that these granulocytes demonstrate some plasticity, as well as highlight the link between inflammation and iNKT cell activation.

Notably, other mechanisms of iNKT cell activation during inflammation have previously been shown. For example, basal autoreactivity of iNKT cells has been shown to be enhanced by the inflammatory cytokines IL-12 and IL-18[105,111-113] and activation increased by greater CD1d expression on APCs[196,197]; the activated iNKT cells in turn respond by enhancing DC and B cell activation. Influencing the frequency of SAA-induced IL-10-secreting granulocytes during the inflammatory response is now another regulatory mechanism of this subset of T cells and no more so was this demonstrated than in the SAA-injected bone marrow chimeras in which the proliferation of the IL-10-secreting immunosuppressive neutrophils occurred only in the $CD1d^{-/-}$ population. Indeed, the relatively small size of the iNKT population suggests that it may be logistically improbable for them to interact with all the MDSCs in the periphery. However, iNKT cells have been shown to reside in the bone marrow[124,198] and our data demonstrating iNKT-GMP crosstalk in Chapter 3 suggest that in addition to acting in the periphery to abrogate the suppressive activity of the MDSCs, iNKT cells may very well be modulating the differentiation and polarization of neutrophils by interacting with the GMPs and influencing the population expansion through amplification of GCSF production.

In conclusion, the results in this manuscript have demonstrated a previously unknown dual reciprocal role of the acute phase protein SAA in affecting the pro- versus anti-inflammatory balance of the immune response, by not only causing the differentiation of suppressive neutrophils but by also promoting the interaction of these suppressive neutrophils with iNKT cells to abrogate

the suppressive activity. Further, the extent of the iNKT-cell influence on myeloid differentiation is highlighted; as in addition to influencing neutrophil plasticity and function in the periphery, we have also shown modulation of granulocyte-macrophage progenitor differentiation through iNKT-GMP crosstalk. Indeed, conceptually MDSCs such as the suppressive neutrophils described in this manuscript may exist as a physiological mechanism to control excessive inflammation; however they are susceptible to exploitation as a mechanism of escaping the immune response by microbes and tumours alike. As such, it stands to reason that harnessing the immunomodulatory capacity of iNKT cells may be a viable and exciting prospect for bolstering vaccination strategies and other therapeutic approaches to infections and tumours, and worthy of increased focus as a subject of future investigations.

CHAPTER 5

Conclusions and future perspectives

With respect to the thesis aims outlined in Chapter 1, the evidence within this manuscript is indeed compelling: (i) the MDSCs expanding in the periphery of flu-infected iNKT-deficient mice were confirmed to be IL-10-secreting suppressive neutrophils, (ii) Granulocyte-macrophage progenitors from the bone marrow of flu-infected iNKT-deficient mice were found to have the ability to suppress T-cell proliferation, (iii) iNKT cells demonstrated the ability to abrogate the suppressive activity of the suppressive GMPs and IL-10-secreting neutrophils and (iv) Serum Amyloid A was shown to be a soluble factor with the reciprocal effects of causing IL-10-secreting suppressive neutrophils differentiation while paradoxically facilitating an iNKT-MDSC crosstalk to abrogate the suppressive activity of the MDSCs.

However, as satisfying as the above results may be, attempting to extrapolate these findings to help further deconstruct the physiology of the immune response (in particular as pertains to the MDSCs-iNKT relationship) has led to even more questions, a few of which include: (i) where exactly are iNKT cells exerting the greatest influence on MDSCs – is it truly in the bone marrow on GMPs, or could it be in the liver (the largest

producer of SAA and the site with the highest prevalence of iNKT cells[199])? (ii) How much are iNKT cell defects (and a resultant aberrant immunosuppressive microenvironment) indeed contributing to the pathogenesis of infections, cancer or autoimmunity? And (iii) how can iNKT cells' influence on MDSCs be harnessed in therapeutic modalities against disease?

5.1 Is the greatest influence of iNKT cells on MDSCs actually in the liver?

In an effort to gain insight into the main sites of iNKT cell influence, a number of studies have investigated the homeostatic distribution of iNKT cells and iNKT cell trafficking, particularly during infections. Using α-GalCer-loaded CD1d tetramer staining, Matsuda et al demonstrated that iNKT cells account for only 0.3-1.5% of lymphocytes in primary and secondary lymphoid tissues in the steady state, while constituting up to 40% of mononuclear cells in the liver[124]. Subsequently Geissman et al, by using intravital fluorescence microscopic imaging and CXCR6-GFP$^+$ knock-in mice, examined the *in vivo* behaviour of liver NKT cells (which account for the majority of CXCR6+ cells in the liver); iNKT cells were shown to mainly occupy the intravascular compartment (liver sinusoids), explained in part by the fact that CXCL16 (the chemokine ligand for CXCR6) is expressed on the sinusoidal endothelial cells. Geissman also showed that CXCR6 regulated the number of hepatic iNKT cells in part by transmitting survival signals[199]. It has been asserted that the expression of CD1d by Kupffer cells, hepatocytes, hepatic stellate cells and sinusoidal-endothelial cells along with the extensive lipid metabolism in the liver may explain why iNKT cells are

particularly suited for surveillance of the liver[199]. Coupling the high frequency of iNKT cells within the liver with the fact that the liver is the major site of SAA synthesis therefore provokes the hypothesis that the liver may indeed be an important site in which iNKT cells are modulating myeloid cell function (in particular that of MDSCs) via the SAA-mediated MDSC-iNKT crosstalk mechanism highlighted in this thesis. As such, it is critical that further investigations are carried out into MDSCs and iNKT cell trafficking, and into the real-time kinetics and location of any *in vivo* interaction between the two populations in order to address whether the liver is not only a major site of production of immunomodulatory serum proteins, but also a possible important site of cell-cell interactions that modulate the immune response.

Although the high representation of iNKT cells in the liver may suggest a special role for these cells in that organ, one must be cautious however not to extrapolate this concept too much without adequate investigations, especially across species from mice to humans. For instance, de Lalla et al pointed out that despite iNKT cells being enriched in chronically inflamed human livers, percentages are much lower than that found in murine livers, in both the pathological and steady state[200]. Moreover, a number of studies in humans have shown the ability of iNKT cells to rapidly accumulate at sites of injury and/or infection[201-206] (as opposed to just remaining/residing in the liver when exerting an immunomodulatory effect). In fact most human iNKT cells express a homing receptor repertoire resembling that of effector memory T cells, lacking CD62L, CCR7 and CXCR5 expression (which

are needed for trafficking through secondary lymphoid organs), while expressing CCR2, CCR5, CCR6, CXCR3 and CXCR4, overall suggesting that the iNKT cells preferentially home directly to non-lymphoid sites of inflammation[125,207,208] as opposed to just residing in one or another organ. Therefore, although along with the size of the liver iNKT cell population there are a few factors that may provoke one to consider the liver to be a crucial site of iNKT cell immunoregulatory influence, more needs to be done to prove this concept.

5.2 iNKT cell defects and disease

Several studies have demonstrated mechanisms through which iNKT cells diminish a host's susceptibility to certain microbial infections[105-107], autoimmune disorders[209-214] and malignancies[119,215-218]. Furthermore, a defect or deficiency in the iNKT cell population has been cited as a possible explanation for the pathogenesis of some disorders in which MDSC expansion has been shown to play a role[145,195].

By comparing Jα18$^{-/-}$ (iNKT-deficient) and/or CD1d$^{-/-}$ (CD1d KO) mice with WT mice, several iNKT-dependent mechanisms integral to the clearance of certain pathogens have been identified. For instance, multiple groups have demonstrated that through the recognition of microbial lipids (as opposed to peptides) iNKT cells aid immunosurveillance and clearance of pathogens such as certain Gram-negative LPS-negative bacteria (like Sphingomonas and Ehrlichia)[105,107,219,220], as well as Borrelia Burgdorferi[106], Mycobacterium tuberculosis[221,222] and Leishmania donovanii[223]. However, as highlighted by

De Santo et al in 2008 (as well as in this manuscript), the up-regulation of endogenous ligands on APCs in response to a flu-infection can also activate iNKT cells which intern abrogate MDSC activity and augment the immune response to the influenza virus[145]. Interestingly, clearance of the gram-negative LPS-expressing bacteria Salmonella, has also been shown to involve iNKT activation that was dependent on self-antigen presentation, as a more fulminant infection was observed when the antigen-presenting cells lacked beta-hexosaminidase B (the enzyme required for the generation of the endogenous iNKT agonist, iGb3)[105]. Of note, IL-12 release by DCs (triggered by LPS in a TLR-MyD88-dependent manner) is also necessary for iNKT activation during Salmonella infection[105,111]. Although iNKT-cell activation during flu infection was shown in this thesis to be SAA-induced, GCSF-mediated and CD1d-dependent, determining whether IL-12- or IL-18-mediated iNKT-activation is also playing a part in the immune response was not explored, and is indeed worth further investigation.

In the pathogenesis of certain autoimmune disorders, such as type I diabetes mellitus and multiple sclerosis, iNKT cells have been identified as playing an immunoregulatory (rather than effector) role[209,210,212-214]. By extrapolating the notion that iNKT cells can modulate immunosuppression by abrogating MDSC activity, one may hypothesize that iNKT activation may propagate autoimmunity. However, iNKT cell activity through a skew towards Th2 cytokine production (and away from a Th1 response), has been found to have a preventative/ protective effect in both type I diabetes mellitus[211] and multiple sclerosis[213,214], demonstrating the complexity of the

regulatory role of iNKT cells. On-the-other-hand, iNKT cells have been linked to the development of asthmatic allergen-induced hypersensitivity, as an otherwise blunted airway hypersensitivity response seen in Jα18$^{-/-}$ (iNKT-deficient) and/or CD1d$^{-/-}$ mice is restored on adoptive transfers of IL-4/IL-13-producing iNKT cells[224]. Although not reported on, it may be worth investigating whether altering the immunosuppressive microenvironment through the inhibition of MDSCs in the lining of the airway may be yet another mechanism through which iNKT cell activation may be contributing to the pathogenesis of asthma.

As it pertains to tumour immunity, the consensus in the field is that iNKT cells play primarily a protective role. For example, Toura et al were able to successfully treat B16 melanoma liver metastases in mice by administering αGalCer-pulsed syngeneic DCs[218]. Hayakawa et al then discovered that chronic administration of αGalCer can even prevent tumour formation in methylcholanthrene treated mice, Her-2/neu oncogene transgenic mice and mice deficient in the tumour suppressor gene p53[217]. In humans however, the importance of iNKT cells in preventing tumours has in many instances been inferred from indirect evidence, for instance through the detection of statistically significant fewer numbers of iNKT cells in the peripheral blood of cancer patients (as shown by Giaccone et al with a variety of tumours[225]) or through the detection of defective iNKT cells (as is suggested by Crough et al's findings that iNKT cells from patients with certain tumours have a decreased responsiveness to αGalCer as compared to those from healthy patients[226]).

In terms of the mechanism of action of iNKT cells in tumour immunity it is worth noting that even though iNKT cells are capable of lysing tumour cells[142], the primary role of the iNKT cells is once again immunoregulatory. The main mechanisms highlighted by investigators on iNKT cell activity in response to tumours have been a skewing of the cytokine mileu towards a Th1 profile[227], inducing IL-12 production by DCs[218], abrogating the immunosuppressive activity of MDSCs[195], and augmenting the activation of effector NK and $CD8^+$ T cells against tumours[228]. Indeed, although the ability of MDSCs to inhibit tumour-antigen-specific responses has been shown by several investigators, the ability of iNKT cells to abrogate the action of MDSCs was only recently highlighted, initially in a flu model[145] and then subsequently in melanoma patients[195], as mentioned and expanded upon in the previous Chapters of this thesis. De Santo et al incorporated several concepts from this manuscript into the publication on MDSCs in melanoma, as in both the flu-infection model outlined in this thesis and in melanoma patients a similar expansion of SAA-induced IL10-secreting neutrophils was observed; furthermore the ability of iNKT cells to relieve the suppressive activity of the MDSCs was also common to both models. However it is important to note that neither the *in-vivo* function nor number of iNKT cells in the setting of flu or melanoma was examined; indeed both these characteristics of iNKT cells in the setting of the respective pathologies could be potential hurdles if future therapeutic modalities that focus on harnessing iNKT cell activity are to be attempted, and therefore are also worthy of further investigation.

5.3 Harnessing iNKT cells' influence in immunotherapy

Based on the murine evidence that activated iNKT cells can increase antigen-specific responses several clinical trials have already been done in an attempt to translate these results to humans. Immunotherapy protocols (mostly for tumour-bearing individuals) involving the administration of intravenous αGalCer[225], DCs pulsed with αGalCer[229,230], or the adoptive transfer of *in-vitro* expanded autologous iNKT cells[231] have all been attempted, each protocol noted to result in varying degrees of change in iNKT cell frequency and/or increased IFNγ levels and/or increase in cytokines associated with DC maturation. However, none of these iNKT cell-harnessing clinical trials managed to achieve significant remission of disease; interestingly neither did any of the clinical trials mention the level of MDSCs before and after the administration of the immunotherapy, which based on the results in this manuscript ought to be another objective parameter incorporated into future protocols.

Several explanations have been put forth as to why the aforementioned clinical trials on harnessing iNKT cells in immunotherapy have not produced results as promising as those seen with the murine experience; one being evidence that humans have lower numbers of iNKT cells when compared to mice[232], and another being the notion that the patients in the clinical trials had much more advanced disease than the mice in which the αGalCer/iNKT-based therapy was successful[227]. Additionally, the induction of iNKT-cell anergy on repeated αGalCer administration has been cited as a

potential problem with immunotherapy protocols[115]. Indeed, to address this latter issue, structure-function relationships among analogs of αGalCer have been investigated in mice, and weaker iNKT cell agonists such as threitolceramide have been shown to be advantageous in comparison to αGalCer with respect to iNKT cells showing quicker recovery from activation induced-anergy[108]. Of note, the concept of manipulating iNKT cell activation with selective ligands in order to optimize immunotherapy goes beyond potential therapies for just tumours, as investigators have also reported on the use of OCH (another weaker iNKT agonist) as being protective against experimental autoimmune encephalitis by inducing a skew in the cytokine production towards a Th2-type profile[213]. Experience however cautions against extrapolating these findings to other diseases or across species until further clinical trials are done.

Therefore, although the results in this manuscript are indeed exciting, much is left to be done to unravel and better define the complex role of iNKT cells in modulating myelopoiesis (particularly with respect to MDSC generation and function) and the relevance of this immunoregulatory effect to different disease processes. There is a large body of murine data supporting the notion that iNKT cell activity can be harnessed to treat certain diseases, however translating the findings into therapeutic modalities in humans has not been particularly successful. To potentially improve the outcomes of therapeutic modalities that incorporate modulating iNKT cell activity as a mechanism, there are several areas that need further investigation, including but not limited to: (i) determining the *in-vivo* trafficking, organ

distribution and number of iNKT cells in both healthy and diseased humans and (ii) genetic and biochemical analyses of GMPs and MDSC to confirm and/or better define the effect of iNKT cells on an axis of differentiation toward a suppressive phenotype (as suggested by the results in this thesis). Moreover, in addition to gaining further mechanistic insight into how iNKT cells may be influencing MDSC differentiation and function, genetic analyses aimed at stratifying patients based on potential quantitative or qualitative iNKT cell defects will give both a better understanding of disease processes and help identify those patients who will benefit most from potential therapeutic modalities geared at harnessing iNKT cell activity.

Materials and Methods

6 Materials

6.1 Mice

Strain	*Description*
C57BL/6 (B6)	These animals are of the H-2^b haplotype
B6.SJL-Ptprc^{a}Pep3^b/BoγJ (B6Lγ5.1)	These congenic mice express the lymphocyte surface antigen CD45.1 (also referred to as Ly5.1) Ptprc^a (protein-tyrosine phosphatase, receptor type c, a allele). This marker allows the discrimination between lymphocytes from these mice and C57BL/6 lymphocytes, which express CD45.2 instead
OT-1	These animals are H-$2K^b$ restricted with a transgenic TCR ($V_\alpha2/V_\beta5.2^+$), specific for $OVA_{257-264}$ (SIINFEKL) peptide. They were provided by M. Merckenschlager (Imperial College London) and backcrossed 8 times onto the B6 background.
B6.CD1d$^{-/-}$	$CD1d^{-/-}$ mice were provided by L. Van Kaer (Vanderbilt University School of Medicine, Nashville, Tennessee, USA) and were backcrossed 10 times onto the B6 background.
B6.NKT$^{-/-}$ (NKT KO)	These mice lack the Jα18 TCR gene segment, and were devoid of Vα14 iNKT cells, while having other lymphoid cell lineages intact.
Vα14 TCR Tg	These transgenic mice have an increased number of Vα14 iNKT cells as compared to WT mice, and were used to harvest iNKT cells.

All mice were maintained in the Biological Services Unit, John Radcliffe Hospital, University of Oxford. Approval of care and use was obtained from the Clinical Medicine Ethical Review Committee, University of Oxford, and procedures were carried out following the Home Office Animals (Scientific

Procedures) Act 1986 (PIL: 30/7588: PPL: 30/2391). Mice were generally used between 6-12 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 cervical dislocation.

6.2 Tissue Culture and Colony Forming Assay Reagents

Reagent	***Supplier***
1x and 10x Phosphate Buffered Saline (PBS)	Lonza

RPMI 1640	Sigma
Isocove's modified Dulbecco's media (IMDM)	Sigma
Dimethyl sulphoxide (DMSO)	Sigma
Fetal Bovine Serum (FBS)	Sigma
L-Glutamine (200mM in 0.85% Azide)	Lonza
PEN-STREP (5000U Penicillin/ml, 5000U Streptomycin/ml)	Lonza
Non-essential Amino-acids (NEAA) x100	Invitrogen
Sodium Pyruvate (100mM)	Invitrogen
Trypan Blue	Sigma
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA)	Sigma
Trypsin/EDTA	Sigma
β-mercaptoethanol (50mM)	GIBCO
MethoCult® (base methylcellulose) M-3134	Stem Cell tech

6.3 Tissue Culture and Colony Forming Unit (CFU) Medium

Medium	***Composition***
R10	RPMI-1640 with L-Glutamine and $NaHCO_3$ (Sigma)
(tissue	supplemented with 10% (v/v) heat-inactivated FCS, Penicillin/

culture)	Streptomycin (x100), L-Glutamine (x100), Non-essential Amino-acids (x100), 1mM Sodium Pyruvate (x100) and 50µM β-mercaptoethanol.
Complete Methyl-cellulose Media (CFU-GM)	40ml Base Methylcellulose M3134 (Stem Cell Technology), 20ml Culture FCS (Thermo Fisher Lot CSG0412), 37ml IMDM (Iscove's modified Dulbecco's media), 1ml L-glutamine, final conc. 2mM, stock conc. 200mM, 1ml penicillin/streptomycin: final conc. 100U/ml stock conc. 10,000U/ml, 1ml 2-β-Mercaptoethanol final conc. 10^{-4} M stock conc. 10^{-2} M (Sigma) and Cytokines as per ***table 6.3.1***

6.3.1 Complete Methylcellulose media cytokine composition

Cytokine	***Stock solution***	***Final conc.***	***Vol. of cytokine added for 100ml complete methylcellulose (µl)***
mGM-CSF	5µg/ml	5ng/ml	110
hFL	5µg/ml	10ng/ml	220
mIL-3	5µg/ml	2ng/ml	44
hG-CSF	10µg/ml	10ng/ml	110

6.4 Solutions

Solutions	***Composition***
0.5% PBST	1× PBS + 0.5% (v/v) Tween-20 (Sigma-Aldrich)
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.
1M DTT stock solution	1.54g DTT (Sigma, M_W = 154.2 g/mol) was dissolved in 10ml of ddH_2O; the solution was dispensed into 100 µl on ice and store at -20 ºC for no longer then 2 month. Samples were not refrozen once thawed.
25x Complete Protease inhibitor stock solution	One complete, EDTA-free Protease Inhibitor Cocktail Tablet (Roche, sufficient for 50ml lysis buffer) was dissolved in 2ml of lysis buffer, dispensed into 100µl aliquots and stored at -20ºC for a maximum of 12 weeks or at 4 ºC for a maximum of 2 weeks.
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.

6.5 Buffers

Buffer	***Composition***
Antibody dilution buffer for Western Blot	0.5% PBST (or 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 Western Blot	0.5% PBST + 5% (w/v) dried skimmed milk powder.
10x Running Buffer for SDS-PAGE	1920mM Glycine, 250mM Trizma Base (all from Sigma) dissolved in 1 l ddH_2O.
1x Running Buffer for SDS-PAGE	75 ml 10x Running Buffer, 7.5 ml 10% (w/v) SDS; buffer was made up to 750 ml with ddH_2O.
1x Transfer Buffer for Western Blot	100 ml 10× Running Buffer, 10 ml 10% SDS (w/v), 150 ml Methanol; buffer was made up to 1 litre with ddH_2O and stored at 4°C until use.
5x Protein Loading Buffer (LB), reducing.	0.313M Tris-HCl (pH 6.8 at 25°C), 10% SDS, 0.05% bromophenol blue, 50% glycerol in ddH_2O. Samples were prepared by adding 1/5 volume of 5x LB and 1/10 volume of 1M DTT.
ELISA coating buffer	0.1 M $NaHCO_3$ (Sigma) in ddH_2O, pH 9, sterile filter.
ELISA stop solution	2 M sulphuric acid.
Substrate solution A for ELISA	10.5 g citric acid monohydrate (Fluka) in 500 ml ddH_2O.
Substrate solution B for ELISA	17 g $Na_2HPO_4 \cdot 2H_2O$ (Fluka) in 500 ml ddH_2O.
ELISA blocking buffer	PBS containing 10% (v/v) FCS, store at 4 °C.
ELISA washing buffer	PBS + 0.1% Tween-20.
Lysis buffer	20mM Tris-Cl pH 7.5, 150mM NaCl, 1mM EDTA-solution pH 8.0, 1% (v/v) Triton x100, 1mM EGTA, 2.5mM Sodium-Pyrophosphate Decahydrate and 1mM β-glycerophosphate

	(all from Sigma) dissolved in ddH_2O. pH was adjusted to 7.5 and solution stored at 4 °C. Prior to cell lysis, 1/25 of 25x Complete Protease inhibitor stock solution was added.
FACS buffer	PBS containing 10% (v/v) FCS, store at 4 °C.
MACS buffer	PBS containing 2 mM EDTA and 10% (v/v) FCS.

6.6 Protein Gels

6.6.1 Separating 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

6.6.2 Stacking Gel

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

6.7 ELISA Reagents

ELISA Kit	*Catalogue No.*	*Supplier*
Mouse (Quantikine) G-CSF Immunoassay	MCS00	R&D Systems
Mouse IL-10 ELISA Kit	88-7104	eBioscience
Mouse SAA Immunoassay Kit	KMA0012	Invitrogen

Note: IL-12 ELISAs were done without a customized kit. See Buffer table for solutions. Antibodies used are listed below.

Antibody	*Clone*	*Isotype*	*Supplier*
Affinity purified ant-mouse IL-12p40 (Capture Antibody)	C15.6	Rat IgG1	eBioscience
Biotinylated ant-mouse IL-12p40 (Detection Antibody)	C17.8	Rat IgG2a, k	eBioscience

IL-12 in serial dilutions was used for the Standard Curve.

6.8 Cytokines

Cytokine	*Catalogue No.*	*Supplier*
Murine G-CSF (mG-CSF)	250-05	Peprotech
Murine GM-CSF (mGM-CSF)	315-03	Peprotech
Murine IL-3 (mIL-3)	210-13	Peprotech
Human Flt3 (hFL)	8985-012	Immunex
Recombinant Human apo-SAA (SAA)	300-13	Peprotech

6.9 Flow Cytometry, Enrichment and Cell Sorting Reagents

Reagent	*Supplier*
CD11b MACS Beads (Human and mouse)	Miltenyi Biotec
CD14 MACS Beads (Human)	Miltenyi Biotec
CD117 MACS Beads (Mouse)	Miltenyi Biotec
Propidium Iodide	Sigma
7-amino-actinomycin-D (7AAD)	eBioscience
5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)	Molecular Probes
Lymphoprep	Axis-Shield PoC AS
RBC Lysis Buffer	Qiagen
Fixation Buffer	eBioscience
Permeabilization Buffer	eBioscience

6.10 Antibodies

6.10.1 Antibodies for Western Blot

Antibody	*Isotype*	*Catalogue No.*	*Supplier*
Primary Antibodies			
Anti-Arginase I (N-20), polyclonal	Goat	sc-18351	Santa Cruz Biotechnology, INC.
Anti-beta 2 Microglobulin, monoclonal	Rabbit	ab75853	abcam

Anti-S100A9	Rabbit	ab75478	abcam
Anti-actin	Rabbit	A 2066	Sigma
Anti-GAPDH (C-terminal), polyclonal	Rabbit	AHP996	AbD Serotec
Secondary Antibodies			
Anti-Goat-IgG-HRP	Donkey	sc-2020	Santa Cruz Biotechnology, INC.
Anti-Rabbit-IgG-HRP	Goat	sc-2030	Santa Cruz Biotechnology, INC.

6.10.2 Antibodies for Flow Cytometry and Cell Sorting

Antibody	***Clone***	***Isotype***	***Conjugate***	***Supplier***
Anti-mouse CD11b	M1/70	Rat IgG2b, k	eFlour™ 450	eBioscience
Anti-mouse CD11b	M1/70	Rat IgG2b, k	PE	eBioscience
Anti-mouse Gr-1	RB6-8C5	Rat IgG2b, k	FITC	eBioscience
Anti-mouse Ly6G	1A8	Rat IgG2b, k	FITC	eBioscience
Anti-human CD11b	ICRF44	Mouse IgG1, κ	PE	eBioscience
Anti-human CD14	TIB 228	Rabbit, IgG2b	PE	ATCC
Anti-human CD15	HI98	Mouse IgM, κ	FITC	eBioscience
Anti-mouse B220	RA3-6B2	Rat IgG2a, k	biotinylated	eBioscience
Anti-mouse B220	RA3-6B2	Rat IgG2a, k	FITC	eBioscience
Anti-mouse CD3e	145-2C11	Armenian Hamster IgG	FITC	eBioscience
Anti-mouse Sca-1	E13-161.7	Rat IgG2a, k	Pacific Blue	BioLegend
Anti-mouse IL-10	JES5-16E3	Rat IgG2b, k	FITC	eBioscience

Anti-mouse TER119	TER-119	Rat IgG2b, k	Purified	eBioscience
Anti-mouse CD11b	M1/70	Rat IgG2b, k	Purified	eBioscience
Anti-mouse Gr-1	RB6-8C5	Rat IgG2b, k	Purified	eBioscience
Anti-mouse CD5	53-7.3	Rat IgG2a, k	Purified	eBioscience
Anti-mouse CD8a	53-7.3	Rat IgG2a, k	Purified	eBioscience
Anti-mouse CD4 (L3T4)	GK1.5	Rat IgG2b, k	Purified	eBioscience
Anti-mouse B220	RA3-6B2	Rat IgG2a, k	Purified	eBioscience
Streptavidin	N/A	N/A	PE-Cy5	eBioscience
Anti-mouse CD40	1C10	Rat IgG2a, k	APC	eBioscience
Anti-mouse CD1d	1B1	Rat IgG2b, k	Biotinylated	eBioscience
Anti-mouse CD127	A7R34	Rat IgG2a, k	PE-Cy5	eBioscience
Anti-mouse CD117	2B8	Rat IgG2b, k	APC-Alexa Fluor® 750	eBioscience
Anti-mouse CD34	RAM34	Rat IgG2a, k	FITC	eBioscience
Streptavidin	N/A	N/A	PE-Cy7	eBioscience
Anti-mouse FcγIII/IIR	2.4G2	Rat IgG2b, k	Purified	BD Pharmingen
Anti-mouse FcγIII/IIR	2.4G2	Rat IgG2b, k	PE	BD Pharmingen

6.10.3 Antibodies for Functional In vitro Assays

Antibody	*Clone*	*Isotype*	*Conjugate*	*Supplier*
Anti-mouse TLR2	T2.5	Mouse IgG1	Purified	eBioscience
Anti-mouse G-CSF	67604	Rat IgG1	Purified	R&D Systems

Note: Anti–mouse CD1d blocking Ab 3C11 was provided by J. Yewdell (NIH, Bethesda, Maryland, USA).

7. Methods

7.1 Preparation of murine cell samples for Flow Cytometry and Functional Assays

7.1.1 Spleen

For single cell preparation from murine spleen samples, whole organs were collected into sterile RPMI, subjected to mechanical disruption and then filtered through a 70μm cell strainer (BD Falcon). Cells were then pelleted (1500 rpm, 5 minutes at 4 ºC) and erythrocytes removed by osmotic lysis by re-suspending pellets in 1ml of 1x red blood cell (RBC) lysis buffer for 5 mins. Cells were then pelleted (1500 rpm, 5 minutes at 4 ºC), washed in FACS buffer, re-suspended in the desired media/buffer and filtered through a 70μm cell strainer (BD Falcon). Viable cell counts were performed by trypan blue exclusion before downstream treatment.

7.1.2 Blood

For blood analysis, mice were bled from the tail vein, collecting between 100-200μl per mouse into an eppendorf tube containing 40μl heparin. Erythrocytes were then removed by osmotic lysis by re-suspending pellets in 1ml of 1x red blood cell (RBC) lysis buffer for 5 mins. Cells were then pelleted (1500 rpm, 5 minutes at 4 ºC), washed in FACS buffer, re-suspended in the desired media/buffer, and filtered through a 70μm cell strainer (BD Falcon). Viable cell counts were performed by trypan blue exclusion before downstream treatment.

Note, when serum was required, 200μl of blood was collected from the tail vein in non-heparinized tubes. Samples were allowed to stand for 90-120mins to enable coagulation and sedimentation. The serum settling above the cells was then pipetted off and frozen at -20ºC until ready for use.

7.1.3 Lung

After euthanasia, the lungs were dissected from the thoraces of the mice and collected in FACS buffer. Once harvested, lungs were teased apart using a scalpel, subjected to mechanical disruption and digested by incubating each in 6ml of type III collagenase (3 mg/ml) for 90 minutes at 37 ºC (re-suspending every 15-20 minutes to aid disruption). Following incubation, cells were filtered through a 70μm cell strainer (BD Falcon) and immediately washed in FACS buffer. Cell suspensions were pelleted (1500 rpm, 5 minutes at 4 ºC), and erythrocytes removed by osmotic lysis by re-suspending pellets in 1ml of 1x red blood cell (RBC) lysis buffer for 5 mins. Cells were then washed in FACS buffer, filtered through a 70μm cell strainer (BD Falcon) and re-suspended in the desired media/buffer before downstream treatment.

7.1.4 Bone Marrow

Hind legs were dissected from each mouse and bone marrow cells flushed from each hind limb with FACS buffer. Cell suspensions were pelleted, and erythrocytes removed by osmotic lysis by re-suspending pellets in 1ml of 1x red blood cell (RBC) lysis buffer for 5 mins. Cells were then washed in FACS buffer, filtered through a 70μm cell strainer (BD Falcon) and re-suspended before downstream treatment.

7.2 Generation of human DCs

DCs were generated as previously described[233]: in short, blood was purchased from the U.K. National Blood Service (Bristol, U.K.). PBMCs were isolated from healthy donors' buffy coats by density gradient centrifugation

over Lymphoprep (Axis-Shield PoC AS). Monocytes were positively selected using anti-CD14 mAb–conjugated magnetic micro-beads (MACS; Miltenyi Biotec). The recovered cells were >99% $CD14^+$, as determined by flow cytometry with the anti-CD14 Ab. DCs were generated by culturing monocytes in RPMI 1640–10% FCS supplemented with 50 ng/ml GM-CSF and 1000 U/ml IL-4 for 5 days.

7.3 Expansion of human iNKT cells

Human iNKT cells were isolated in our lab by Paolo Polzella, as previously described by McCarthy et al[234]. In short, PBMCs were isolated from healthy donors' buffy coats by density gradient centrifugation over Lymphoprep (Axis-Shield PoC AS). Monocytes were positively selected using anti-CD14 mAb–conjugated magnetic micro-beads (MACS; Miltenyi Biotec), and monocyte-depleted lymphocyte fractions (CD14 negative) were frozen until needed. Monocytes were cultured with 50ng/ml GM-CSF and 1,000 U/ml IL-4 in six-well plates at 4×10^5 cells/ml (3 ml/well). After 5 d, maturation was induced using bacterial LPS (1µg/ml LPS of *Salmonella abortus equi*; Sigma-Aldrich). Immature and mature monocyte-derived DCs were phenotypically analyzed for maturation markers. 2×10^5 monocyte-derived DCs were pulsed with αGalCer for 2 h in 24-well plates in 200µl RPMI 1640. 2×10^6 autologous lymphocytes were added in 1.8 ml of medium (5% human serum). After 3 d, 25 IU/ml IL-2 was added to cultures. Thereafter, cultures were fed every 3–4 d with fresh medium containing 1,000 U/ml IL-2. iNKT cell frequencies were determined using APC CD1d tetramer and Vα24 antibody (Serotec).

7.4 Antibody and tetramer staining for Flow Cytometry

Cells for staining were made up to suspensions of maximum 1 x 10^6 cells/100μl in FACS buffer. 100μl of cell suspension was transferred to each well of a round bottom 96-well plate and pelleted (1500 rpm, 5 minutes at 4ºC). Supernatant was removed by inversion of the plate; plates were then gently vortexed to homogenize the cell pellets before adding antibody mixes.

Antibody mixes were prepared by mixing 50μl/sample FACS buffer multiplied by the total number of samples to be stained, and the antibodies added at the final desired concentration to this total volume of FACS buffer. 50μl of the relevant antibody mixture was added to each well of cells and the cells stained for 20min at 4ºC. Cells were then washed in FACS buffer, re-suspended in 200μl FACS buffer, transferred to FACS tubes and directly analyzed or kept at 4ºC in the dark for future analysis. Non-stained cells and single colour-stained cells were always included as controls. A complete list of antibodies and reagents used for flow cytometric analyses can be found in the Materials section.

For CD1d/α-GalCer tetramer staining, cells were first stained in an excess of CD1d/α-GalCer tetramer in 50μl volume at 37ºC for 20mins. Cells were then washed twice in FACS buffer and stained with monoclonal antibodies, as outlined above, before standard flow cytometric analysis.

Once prepared for direct analysis, a cell-impermeable nucleic acid dye, such as 7-amino-actinomycin-D (7AAD) at a 1:300 final concentration, or Propidium Iodide (PI) at a 1:200 final concentration, was added to the samples immediately prior to running on the FACS machine to allow exclusion of dead cells. Samples were run on Dako Cyan machines, unless otherwise stated. Data analysis and gating was then performed using FlowJo 8.8 for Mac Tree Star Inc.

7.5 Intracellular staining for IL-10

1 x 10^6 cells of homogenized lung cell suspensions were first stained with CD11b and Ly6G monoclonal antibodies in 96-well round-bottom plates according to the protocol outlined above. Cells were then pelleted (1500 rpm, 5 minutes at 4ºC) and re-suspended in 50μl Fixation buffer for 1 hour at 4ºC. Cells were then washed twice in Permeabilization buffer and re-suspended in 50μl of FITC conjugated rat anti-mouse IL-10 (1:100) and incubated on ice for 20 minutes. Cells were washed once more in Permeabilization buffer, re-suspended in 200μl FACS buffer per well, and analyzed on a FACS Caliber Cytometer (Becton Dickenson). Data analysis and gating were performed using FlowJo 8.8 for Mac Tree Star Inc.

7.6 Staining of Granulocyte-Macrophage Progenitors (GMPs) for Analysis and cell sorting

Bone marrow cell suspensions were prepared as outlined in 7.1.4. Staining protocol for GMPs was adopted from Akashi et al[11], as depicted in **Figure 14**.

Notably, FMO ("fluorescence-minus-one") controls were used throughout the staining protocol.

Both samples and controls were first stained with the bone marrow lineage cocktail (10x) at concentrations of $25x10^6$ cells/100μl (the lineage cocktail comprising of anti-CD4, CD5, CD8α, CD11b, Gr-1, TER119, B220 and the lymphoid marker CD127). Each sample was enriched for c-kit with CD117-beads and MACS cell-separation columns on a magnet prior to staining with the appropriate markers for myeloid progenitors as described by Akashi et al[11], before analysis was done on an LSRII and sorting of GMPs on a FACSAria cell sorter. See **Figure 14** for the sequential gating and definition of the GMPs, CMPs and MEPs.

7.7 Reverse Transcription and Real-time quantitative PCR

RNA extraction from GMPs sorted from the bone marrow of naïve and flu-infected WT and iNKT-deficient mice was kindly done by Dr. Babak Baban at the Peter Medawar Building of Pathogen Research, University of Oxford.

cDNA was obtained by denaturation of RNA followed by reverse trancription using the Superscript III Reverse transcriptase kit (Invitrogen). In short, the following components were added to a nuclease-free microcentrifuge tube:

- 1μl of oligo$(dT)_{20}$ (50μM); or 200-500ng of oligo$(dT)_{12-18}$

- 10pg - 5 μg total RNA
- 1μl 10mM dNTP Mix (10mM each dATP, dGTP, dCTP and dTTP at neutral pH)
- sterile distilled water to 13μl

The above mixture was heated at 65˚C for 5 minutes and incubated on ice for at least 1 minute. The tubes were centrifuged briefly and the following contents added:

- 4μl 5X First Strand Buffer
- 1μl 0.1M DTT
- 1μl RNaseOUT™ Recombinant RNase Inhibitor (Cat. No. 10777-019, 40units/μl)
- 1μl of Superscript™ III RT (200units/μl)

The mixture was pipetted gently up and down and then incubated at 50˚C for 30-60 minutes, increased to 55˚C for gene specific primers and the reaction inactivated by heating at 70˚C for 15 minutes. The cDNA obtained at this point was then used as a template for amplification in PCR.

In order to use the comparative Ct method ($\Delta\Delta C_T$) to relatively quantify gene expression, the amplification efficiencies of the target and endogenous control probes must be approximately equal. As TaqMan primer/probes (GAPDH, Actin, S100A9) from Applied Biosystems are validated for such relative gene expression quantification, these were directly used in

quantitative PCR (qPCR) experiments determining relative gene expression by the $\Delta\Delta C_T$ method.

Real-time quantitative PCR was performed in a MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, covered with MicroAmp Optical Adhesive Film (both from Applied Biosystems) and with the TaqMan Gene Expression Master Mix (Applied Biosystems). qPCR reactions were setup by mixing 11μl of qPCR master mix, 9μl of cDNA (10-100ng) and 1μl of the relevant 20X TaqMan probe.

All reactions were run in standard mode in a 7500 Fast Real-time PCR system (Applied Biosystems). The cycling conditions used were:

1. 50°C, 20 seconds
2. 95°C, 10 minutes
3. 95°C, 15 seconds
4. 60°C, 1 minutes
5. Back to step 3, 40 cycles.

Data acquisition was performed at step 4 of each cycle.

7.8 Microarray

After RNA extraction was done from the GMPs acquired from the bone marrow of naïve and flu-infected WT and iNKT-deficient mice, microarray was kindly carried out by Dr. Babak Baban at the Peter Medawar Building of

Pathogen Research, University of Oxford. Analysis of the results of the microarray was done in conjunction with the Computational Biology Research Group at the Weatherall Institute of Molecular Medicine, University of Oxford.

7.9 Western Blotting

The general steps for all the Western Blots done in this manuscript were as follows:

- A SE 600 vertical slab unit in a dual gel-casting stand was assembled using 1.5 mm spacers.
- A 10% running gel solution was mixed; recipe as outlined in 6.6.1
- The solution was pipetted down the spacer via one corner into each sandwich to a level about 4cm from the top, being careful not to introduce air bubbles.
- Industrial methylation spirit (IMS) was then pipetted gently from the top corner of the spacer into the sandwich to cover the running gel solution, filling the spacer to the top, creating an even layer across the surface of the running gel solution. This was left for 30 minutes, until polymerisation of the gel had taken place and there was a very sharp liquid-gel interface visible.
- Stacking gel solution was mixed in a similar fashion to step 2; recipe as outlined in 6.6.2.
- The IMS overlay was then poured off by tilting the casting stand and the stacking gel solution pipetted in a similar fashion to step 4, filling

the spacer to the brim. A comb was then carefully inserted into each sandwich, taking care not to trap any bubbles below the teeth of the combs, and the gel allowed to sit for 1 hour for polymerisation to take place.

- Equal parts of lysate sample and 3X treatment buffer were aliquoted and mixed in eppendorfs, ensuring that each sample had the same amount of protein; a hole was created in the lid of each sample, which was then boiled at 100°C for 5 minutes.
- The combs were removed slowly from the gels, being careful to avoid the well dividers.
- The sandwich was placed in the electrophoresis tank (gel unit), and the apparatus filled with tank buffer, ensuring the wells were filled as well.
- Equal mass of samples were loaded into the wells by pipetting them slowly through the tank buffer to the base of each well, avoiding swirling and diffuse loading, thereby ensuring a sharp interface between sample and tank buffer, and by extension band sharpness. Sample buffer with standard protein (ladder) was loaded adjacent to either end of the series of wells with protein samples.
- The lid was placed on the gel unit, the leads connected black/black and red/red; gel was run for 1 hour at 150V (until the loading dye has run off the bottom of the gel).
- The apparatus was dismantled, the gel carefully lifted away from the glass plates, and the stacking gel removed and discarded. The top corner of the side of the running gel from which loading was

commenced was cut, to avoid ambiguity of identifying lanes and the gel placed in a container with transfer buffer while the tank transfer system was set up.

- The blotter paper and nitrocellulose membrane were cut to fit the transfer cassette and all soaked in transfer buffer.
- The components of the transfer sandwich was assembled as shown below; as each layer was added, great care was taken to remove all air pockets by rolling a clean pipette over the layer.

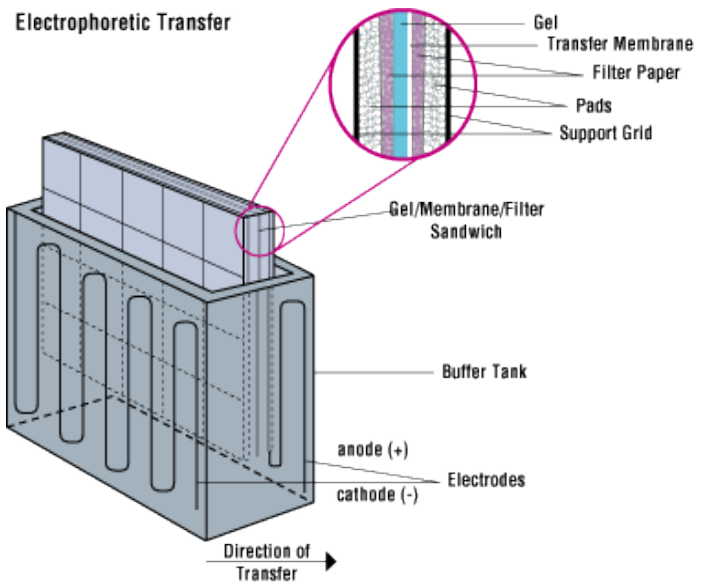


Figure 36: Diagram showing the components of the electrophoretic transfer sandwich used in a Western Blotting protocol

- The tank was filled with transfer buffer, ensuring electrodes were completely covered, the lid put on the transfer unit (black/black and red/red) and power supply connected to run at 30V overnight.
- Once the proteins were transferred onto the nitrocellulose membrane, the membrane was blocked in 5% Marvel (skimmed milk powder) in TBST for at least 1 hour at room temperature.

- The primary antibody was prepared by adding antibody to 5% Marvel in TBST; starting dilution 1:200.
- The primary antibody was applied to the membrane and this was incubated at 4°C overnight.
- The membrane was then washed 4 x 10mins in TBST (on a rocker oscillating at 50 revs/min), while the secondary antibody was prepared (starting dilution 1:2000 in just TBST, i.e 10µl in 20mls of TBST).
- The secondary antibody was applied to the membrane and incubated at room temperature for 1 hour.
- The membrane was washed again 4 x 10mins in TBST.
- The ECL reagent was prepared according to the manufacturers instructions; a 1:1 ratio of solution A and solution B (thus for each blot 0.7mls of solution A to 0.7mls of solution B was prepared).
- The ECL reagent was left on the membrane for 1 minute before taking it to the reading room for the development of the film.

7.10 OT-I proliferation assays

Splenocytes from OT-I mice were pulsed for 1 h at 37 °C with SIINFEKL peptide (2µg/ml) and washed and then were labeled with 5µM CFSE (carboxyfluorescein succinimidyl ester). Sorted $CD11b^{+}Ly6G^{+}$ cells (2×10^{4}) were cultured in 96-well flat-bottomed plates with 2×10^{5} CFSE labeled OT-I splenocytes. Cells were analyzed 4 d later with a FACS Caliber with CellQuest software. Data are presented as the percentage of proliferation of

SIINFEKL-pulsed, CFSE-labeled OT-I splenocytes in the presence of $CD11b^{+}Ly6G^{+}$ cells relative to the proliferation of SIINFEKL pulsed, CFSE-labeled-OT-I splenocytes in the absence of $CD11b^{+}Ly6G^{+}$ cells (set as 100%).

7.11 Mixed Leukocyte Reaction

PBLs (2×10^5) were mixed with allogeneic irradiated (50Gy) DCs (5×10^4) in 200µl of RPMI 5% human AB serum in 96-well flat-bottom plates. Cells were incubated at 37°C, 5% CO2 for 5 days, and then 1µCi/well [^{3}H]thymidine (PerkinElmer) was added for 15–18 hours. [^{3}H]thymidine incorporation was measured using a Wallac MicroBeta JET 1450 reader (PerkinElmer). MDSC-mediated inhibition of lymphocyte proliferation was carried out by co-culturing irradiated SAA-differentiated MDSCs (5×10^5), from either healthy donors, together with PBLs and irradiated DCs. The data are expressed as the percentage of PBL proliferation driven by allogeneic irradiated DCs in the presence of irradiated MDSCs compared with alloreactive PBL proliferation in the absence of MDSCs (100%).

7.12 ELISA.

For measurement of cytokine production, supernatants of MDSCs (or GMPs) co-cultured with BM-derived iNKT cells were collected at different time points. The amount of IL-12p40 and IFN-γ was measured using an ELISA kit (eBioscience).

7.13 Granulocyte-Macrophage Colony-Forming Unit (CFU-GM) Assays

Materials and cytokines for the complete methylcellulose medium are outlined in 6.3 above. Preparation entailed first thawing M-3134 methylcellulose flasks overnight in a refrigerator. A filtered mix of FCS, IMDM, cytokines (as outlined in 6.3.1), glutamine, Penicillin/Streptomycin and mercaptoethanol solution at the volumes and concentrations in table 6.3 was added to a flask containing 40ml of methylcellulose, thereby constituting complete methylcellulose medium.

The media was mixed well by vortexing and then left to stand for 15 minutes in the incubator to allow bubbles to come up to the surface of the media and dissipate. Flasks of complete media were then placed in a 37℃ water bath for ten minutes and then divided into 2.25ml aliquots in falcon tubes. A fresh flask of complete medium was prepared for every colony-forming assay done, as M3134 is not stable at 4℃ and therefore not amenable to repeat freezing and thawing.

Splenocytes and bone marrow cells of sample mice were harvested as described in 7.1.1 and 7.1.4, viable cells counted using trypan blue exclusion dye on a Neubauer haemocytometer, and cell suspensions made up (1.0 x 10^5/ml of splenocytes and 4 x 10^4 of bone marrow cells separately) in RPMI. 250μl of either the splenocyte or bone marrow cell suspensions were added to aliquots of the complete methylcellulose media so as to have final volumes of 2.5mls and either 25,000 splenocytes or 10,000 bone marrow

cells per aliquot. The tubes with media and cells were then vortexed vigorously, left in the incubator for 15 minutes to allow bubbles to come up to the surface of the media and dissipate.

Each 2.5ml methylcellulose-cell suspension was then dispensed as 1ml aliquots into separate 2mm gridded cell culture dishes using a 1ml syringe (a separate sterile new syringe fitted with a needle used for each tube plated). The viscous methylcellulose mixture was spread evenly across the surface of each dish by rotating and tilting the dish. The plates were labelled and then placed inside a plastic humidity culture box within a 37$^{\circ C}$ incubator.

After 7 days of incubation the culture dishes were removed one at a time and placed inside a 60mm dish to score them using an inverted microscope; a colony was considered to be any cluster of fifty or more granulocytes and macrophages.

7.14 Generation of Bone Marrow Chimeric Mice

B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (B6-SJL) mice were irradiated with two doses of 450rads, 3 hours apart at the Biomedical Services Unit, John Radcliffe Hospital, University of Oxford. Mice were then immediately reconstituted with bone marrow cells from donor mice by intravenous injection of 5 x 10^{6} bone marrow cells in an inoculum of 100ml using a 30G needle. Sample mice were reconstituted to have a 50:50 ratio of CD1d^{-}CD45.2: CD1d^{+}CD45.1

hematopoietic cells (as shown in **Figure 11**) while control mice were reconstituted to have a 50:50 ratio of $CD1d^{+}CD45.2$: $CD1d^{+}CD45.1$ hematopoietic cells. The mice were kept in micro-isolator cages with sterile sawdust, food and water while they were allowed to reconstitute for eight weeks. The blood of all animals was thereafter examined for reconstitution by using antibodies and FACS.

APPENDIX

Abbreviations

APC	Antigen presenting cell
ARG	Arginase
BMT	Bone Marrow Transplant
CMP	Common myeloid progenitor

CSF	Colony-stimulating factor
DC	Fluorescent Antibody Cell Sorting
Flu	Influenza A/Puerto Rico/8/34 virus
GalCer	Galactosylceramide
G-CSF	Granulocyte Colony-stimulating factor
GM-CSF	Granulocyte-Macrophage Colony-stimulating factor
GMP	Granulocyte-macrophage progenitor
HIU	Hemagglutinin Units
IFN	Interferon
IL	Interleukin
IMC	Immature myeloid cells
M-CSF	Macrophage Colony-stimulating factor
MDSC	Myeloid-derived Suppressor Cells
MEP	Megakaryocyte-erythrocyte progenitor
MHC	Major Histocompatibility Complex
NOS	Nitric oxide synthase
OSM	Oncostatin M
PBMC	Peripheral Blood Mononuclear Cells
SAA	Serum Amyloid A
STAT	Signal transducer and activator
TCR	T cell receptor
TGF	Transforming Growth Factor
TLR	Toll-like receptor
WT	Wild type

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