B CELLS AS REGULATORS OF NATURAL KILLER CELL IFN-γ PRODUCTION IN MICE

by

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Abstract

Natural killer (NK) cells are a potent source of interferon (IFN)-γ which is required for the protection and clearance of microbial pathogens and tumours. Interleukin (IL)-12 is a cytokine released by dendritic cells (DC), macrophages (Mϕ), and B cells during early pathogenic infection in response to TLR signalling. It is a key inducer of IFN-γ production and is thought to execute this primarily via the stimulation of NK cells. The focus of this thesis was to elucidate the cells involved in regulating mouse NK cell IFN-γ release using stimulation with exogenous or endogenously produced IL-12. Our results show that IL-12 is able to stimulate IFN-γ elaboration primarily from NK cells in cultures of unfractionated splenocytes, yet it is unable to stimulate highly purified NK cells unless exogenous IL-18 is also added. Macrophages, dendritic cells, or NKT cells do not act as partners to co-stimulate NK cell IFN-γ production. Rather, our work uncovers a necessary and sufficient role for B cells in providing stimulatory help for NK cell IFN-γ release during IL-12 stimulation. We show that B cells provide secreted IL-18 and contact dependent factors which remain undetermined. We next used the TLR9 ligand CpG-ODN (unmethylated cytokine-guanine dinucleotides) in order to test the ability of B cells to stimulate NK cell IFN-γ production in the presence of endogenously produced IL-12. While NK cells were again the primary source of IFN-γ in unfractionated splenocyte cultures, their activation was suppressed rather than enhanced by B cells. This suppressive activity was attributed to a small subset of splenic B cells that express CD5 and secrete IL-10 when directly activated with CpG. IL-10 suppressed the production of IFN-γ from NK cells by reducing the levels of IL-12 available for NK cell stimulation, and by also acting on the NK cells directly and blunting their release of IFN-γ. Overall, the research presented herein elucidates a novel role for B cells as both positive and negative regulators of resting NK cell IFN-γ production in mice.

Preface

A version of Chapter 2 has been published. Haddad, E., Senger, L., and Takei, F. (2009) An accessory role for B cells in IL-12 induced activation of resting mouse NK cells. J. Immunology. 183:3608-3615. I designed and conducted the experiments and analyzed the results, and wrote the manuscript. L. Senger conducted the experiment presented in Fig. 9A (right). Dr. F.Takei designed experiments and wrote the manuscript.

I designed and conducted the experiments and analyzed the results for the work presented in Chapter 3. L. Senger designed and conducted experiments and analyzed the results for Fig. 16 A, C, and F. Dr. C. Cohen designed primers and conducted qPCR for Fig. 15B. M. Chow conducted experiments and analyzed results. Dr. F. Takei designed experiments.

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Table of Contents

Abstract ii

Preface iii

List of tables viii

List of figures ix

List of abbreviations x

Acknowledgements xiii

Dedication xv

Chapter 1 Introduction 1

1.1 Natural killer cells 3
1.1.1 NK cell function 5
1.1.2 Cytokine producing NK cells 8
1.1.3 NK cells and IFN-γ 11
1.1.3.1 IFN-γ 11
1.1.4 The regulation of NK cell IFN-γ synthesis 16
1.1.4.1 Soluble signals that enhance NK cell IFN-γ production 18
1.1.4.2 Soluble signals that suppress IFN-γ from NK cells 22
1.1.4.3 Contact dependent factors 24
1.1.5 DCs as partners to NK cell activation 27
1.1.6 Microbial products that enhance NK cell IFN-γ 30

1.2 B Cells 34
1.2.1 B cell subsets 34
1.2.2 B cell function 37
1.2.2.1 Antibody production 37
1.2.2.2 Antigen presentation 40
1.2.2.3 Cytokine production 41
1.2.3 B cell activation 44

1.3 B cell and NK cell interaction 47

1.4 Objective of the thesis 53

Chapter 2 An accessory role for B cells in the IL-12 induced activation of resting mouse NK cells

2. 1 Introduction 56

2.2 Materials and methods 58
2.2.1 Mice 58
2.2.2 Antibodies, cytokines and media 58
2.2.3 Preparation of splenocytes and unfractionated cultures 59
2.2.4 Intracellular cytokine staining 59
2.2.5 Cell depletion experiments 59
2.2.6 DC isolation and co-culture 60
2.2.7 B and NK cell co-cultures 60
2.2.8 In vivo IL-12 injection 61
2.2.9 IFN-γ and IL-18 ELISA 61
2.2.10 Statistical analysis 61
2.3 Results 62
2.3.1 $NK1.1^+$ cells are the primary producers of IFN-γ among IL-12 stimulated unfractionated spleen cells 62
2.3.2 Purified NK cells are not stimulated with IL-12 alone 65
2.3.3 DC and macrophages are not required for the stimulation of NK cells by IL-12 67
2.3.4 B cells are required and sufficient for IFN-γ induction from IL-12-stimulated NK cells 69
2.3.5 B cell provide endogenous IL-18 and cell contact in NK cell stimulation by IL-12 71
2.3.6 B cell KO mice injected with IL-12 produce lower levels of IFN-γ and IL-18 than WT 73
2.4 Discussion 74
Chapter 3 IL-10 secreting B cells suppress CpG ODN induced NK cell activation 79
3.1 Introduction 80
3.2 Materials and methods 82
3.2.1 Mice 82
3.2.2 Antibodies and reagents 82
3.2.3 CpG ODN stimulation of unfractionated splenocyte 83
3.2.4 B cell isolation and depletion 83
3.2.5 RNA extraction, reverse transcription and quantitative PCR 84
3.2.6 NK cell purification 85
3.2.7 Cytokine ELISA 85
3.2.8 Statistical analysis 85
3.3 Results 86
3.3.1 $NK1.1^+$ cells are the primary source of IFN-γ in CpG ODN stimulated unfractionated splenocytes 86

3.3.2 CpG-2216 stimulation of B cell deficient spleen cells induces more IFN-γ^+ NK cells and higher levels of IFN-γ secretion 88
3.3.3 B cells suppress NK cell production of IFNγ induced by CpG-2216 89
3.3.4 IL-10 inhibits CpG-2216 induced IFN-γ production 90
3.3.5 $CD5^+$B cells produce IL-10 in response to CpG-2216 stimulation 92
3.3.6 IL-10 directly and indirectly inhibits IFN-γ production by NK cells 94
3.4 Discussion 98
Chapter 4 General summary and discussion 104
4.1 Summary 106
4.2 Significance of the work 107
4.3 Strength and limitations 107
4.4 Future directions 109
4.4.1 B cell subsets 110
4.4.2 B-NK cell contact 110
4.4.3 NK cell activation and suppression 111
References 116
Appendices 162
Appendix 1 Populations within spleen cells that are IFN-γ^+ after 48 or 72 h of stimulation with IL-12 163
Appendix 2 $CD11c^+$ NK cells are the main producers of IFN-γ among IL-12 stimulated NK cells 164
Appendix 3 Percentage of NK cells found in WT and BKO non-infected mice 165
Appendix 4 B cells do not promote NK cell cytotoxicity against YAC-1 cells in IL-12 stimulated splenocytes 166
Appendix 5 The concentration of IL-18 required to stimulated IFN-γ from IL-12 activated NK cells is below that of ELISA detection limits of IL-18 in supernatants 167
Appendix 6 IL-18Rα is not needed for NK cell stimulation by B cells during IL-12 activation 168
Appendix 7 CpG ODN 1826 is a poor stimulator of WT IFN-γ even in the presence of IL-10 neutralization 169
Appendix 8 CpG ODN 2216 induces the production of IL-10 from WT and BKO spleen cells 170
Appendix 9 B cells secrete IL-10 in response to CpG ODN 2216 and 1826 171

Appendix 10 Strain comparisons of IFN-γ production after CpG ODN 2216 in the absence of IL-10 and/or IDO activity 172

Appendix 11 WT and BKO mice differ in the response to HSV-1 infection with respect to draining lymph node lymphocyte numbers and NK and T cell activation status 174

List of tables

Table 1 TLRs common to human and mouse DC 28
Table 2 Role of DC and M in the NK cell response to various pathogens..... 30
Table 3 B cell subsets and functions..... 37
Table 4 Antibody class roles and triggers for Ig-class switching..... 39

List of figures

Figure 1 IFN-γ signalling impacts both innate and adaptive immunity 15
Figure 2 Summary of signals that enhance NK cell IFN-γ production 33
Figure 3 Cytokine secreting subsets of B cells 44
Figure 4 TD and TI antigen activation of B cells 47
Figure 5 IL-12 stimulates IFN-γ production mainly from NK1.1$^+$ cells in unfractionated spleen 65
Figure 6 NK1.1$^+$ T cells are not required for IL-12 induced IFN-γ production by unfractionated splenocytes 66
Figure 7 DC and Mϕ are not required for IL-12 induced IFN-γ production by unfractionated splenocytes 68
Figure 8 B cells are required for IFN-γ from IL-12 treated unfractionated splenocytes and purified NK cells 70
Figure 9 B cells provide IL-18 and contact dependent NK cell stimulatory signals during IL-12 activation 73
Figure 10 B cells are required for optimal IFN-γ and IL-18 production in vivo induced by IL-12 injection 74
Figure 11 CpG ODN induces IFN-γ primarily from NK1.1$^+$ cells in unfractionated spleen 87
Figure 12 CpG ODN induces more IFN-γ production by B cell deficient splenocytes than WT splenocytes 89
Figure 13 B cells supress CpG ODN induced NK cell production of IFN-γ 90
Figure 14 IL-10 is involved in the suppression of CpG ODN induced IFN-γ production 92
Figure 15 B cells are a source of CpG ODN induced IL-10 94
Figure 16 IL-10 suppresses NK cell production of IFN-γ in a two pronged manner 97
Figure 17 B cells act as enhancers or suppressors of NK cell IFN-γ in response to IL-12 115

List of abbreviations

ADCC	antibody dependent cell mediated cytotoxicity
APC	antigen presenting cell
B10	IL-10 producing B cells
BCG	Bacillus Calmette Guerin
BCR	B cell receptor
Be	B effector cell
COX-2	Cyclo-oxygenase
CpG ODN	cytosine phosphate guanine oligonucleotide
CTL	cytotoxic T cell
DAP	DNAX-activating protein
DC	dendritic cell
EAE	experimental autoimmune encephalitis
EBV	Epstein Barr virus
ELISA	enzyme linked immunsorbent assay
FACS	florescence activated cell sorting
FcR	receptor for Fc portion of antibodies
FDC	follicular dendritic cell
GC	germinal center
GM-CSF	granulocyte macrophage cell stimulating factor
HER2	human epidermal growth factor-2
HSV	Herpes simplex virus
i.p.	intraperitoneal injection
IBD	inflammatory bowel disease
ICAM	intracellular cell adhesion molecule
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IFNGR	IFN-γ receptor
Ig	immunoglobulin
IL	interleukin
IL-12R	IL-12 receptor
IL-18R	IL-18 receptor
ITAM/ITIM	immunoreceptor activating/inhibitory motifs
JAK	Janus activated kinase
KIR	killer cell immunoglobulin like receptor
KLRG1	killer cell lectin like receptor subfamily G, member 1
KO	knock out
LFA	lymphocyte function associated antigen
LN	lymph node
LPS	lipopolysaccharide
LT	lymphotoxin

Ly49	lymphocyte antigen 49 complex
Mϕ	macrophage
CMV/MCMV	cytomegalovirus/murine cytomegalovirus
MHC	major histocompatbility complex
MICA	MHC class I polypeptide-related sequence A
MS	multiple sclerosis
MULT-1	murine ULBP-like transcript 1
MyD88	Myeloid differentiation primary response gene 88
MZ	marginal zone
N-CAM	neural cell adhesion molecule
NCR	natural cytotoxicity receptor
ND	not determined
NK	natural killer
NKG2D	NK group 2 member D
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PHA	phytohemagluttinin
PI-3 kinase	phosphoinositide 3-kinase
PMA/Iono	phorbol 12-myristate 13-acetate/ionomycin
Poly IC	Polyinosinic:polycytidylic acid
q-PCR	quantitative PCR
RA	rheumatoid arthritis
Rae-1	retinoic acid early inducible gene-1
RPMI	Roswell Park Memorial Institute
SAP	slam-associated protein
SCID	severe combined immunodeficiency
SHP	Src homology 2-containing phosphatase
SLAM	signaling lymphocyte activation molecule
SLE	systemic lupus erythematosus
SOCS	suppressor of cytokine signaling
STAT	signal transducers and activator of transcription
TCR	T cell receptor
TD	T cell dependent antigen
TGF-β	transforming growth factor-β
Th	T helper
TI	T cell independent antigen
TLR	toll like receptor
TNF-a	tumour necrosis factor
Treg	regulatory T cells
TTP	tristatetraprolin
ULBP	UL-16 binding protein
WT	wild-type

ZAP-70	ζ-chain associated protein kinase 70

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Chapter 1 Introduction

The immune response is the body's key mechanism in keeping pathogens at bay. The initial and prompt defensive activity of mounting an inflammatory response against an invading pathogen and limiting its spread falls within the domain of innate immunity. This evolutionary ancient arm of host defence relies on germ-line encoded receptors that recognize broadly conserved signatures associated with most microbial pathogens. In vertebrates a second type of immune response results in the production of antibodies and the development of long lasting immune protection and memory to the invader. Known as adaptive immunity, it makes use of receptors which undergo variable rearrangements to allow for a more specific and diverse recognition of foreign antigens. The interaction between the two arms of the immune response is necessary for effective immunity and is highly dependent upon cellular communication. This communication is achieved through the expression of cell-surface ligands, and through the release of, and response to, soluble immune modulating molecules like chemokines and cytokines.

Interferon-γ (IFN-γ) is an inflammatory cytokine that is secreted during both the innate and adaptive immune response. While IFN-γ is required for the clearance and control of intracellular microbial pathogens and tumours, if left unchecked, its increasing levels can lead to chronic inflammation resulting in disease rather than protection. Exquisite regulatory processes must be put in place by the immune system and its players to ensure that the IFN-γ response is appropriate.

This thesis focuses on the regulatory processes that influence the production and release of IFN-γ from innate lymphocytes known as 'natural killer' (NK) cells which are the principle source of IFN-γ during the very early immune response.

1.1 Natural killer cells

In 1975 a population of lymphocytes capable of destroying cultured leukemia cells was isolated from the spleens of adult mice (Kiessling, Klein, Pross, & Wigzell, 1975). The rapid kinetics and seemingly 'spontaneous' or 'natural' cytotoxicity they displayed against their targets inspired researchers to term these cells natural killers (NK). We now know that besides being potent killers, NK cells are also powerful producers of immune modulating cytokines and have been shown to play a crucial role in the host defence response against infections with viral, bacterial, fungal, and parasitic species, and also in the control and clearance of tumours. While their effector functions may overlap with those of the cytokine producing $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T (CTLs) cells, NK cells are very early acting cells and flex their functional muscles during the innate phase of the immune response, long before T cells are fully activated.

The earliest adult NK progenitors arise from bone marrow stem cells and develop into functionally mature NK cells in the various tissues in which they reside (Yokoyama, Kim, & French, 2004). In humans, mature NK cells have been described in peripheral blood, lymph node, and uterus, as well as in various fetal tissues (Farag & Caligiuri, 2006). Studies with mice, which do not have the limits upon tissue availability that human studies do, have shown that they are primarily found in the blood, spleen, and liver, and also detected in the uterus, bone marrow, lung, lymph nodes, and thymus. At steady state, the spleen is the largest reservoir of mouse NK cells (Gregoire et al., 2008).

Peripheral blood NK cells can be identified morphologically as large lymphocytes, and in humans contain pre-formed granular proteins associated with cytotoxicity (Fehniger et al.,

1999; Ma et al., 2004). This is not seen in human T cells which require activation to assemble granules, and only observed in mouse NK cells after they have been primed or fully activated. Flow cytometry can be used to identify NK cells based on their expression of surface proteins. CD56 is used to identify human NK cells (human isoform of N-CAM), while in mice NK cells are identified using either NK1.1 (NKR-P1, killer cell associated c-type lectin like receptor) or DX5 (CD49b), the α2-integrin. Whereas DX5 marks NK cells found in all inbred laboratory mouse strains, NK1.1 is specific to some mouse strains, such as C56BL/6 and New Zealand Black mice. All mature NK cells, regardless of their source, are negative for the surface expression of CD3, a component of the T cell receptor complex (TCR). Lymphocytes found in humans and mice that co-express the classical NK cell markers (CD56 or NK1.1/DX5) along with CD3 are termed NKT cells and can be found wherever NK cells exist but generally at lower frequencies per tissue. NKT cells are functionally similar to NK cells in that they are an early source of cytokines, like IFN-γ and IL-4, but are thought to belong to the T cell lineage (Godfrey, Stankovic, & Baxter, 2010). Recent work by Walzer et al. (Walzer, Blery, Chaix, Fuseri, Chasson, Robbins, Jaeger, Andre, Gauthier, Daniel, Chemin, Morel, Dalod, Imbert, Pierres, Moretta, Romagne, & Vivier, 2007a) has proposed the use of NKp46, a member of the highly conserved natural cytotoxicity receptor (NCR) family of NK-activating receptors, as an NK cell specific marker. It is however expressed by a very small subset of human and mouse T lymphocytes, including NKT cells (Caligiuri, 2008).

Human and mouse NK cells can also be identified by the surface expression of specialized triggering receptors for MHC class I and –class I related molecules or viral proteins. In humans these include the KIR (killer immunoglobulin like) receptors, and the C-type lectin Ly49 (lymphocyte antigen 49 complex) family in mice. In mice, ligation of the inhibitory Ly49

A, C, E, and I receptors triggers their cytoplasmic ITIM (immunoreceptor tyrosine inhibitory motifs) and triggers the inhibition of NK cell function. The activating Ly49 receptors (D and H) do not contain activating motifs themselves but can associate with the ITAM containing DAP12 protein. The CD94/NKG2A, and the activating CD94/NKG2C and NKG2D molecules are common between mice and human NK cells (Lanier, 2008a; A. Moretta et al., 2001).

1.1.1 NK cell function

NK cells are dual effector cells, in that they can effectively destroy target cells through cytotoxic programs, as well as rapidly secrete immune modulating cytokines. Mature NK cells constantly monitor the tissues through which they circulate or reside in for the presence of self-cells that have either become infected with viruses and intracellular bacteria, or have undergone a malignant transformation. These aberrant cells express stress-related (MICA/B on human cells, and Rae-1 and H60 on mouse cells) or pathogen associated proteins which NK cells detect via their activating receptors. NK cell activating receptors lack intrinsic signalling activity and need to couple with ITAM bearing adaptor proteins such as the FcεR1γ and DAP12 proteins found in NK cells. In mice Ly49D, Ly49H, and NKG2D activating receptors are associated with DAP12. Receptor engagement leads to the phosphorylation of the ITAM tyrosines, most likely by the Src family kinases, which in turn results in the recruitment of Zap-70 or Syk signalling kinases (Lanier, 2008a). Mouse, but not human, NKG2D can also associate with DAP10 another adaptor molecule that can induce cytotoxicity in a Syk-independent but PI-3 kinase dependent pathway (Zompi et al., 2003).

NK cells also gauge the interacting cell for its levels of surface major histocompatibility class I (MHC class 1) proteins. ITIM containing inhibitory receptors, such as mouse NK cell

Ly49s A, C, G and I, are specific to MHC class I and MHC class 1 like proteins, and when engaged, prevent NK cell activation (Lanier, 2008a). ITIM tyrosine phosphorylation after receptor engagement recruits SHP-1/2 (SH2 domain containing) phosphatases which accumulate near the interface of the NK cell and its target, and de-phosphorylate key signalling molecules, leading to the termination of Ca^{2}+ influx, granzyme/perforin degranulation, cell proliferation, and cytokine release. Whether or not an NK cell kills depends on the relative strength of the activating and inhibitory signals that they receive (Lanier, 2008a).

NK cells are more than just killers. Once activated, they are a quick and robust source of an ever-broadening array of cytokines. To date they have been shown to produce pro-inflammatory cytokines such as IFN-γ, TNF-α, GM-CSF (granulocyte-macrophage colony stimulating factor), lymphotoxin (LT), and IL-8 (Caligiuri, 2008). The production of such cytokines, notably IFN-γ, is as important as NK cell cytotoxicity, and arguably more so during early infection. NK cells have also been shown to secrete anti-inflammatory cytokines such as IL-4, IL-5, IL-10, IL-13 and IL-22 (Babu, Blauvelt, & Nutman, 2007; Satoh-Takayama et al., 2008){{}}. Overall, through their release of varied cytokines NK cells have the ability to strengthen and shape the innate immune response and drive and define the specialized adaptive responses of T and B cells. The fact that there are no reports of constitutive cytokine production from NK cells suggests that this function, like cytotoxicity, must also be under tight regulation.

Natural NK cell deficiencies in humans have been described and have highlighted an in vivo role for NK cells in the protection against viruses from the Herpes family, and mycobacterial infections (Biron, Nguyen, Pien, Cousens, & Salazar-Mather, 1999a). In mice the majority of studies showing the role of NK cells against pathogenic infection as well as tumours

have been shown with in vitro studies. Most in vivo studies have relied upon antibodies to eliminate NK cells. Anti-asialo GM1 mAb is commonly used to deplete NK cells in mice; it binds to a glycolipid that is found on NK cells but is also present on monocytes and Mϕ. The anti-NK1.1 mAb is also used to deplete NK cells in C57Bl/6 mice but may simultaneously eliminate NKT cells, as they also express NK1.1. While such mAb treated mice have presented with disseminated bacterial infection, problems controlling infections with certain virus (Biron, Nguyen, Pien, Cousens, & Salazar-Mather, 1999a), and difficulty inducing Th1 polarized adaptive responses, the concomitant loss of other cell types makes it difficult to cleanly define NK cell specific effects in vivo. The primary obstacle to in vivo mouse work has been the lack of a uniquely NK cell deficient animal. Kim et al. (Kim, Iizuka, Aguila, Weissman, & Yokoyama, 2000) produced transgenic mice that expressed Ly49A cDNA under the control of the mouse granzyme A (protein associated with cytotoxicity in NK and CTLs) genomic sequence derived by pronuclear injection of C57BL/6 fertilized eggs. One founder transgenic mouse had Ly49A on all NK cells but had a marked reduction specifically in $NK1.1^{+}$ $CD3^{-}$ (NK) cells. The NK cells found in these mice had specific deficits with splenic NK cells unable to kill typical NK cell sensitive targets such as YAC-1, and RMA-S cells in vitro. These mice were used to examine the role of NK cell mediated killing in vivo against typical mouse tumour models (B16) and found similar defects with observed uncontrolled tumour metastasis and outgrowth when compared to mice in which the NK compartment was unaltered. IFN-γ production in response to in vivo injection of LPS was reduced but not completely lost. Their subsequent study (Kim et al., 2006) showed that the reduction of NK cells in these mice was attributed to a defect in a ubiquitous transcription factor (ATF-2). The continued search for a selective in vivo means of eliminating NK cells led to a study by Walzer and colleagues (Walzer, Blery, Chaix, Fuseri, Chasson, Robbins, Jaeger, Andre, Gauthier, Daniel, Chemin, Morel, Dalod,

Imbert, Pierres, Moretta, Romagne, & Vivier, 2007a) who reported that NKp46 was the unifying NK cell marker across species. They identified a functional NKp46 promoter which they used to drive the expression of the human diphtheria toxin (DT) receptor and found that subsequent DT injection led to a complete and selective loss of NK cells (but not $NKp46^{+}$ T cells which express low levels of NKp46) which was shown to persist for at least seven days. Splenocytes from these mice were unable to destroy NK cell sensitive YAC-1 target cells compared to those from WT mice. A 2009 study by Gascoyne et al. (Gascoyne et al., 2009) has shown that another transcription factor, E4BP4 is essential to NK cell development and its loss results in mice with severe impairments in NK cell mediated cytotoxicity. The use of the E4BP4 or NKp46 deficient mice to reveal the in vivo role of NK cells in disease is yet to be reported.

1.1.2 Cytokine producing NK cells

NK cell subsets with a greater propensity/efficiency for one function over the other exist in humans and mice (Cooper et al., 2001; Hayakawa, Huntington, Nutt, & Smyth, 2006). CD56 surface expression distinguishes two functional subsets of human NK cells in peripheral blood, lymph node, and placenta; other tissues have not been investigated. $CD56^{dim}$ NK cells have a high expression of cytotoxic granules as well as CD16, the low affinity FcγRIII which binds to the constant portion of immunoglobulin (Ig, antibodies) and mediates antibody dependent cell-mediated cytotoxicity (ADCC); thus these cells are highly cytotoxic but have been shown to be inefficient cytokine producers (Batoni et al., 2005). In contrast $CD56^{bright}$ NK cells are potent producers of type 1 (IFN-γ, TNF-β, GM-CSF) and type 2 cytokines (IL-10, IL-13) after stimulation with cytokines, microbial pathogens, or Toll like receptor (TLR) ligands (Batoni et al., 2005; Cooper et al., 2001) and are enriched in secondary lymphoid organs (Ferlazzo et al., 2004). They are highly migratory cells owing to their expression of CXCR3 and CCR7 but they

are poor cytotoxic cells and have low levels of granzyme/perforin proteins, which Chan et al. have attributed to their less mature developmental status when residing in secondary lymphoid tissues (A. Chan et al., 2007).

Hayakawa and Smyth were the first to show that mouse NK cells, like their human counterparts, are also functionally heterogeneous. They found CD27, a TNF receptor family member, could be used to dissect mature naive mouse NK cells into high and low expressing subsets with distinct functions (Hayakawa et al., 2006). $CD27^{low}$ NK cells were found to possess an overall higher threshold for activation of both cytokine production and cytotoxicity, which has been attributed to a higher frequency of cells expressing inhibitory Ly49C/I (recognizing self-MHC class I molecules in C57BL/6 mice) and killer cell lectin-like receptor G1 (KLRG1) (Hayakawa & Smyth, 2006). In contrast $CD27^{high}$ cells were much better killers of target cells, and potently secreted IFN-γ in response to high dose IL-12 or IL-18 alone or in combination, while $CD27^{low}$ NK cells only responded to the combination dose and secreted modest levels of IFN-γ. Additionally, $CD27^{high}$ cells had a greater ability to produce IFN-γ in response to DC (Hayakawa & Smyth, 2006)and like $CD56^{bright}$ NK cells, were also enriched in secondary lymphoid organs (spleen, liver, and lymph node).

Cytokine producing $CD4^{+}$ T helper (Th) cells separate into type 1 (Th1) cells that primarily secrete IFN-γ and are involved in cell mediated inflammatory reactions, and type 2 (Th2) cells which secrete IL-4, IL-5, and IL-13 and encourage antibody production and are associated with allergic responses (T. R. Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986; T. R. Mosmann & Sad, 1996). The differentiation of these two subtypes depends on cytokines; IL-12 secreted from APCs required for Th1 development, and IL-4 needed for Th2

development, with each cytokine activating specific transcription factors in the T cells. Furthermore, Th1 cells are able to respond to IL-12 in the local microenvironment as they express a functional IL-12 receptor whereas the Th2 subset does not (Rogge et al., 1999). The Th1/Th2 paradigm has since been applied to DC, NKT cells (Colonna, 2001), and recently B cells (T. Mosmann, 2000). Peritt et al. (Peritt et al., 1998) were the first to apply the paradigm to NK cells and demonstrated that human NK cells, isolated from peripheral blood, cultured in the presence of IL-12 produced IL-10 and IFN-γ (NK1), while those cultured in the presence of IL-4 produced IL-5 and IL-13 (NK2). A similar distinction has also been made for cytokine producing NK cells in mice where NK1 cells that were differentiated with IL-12/IL-2 produced high levels of IFN-γ with hyperacteylation of the IFN-γ promoter locus, while NK2 cells that were differentiated with IL-2/IL-4/anti-IFN-γ mAb produced IL-13 and IL-5 in a STAT6 dependent manner (Kimura & Nakayama, 2005). Chakir et al. (Chakir, Camilucci, Filion, & Webb, 2000) showed that mouse NK cells cultured with IL-2 differentiate into cells that express either a high or low level of the β-subunit of the IL-12 receptor and are skewed towards IFN-γ production. These cells did not however express mRNA for NK2 associated cytokines, like IL-5 and IL-13.

Finally, mouse secondary lymphoid organs possess a subset of NK cells known as NKDC that express $CD11c^+$, the classical surface marker for DC. NKDC ($CD11c^+NK1.1^+$) or IKDC ($CD11c^+NK1.1^+B220^+$) were initially reported to be functional and phenotypic hybrids between NK and DC cells. While they possessed the NK like characteristics of IFN-γ secretion and were cytotoxic against NK cell sensitive targets, they also resembled DC in their ability to present antigens to T cells in the context of MHC class II (Shortman & Villadangos, 2006). Later reports documented these cells to be developmentally related to NK cells and not DC, and

to actually represent a pool of activated NK cells which have upregulated CD11c (Vosshenrich, Lesjean-Pottier, Hasan, Richard-Le Goff, Corcuff, Mandelboim, & Di Santo, 2007). Importantly, the antigen presenting ability of these NKDC/IKDCs noted initially could be attributed to contaminating DC cells which were typically associated with isolation (Caminschi et al., 2007; Vosshenrich, Lesjean-Pottier, Hasan, Richard-Le Goff, Corcuff, Mandelboim, & Di Santo, 2007). In terms of cytokine production by the NK cell compartment the NKDC/IKDC subset has been demonstrated to be an early and important source of IFN-γ. Their IFN-γ release occurs during the immune response to intracellular bacteria such as *Listeria* and *Leishmania* (Plitas, Chaudhry, Kingham, Raab, & DeMatteo, 2007; Vosshenrich, Lesjean-Pottier, Hasan, Richard-Le Goff, Corcuff, Mandelboim, & Di Santo et al., 2007), and can also be induced by bacterial cell components such as CpG ODN, which is a synthetic mimic of bacterial DNA (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006b; Pillarisetty, Katz, Bleier, Shah, & Dematteo, 2005; Vremec et al., 2007).

1.1.3 NK cells and IFN-γ

1.1.3.1 IFN-γ

Interferons were initially described as secreted proteins capable of interfering with viral replication and protecting cells against viral infection (Watanabe, 2004). The interferon family of proteins is divided into three classes based on criteria such as gene and protein structure, biochemical properties, and cellular source. The Type I IFNs consists of the IFN-α and IFN-β proteins secreted largely by leukocytes and fibroblasts, respectively, in response to viral infection; a subset of DCs known as plasmacytoid DCs (pDCs) have also been shown to be a potent source of IFN-α during viral infection. With respect to NK cells, Type I IFNs are important stimulators of NK cell cytotoxicity against virally infected cells. The Type II IFN

class consists solely of IFN-γ, which was initially termed 'immune IFN' as it could be induced by immune and inflammatory stimuli as well as mitogens such as PHA (Boehm, Klamp, Groot, & Howard, 1997). Recently, IFN-λ proteins have also been described and have been classed as the Type III IFNs (Kotenko et al., 2003).

IFN-γ is primarily produced by activated CD4 T, CD8 T, γδ T cells, NKT, and NK cells. While the T cell production of IFN-γ is delayed, roughly 3-5 days after initial infection or stimulation, NK cell IFN-γ production can occur within the first twelve hours and it peaks within the first 48 hours. SCID (severe immune compromised disease) mice and humans lack all T (including NKT cells) and B cells, and thus are incapable of mounting an adaptive immune response against microbial infection and establishing immunity against pathogen re-challenge. Yet these mice possess an effective early innate response and are able to secrete IFN-γ owing to their intact NK cell compartment (Dorshkind, Pollack, Bosma, & Phillips, 1985).

There are reports which suggest that APCs (Frucht et al., 2001; Munder, Mallo, Eichmann, & Modolell, 1998), including B cells (Harris, Goodrich, Gerth, Peng, & Lund, 2005), may also represent important sources of IFN-γ after stimulation. These findings were reported prior to the identification of the NKDC/IKDC subset that is found within preparations of Mϕ and DCs. Indeed, Schleicher et al. (Schleicher, Hesse, & Bogdan, 2005) show that even small numbers of $CD11c^{+}/CD11b^{+}$ expressing NK cells found in bone marrow derived Mϕ cultures are the predominant source of IFN-γ. While a physiological role for NK cells as the primary producers of IFN-γ has been shown in vivo and in vitro, the same cannot be said for DC, Mϕ, or B cells (Thale & Kiderlen, 2005).

Biologically active IFN-γ is a homodimer that signals by binding to its IFN-γ receptor (IFNGR) which is composed of two ligand binding IFNGR1 (α) chains associated with two signal-transducing IFNGR2 (β) chains (Schroder, Hertzog, Ravasi, & Hume, 2004). The following activation of the Janus Kinases (JAK1 and JAK2) leads to the phosphorylation of STAT1 homodimers which transduce the signal to the nucleus, bind IFN-γ-activation site (GAS) elements, and ultimately lead to the expression of over 200 IFN-γ regulated genes (Boehm et al., 1997). The majority of these gene products are involved in establishing and maintaining a protective inflammatory innate response and inducing a downstream Th1 polarized adaptive response. IFN-γ is a multi-tasking inflammatory cytokine with pleiotropic effects on both innate and adaptive immune cells (Figure 1). It has been demonstrated to act as an enhancer of APC function and maturation via the stimulation of phagocytosis and the upregulation of the MHC class I and II proteins needed for antigen presentation, a promoter of the anti-microbial oxidative burst in macrophages, a promoter of Th1 cell differentiation and maturation, stimulator of cytotoxic $CD8^+$ T (CTL) cell activity, driver of B cell antibody differentiation and release, and initiator of signalling cascades that lead to the release of various cytokines and chemokines. A role for IFN-γ has also been demonstrated in the prevention, destruction, and clearance of various tumours through its induction of the caspases that drive apoptosis, its anti-angiogenic properties, and its stimulation of CTL and NK cell mediated cytotoxicity against transformed cells. Inborn or experimentally generated deficiencies in IFN-γ protein or in its receptor do not lead to any outright developmental problems, but humans/mice with such defects suffer from recurrent and systemic bacterial infection, and specifically have trouble preventing and clearing mycobacterial species such as *M. tuberculosis*, and the usually harmless *M. bovis* (Biron, Nguyen, Pien, Cousens, & Salazar-Mather, 1999b). As an example, the Bacille

Calmette- Guerin (BCG) tuberculosis vaccine is prepared from live but attenuated *M. bovis* and commonly administered with very little side-effects, but in infants and young children with IFN-γ deficiencies this vaccine leads to disseminated mycobacterial infection, full-fledged tuberculosis, and in many cases death (Jouanguy et al., 1997).

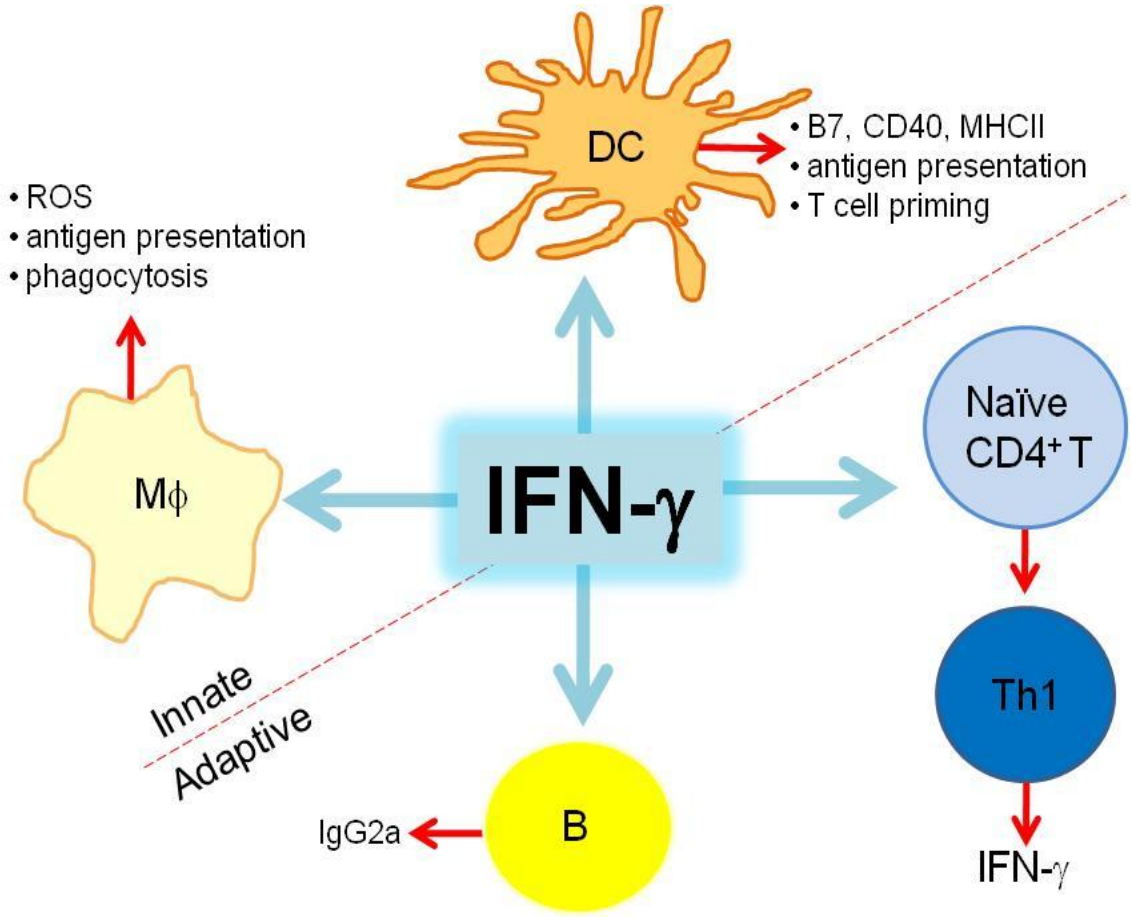

Figure 1 IFN-γ signalling impacts both innate and adaptive immunity

IFN-γ promotes the production of reactive oxygen (ROS) and nitrogen species from monocytes/Mϕ that help the cell clear intracellular pathogens. In both DC and Mϕ IFN-γ leads to the upregulation of CD40, CD80 and CD86, and MHC II which along with an enhanced uptake of potential antigens makes these cells excellent partners for antigen-specific T cell priming and activation. The differentiation of naive T cells to more a polarized pro-inflammatory inducing Th1 cell is also driven by IFN-γ. It plays a role in the maturation and differentiation of B cells as they not only upregulate costimulatory markers for better T cell interaction, but also undergo isotype switching of their Ig proteins which leads to the release of higher affinity Ig with specific functions. IFN-γ favours IgG2a release, this antibody is a good activator of complement, phagocytosis, and ADCC and helps clear intracellularly replicating pathogens.

The unregulated production of IFN-γ can lead to chronic inflammation and irreparable tissue damage over time. Increased levels of IFN-γ have been implicated in various pathologies including autoimmune disorders like systemic lupus erythematosus (SLE) (Viallard et al., 1999), Sjogren's Syndrome, multiple sclerosis (MS) and its animal model EAE (experimental autoimmune encephalitis) (Theofilopoulos, Baccala, Beutler, & Kono, 2005), and myasthenia gravis (Balashov, Olek, Smith, Khoury, & Weiner, 1998), as well as in rheumatoid arthritis (RA) (Lamacchia, Palmer, Seemayer, Talabot-Ayer, & Gabay, 2010)(Theofilopoulos et al., 2005), allergy and asthma (Li et al., 2010), and Crohn's disease/inflammatory bowel disease (IBD) (Abraham & Cho, 2009). Furthermore, while the localized production of IFN-γ at sites of active inflammation helps to prevent the dissemination of pathogens, if there is a systemic exposure to pathogens or their products it leads to septic shock which is attributed to multi-organ failure and a global cytokine storm in part driven by IFN-γ (Heinzel, 1990). Therefore it is crucial that the levels of IFN-γ in the host at any time should be fine-tuned and controlled.

1.1.4 The regulation of NK cell IFN-γ synthesis

Both cytokine production in NK cells and cytotoxicity can be regulated through signals coming to the cell via ITIM-containing or ITAM-associated receptor engagement and/or cytokine signaling; however, the two NK cell effector programs are not mutually inclusive. For example, while IL-2 is a potent inducer of NK cell proliferation and lytic activity, on its own it is not an optimal signal for IFN-γ secretion. IL-2 does however upregulate IFN-γ mRNA and may prime the NK cell for cytokine production after it receives other activating signals. Signalling through the CD45 (Hesslein, Takaki, Hermiston, Weiss, & Lanier, 2006) and CD72 (Alcon, Luther, Balce, & Takei, 2009) proteins found on NK specifically initiates cytokine production rather than cytotoxicity.

The Src kinase activity that is required for ITAM-based signaling is partly controlled by CD45. This protein tyrosine phosphatase generates a pool of primed Src family kinases within the NK cell that appears to be crucial for ITAM-based cytokine and chemokine production but not cytotoxicity. This was shown by Lanier and colleagues (Hesslein et al., 2006) who found that NK cells from CD45 deficient mice presented with dramatically abrogated IFN-γ and MIP-1β (macrophage inflammatory protein) during in vitro stimulation with NKG2D, Ly49D, CD16 or NK1.1 (all ITAM-based signaling) cross-linking. Yet the lytic ability, via NKG2D engagement (m157, or Rae-1 expressed on mouse Ba/F3 cells), of these CD45 deficient NK cells was comparable to WT NK cells.

CD72 is an ITIM containing C-type lectin typically associated with the negative regulation of B cell receptor signaling. Work from our lab has shown that mouse splenic NK cells expressing high levels of CD72 ($CD72^{hi}$) are fully cytotoxic, but are low producers of IFN-γ when stimulated with IL-12/IL-18. Conversely, $CD72^{lo}$ NK cells were normal with respect to their release of IFN-γ (Alcon et al., 2009).

Taken together, the above results show that NK cell function can be skewed to cytokine production rather than cytotoxicity if the right receptors are engaged.

The concept of NK cells as perpetually trigger happy cells, ready to assert their functional programs with very little guidance, was challenged with reports that showed that NK cells were only able to respond to microbial pathogens or their products when in unfractionated preparations of secondary lymphoid tissue. In the absence of activating signals NK cells are

resting cells and do not express IFN-γ mRNA, nor constitutively release the protein. We know now that NK cells require the integration of soluble (indirect) signals and contact dependent (direct) signals provided by other immune cells, specifically APCs (Newman & Riley, 2007) for optimal functional responses, including IFN-γ release.

1.1.4.1 Soluble signals that enhance NK cell IFN-γ production

1.1.4.1.1 IL-2/IL-15

IL-2 and IL-15 have several similar functions on account of their shared receptor components and common use of signaling molecules. Both cytokines use a heterotrimeric receptor composed of the shared IL-2/15Rβ (CD122) and γc subunits, and a third unique α (CD25) subunit. IL-2 is a soluble cytokine and binds a pre-formed trimeric receptor; IL-15 is a membrane associated cytokine and when bound on the IL-15Rα subunit of DC is presented in trans to the IL-2/15Rβ-γc dimeric receptor on the NK (Koka et al., 2004). The use of IL-2/IL-15 facilitates NK cell differentiation and promotes mature NK cell homeostasis in the periphery (Becknell & Caligiuri, 2005). NK cells are routinely propagated in IL-2/IL-15, and are fully activated with respect to cytotoxicity against target cells but are only primed with respect to cytokine production. Purified NK cells cultured in IL-2 have increased IFN-γ mRNA and only release appreciable amounts of IFN-γ if other stimulatory signals are present, for instance cytokines like IL-12 or IL-18, or signaling through any of the activating receptors (CD16, Ly49/H in mice, NKG2D) (Ortaldo & Young, 2006; Ortaldo et al., 2006). Likewise IL-15 has only been shown to induce IFN-γ from NK cells in the presence of IL-12 (Carson, Ross et al., 1995), a combination which can override the experimental cross-linking of Ly49G2 inhibitory receptor signalling that would otherwise prevent NK cell activation (Ortaldo & Young, 2003).

1.1.4.1.2 IL-12

IL-12 is the master pro-inflammatory cytokine and primary inducer of IFN-γ production from activated T and NK cells during an immune response. DC derived IL-12 has been shown to induce NK cell IFN-γ and together these cytokines create a positive feedback loop that drives T cell differentiation and establishes Th1 cell mediated immunity (Martin-Fontecha et al., 2004). While IL-12 is not necessary for normal development of the immune system, mice with targeted disruptions in the IL-12 protein or receptor show susceptibility to *Listeria*, *Leishmania*, as well as *Salmonella* and *Mycobacteria* species (Trinchieri, 2003). These mice secrete normal levels of IL-10 and IL-4 but their NK and $CD4^+$ Th1 cells produce very low levels of IFN-γ when challenged with systemic LPS (Magram, Connaughton, Warrier, Carvajal, Wu, Ferrante, Stewart, Sarmiento, Faherty, & Gately, 1996b).

The biologically active IL-12p70 heterodimer is composed of covalently linked p35 and p40 subunits and signals through a functional IL-12 receptor (IL-12R), comprised of the IL-12Rβ1 and IL-12Rβ2 subunits (Presky et al., 1996). IL-12 itself, TNF-α, IL-18, IL-2, and IFN-γ, but not IFN-α or-β, have all been demonstrated to regulate the levels of the IL-12Rβ chain (Gately et al., 1998) and as this subunit is responsible for transducing IL-12 signals, these cytokines play a role in controlling the cell's sensitivity to IL-12. IL-2 propagated human or mouse NK cells have increased expression of the functional IL-12R and can respond to IL-12 alone to secrete IFN-γ (Wang, Frank, & Ritz, 2000). Conversely, NK cells, as well as T cells, freshly isolated from normal human peripheral blood or mouse secondary lymphoid organs are poor responders to IL-12 alone. This can be attributed to their very low expression of the functional IL-12 receptor (IL-12R) comprised of both IL-12Rβ1- and -2 subunits (Chaudhry,

Kingham, Plitas, Katz, Raab, & DeMatteo, 2006a). NKT cells express high levels of the IL-12 receptor even at resting conditions (Kawamura et al., 1999), and can respond to even sub-optimal doses of IL-12 alone (Kawamura et al., 1999; Smyth et al., 2002). The expression of the IL-12 receptor might be attributed to a basally primed state of resting NKT innate cells, similar to that that of memory T cells.

A role for endogenous IL-12 as an inducer of Th1 responses leading to the subsequent clearance of diverse pathogens has been shown in mouse infection models of *Leishmania major*, *Trypansoma cruzi*, *Cryptococcus neoformans*, *M. tuberculosis* and *M. bovis*, and *Listeria monocytogenes* (Brombacher, Kastelein, & Alber, 2003). It has also been shown to aid in the clearance of murine cancers (Kawamura et al., 1999; Labbe et al., 2009), and prevention of metastasis, and to act as an inhibitor of tumour angiogenesis (Del Vecchio et al., 2007). While there are studies that have suggested that IL-12 mediates its anti-tumour effects via the induction of IFN-γ release from NK cells in vivo (Takeda et al., 2000), the mechanism behind its activation of NK cells is not clear.

1.1.4.1.3 IL-18

IL-18 belongs to the IL-1 cytokine family comprised of IL-1, IL-18 and IL-33 which are related in their receptor structure, and signal transduction pathways (Arend, Palmer, & Gabay, 2008). IL-18, like its family members, is synthesized as a precursor (pro-IL-18) which requires cleavage by the protease, IL-1β converting enzyme (ICE)/caspase-1(Dinarello, 1998). Macrophages, DC, B cells, and neutrophils have all been shown to possess a substantial pool of pro-IL-18 which can be released as the mature protein after cellular activation (Lorey, Huang, &

Sharma, 2004; Nakanishi, Yoshimoto, Tsutsui, & Okamura, 2001; Sporri, Joller, Hilbi, & Oxenius, 2008). The caspase-1 independent processing of pro-IL-18 has been suggested to occur through other enzymes such as proteinase 3 (Dinarello & Fantuzzi, 2003) and has also been shown with Fas Ligand triggering in mouse macrophages (Tsutsui et al., 1999) but it is still unclear whether similar mechanisms exist in all IL-18 producing cell types, in humans, or are physiologically relevant.

IL-18 mRNA is also present constitutively but, unlike pro-IL-18, is not restricted to any specific cell type and is ubiquitously expressed by most body cells. Interestingly, inflammatory signals have little impact on the production of pro-IL-18 and most of the regulation associated with the production of IL-18 is controlled by capase-1 activity (Arend et al., 2008), and its effects upon the cells can be neutralized after the IL-18 binding protein (IL-18bp) binds to soluble IL-18 and prevents it from inducing its signal (Boraschi & Dinarello, 2006).

IL-18 was initially described as an IFN-γ inducing factor due to its ability to induce potent levels of IFN-γ in IL-2 treated bulk mouse spleen (Nakamura, Okamura, Wada, Nagata, & Tamura, 1989). The functional IL-18 receptor (IL-18R) is comprised of the ligand binding IL-18Rα chain and the signal transducing IL-18Rβ chain. The expression of the IL-18Rβ chain can be modulated by IL-2, IL-12, and IL-15, and IFN-γ (Yoshimoto, Takeda et al., 1998). In pre-immune mouse spleen, IL-18R is found on resting NK cells but on its own is not capable of inducing IFN-γ. Andoniou et al. (Andoniou et al., 2005) found that the production of IFN-γ by NK cells in response to MCMV infected conventional DCs relied upon the production of IL-18 from the infected DCs. IL-18 deficient mice exhibit impaired NK cell activity as seen by reduced IFN-γ production, and an abrogated Th1 cell response to infection with

Propionibacterium acnes and BCG. In mice that were deficient in IL-18 and IL-12, NK activity and Th1 responses were further impaired highlighting the importance of these cytokines and their synergistic effects during host defence and NK cell activation (Takeda et al., 1998).

The effects of IL-18 upon the course of the immune response depend strongly on what other cytokines can be found in the surrounding milieu (Dinarello, 1999; Dinarello, 2006). The stimulation of IL-18 alone does not stimulate NK cell IFN-γ elaboration, but it does synergize with IL-12 and act as a potent co-stimulator of IFN-γ both in vitro and in vivo (Nakahira, Ahn, Park, Gao, Tomura, Park, Hamaoka, Ohta, Kurimoto, & Fujiwara, 2002a). It has also been shown to synergize with CpG ODNs and activate both IFN-γ release and the cytotoxicity of the NK cell compartment to prevent the growth of tumours in vivo (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006a).

1.1.4.2 Soluble signals that suppress IFN-γ from NK cells

1.1.4.2.1 TGF-β

TGF-β is perhaps best known for its roles in the differentiation and induction of regulatory T cells (Treg) and in the process of carcinogenesis, but it has also been shown to be a key suppressor of inflammatory responses exerting its action on a broad range of immune cells. With respect to NK cell activity, TGF-β has been shown to inhibit both cytotoxicity and IFN-γ production through indirect and direct means. It can downregulate the production of IL-12 and other immune stimulating factors from DCs and thus reduce the stimulatory signals that are needed to turn on NK cell IFN-γ production. It also acts on NK cells directly by suppressing T-bet, a transcription factor that is required for IFN-γ production, through the induction of SMAD

proteins (Flavell, Sanjabi, Wrzesinski, & Licona-Limon, 2010; Yu et al., 2006). The direct action of TGF-β upon NK IFN-γ production, as well as cytotoxicity, was also shown by Ghiringelli et al. (Ghiringhelli et al., 2005). Their work showed that membrane bound TGF-β was expressed by human Treg cells and when presented to stimulated human NK cells decreased IFN-γ release and killing against tumour cell lines. Besides directly inhibiting the production of IFN-γ, TGF-β has also been shown to destabilize IFN-γ mRNA and lead to reductions in IFN-γ anti-viral activity (Hayashi et al., 2003) in human NK cell lines stimulated with IL-18 alone. TGF-β has been detected in all cell lineages deriving from bone marrow, and this includes NK cells (Horwitz, Gray, & Ohtsuka, 1999). While exogenous TGF-β is able to prevent NK cell IFN-γ release, the endogenous production of TGF-β by stimulated NK cells has not been shown to act back upon the NK cell itself (Bellone, Aste-Amezaga, Trinchieri, & Rodeck, 1995).

1.1.4.2.2 IL-10

Like TGF-β, IL-10 is also expressed by a wide variety of immune cells. It has been shown to be a growth and differentiation factor for Treg cells, is upregulated in various disease states including cancer, and is best known for its suppression of Th1/Th2 driven inflammatory responses (Fiorentino, Bond, & Mosmann, 1989). IL-10 is considered to be a key player in striking the balance between immune protection and pathology. In the gut the production of IL-10 from activated Tregs, and subsets of B cells, helps keeps immune responses to bacterial flora and dietary antigens in check, as seen in the inflammatory bowel disease of IL-10 deficient mice (Kuhn, Lohler, Rennick, Rajewsky, & Muller, 1993). The absence of IL-10 during pathogenic infection similarly leads to the over-exuberant production of IFN-γ, leading to an uncontrolled

inflammatory response which while detrimental to the host doesn't seem to affect the pathogen (Trinchieri, 2007). With respect to the control of NK cell IFN-γ levels, IL-10 is believed to work indirectly through its ability to antagonize the production of IL-12 from activated Mϕ and DC. Although NK cells can secrete IFN-γ in an IL-12 independent manner, IL-10 has also been shown to limit the release of other NK cell activating cytokines like IL-2, IFN-α, and IL-18 (Mocellin, Marincola, Rossi, Nitti, & Lise, 2004; Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). In addition, IL-10 downregulates co-stimulatory B7 proteins and ICAM-1 which are involved in the induction of IFN-γ from NK cells during cell-cell interaction with infected APCs. IL-10 has also been implicated in the reduction of MICA expression on human melanoma cells (Serrano et al., 2010)(Serrano et al., 2010), therefore NKG2D mediated induction of IFN-γ would also be impinged upon by IL-10.

1.1.4.3 Contact dependent factors

Cytotoxic granules released by an activated NK cell need to be directed at the target cell in order to avoid the bystander killing of healthy cells. To this end, NK cells form tight immunological synapses at the cellular interface between their targets and themselves (Orange, 2008). Similar synapses have been shown to form between NK cells and DCs in vitro (Borg et al., 2004). The contact dependency of DC delivered help required for optimal NK cell IFN-γ production likely reflects the action of membrane bound receptor/ligand pairs, as well as the need for a local delivery of cytokines at high concentration from DC toward the NK cell (Walzer, Dalod, Robbins, Zitvogel, & Vivier, 2005). An example of this is the contact dependent presentation of IL-15 by DC to NK cells (Koka et al., 2004; Schluns, Stoklasek, & Lefrancois, 2005).

2B4 (CD244) is expressed on most human and mouse NK cells. CD48 can bind to other CD2/SLAM proteins like CD2 and CD48, but CD48 which is broadly expressed on all hematopoietic cells is the higher affinity ligand (Lanier, 2001). In human cells SAP binding to the 2B4 cytoplasmic domain after 2B4 ligation induces NK cell lytic activity and IFN-γ production, but this has not been shown in mice. Mouse 2B4 is expressed as either the 2B4 short or 2B4 long isoforms which have opposing functions. The ligation of 2B4 on NK cells with anti-2B4 mAb has been shown to decrease or increase IFN-γ production; with the predominant inhibitory function of murine 2B4 correlating with an increased 2B4 long isoform level over that of 2B4 short (Mooney et al., 2004). CD2 and CD48 have been found on various immune cells incuding DC, B, T, NKT, as well as on NK cells themselves.

Davis and colleagues have likened cytokine secretion by T cells to occur in 'shouts' – multidirectional release, or 'whispers'- polarized synaptic secretion, with the mode of secretion depending upon the intended function of the cytokine (Huse, Quann, & Davis, 2008); similar choices for cytokine release likely exist for other immune cells. Indeed, the release of IL-12 and IL-18 from activated DCs has been shown to be directed within the synaptic cleft formed between human DC and NK cells (Borg et al., 2004; Semino, Angelini, Poggi, & Rubartelli, 2005). NK cells might also be able to dictate whether their release of cytokines should be exclusive to the target cell they interact with, or be more globally distributed and have greater influence on other cells in the surrounding environment.

The recognition of NKG2D ligands enables an NK cell to gauge the health of host cells. The MHC class I like MICA and MICB (MHC class I polypeptide related sequence A and B) proteins, and the Rae-1 (retinoic acid early transcript 1), H60, and MULT-1 (murine ULBP-like

transcript 1) (Carayannopoulos, Naidenko, Fremont, & Yokoyama, 2002) ligands which are weakly homologous to MHC class I molecules are inducible ligands for human and mouse NKG2D respectively (Lanier, 2008b). In murine cells, infection with *Staphylococcus aureus*, *Listeria monocytogenes,* and *Mycobaterium.bovis* can all upregulate Rae-1 on infected cells. The expression of these ligands on a cell renders it susceptible to NK cell lysis, but NKG2D ligation also enhances IFN-γ. The induction of human NK cell IFN-γ by influenza infected DCs has been shown to depend on NKG2D and NKp46 (also associated with ITAM containing ζ or FCεR1) as antibodies to either receptor blocked cytokine release by 90% (Draghi et al., 2007).

Most tumour cell lines constitutively express NKG2D ligands and are therefore susceptible to NK cell cytolysis, and in some instances can induce IFN-γ as well. Raulet and colleagues retrovirally expressed high levels of Rae-1 and H60 on various mouse tumour cell lines, including RMA cells, that usually do not express these ligands and found an increased percentage of NK cells positive for intracellular IFN-γ in vitro (Diefenbach, Jensen, Jamieson, & Raulet, 2001). Another study by Held and colleagues (Coudert et al., 2005) used RMA cells (usually not sensitive to NK cell killing) stably transfected with H60 in co-culture with mouse splenic NK cells in the presence of IL-2 for 72h. When NK cells were removed from these cultures they showed abrogated lysis against RMA-H60 cells, whereas NK cell cultured with IL-2 in the presence of untransfected RMA could kill the RMA-H60 cells. Surprisingly, the chronic NKG2D-H60 engagement did not impact IFN-γ production from the NK cells. The reduction in cytotoxicity and the increase in IFN-γ, which has been shown to reduce NKG2D ligands on mouse tumour cells (Bui, Carayannopoulos, Lanier, Yokoyama, & Schreiber, 2006), were suggested to be a possible mechanism for a tumour to evade the NK cell response and speak to the importance of this signalling pathway in the control of tumour growth.

1.1.5 DCs as partners to NK cell activation

Accessory cells which are primarily monocytes/Mϕ and DCs, but can include other immune (NKT, neutrophils) and non-immune (stromal) cells, offer the coordinated contact dependent and soluble signals needed for the optimal NK cell response to pathogens or disease. The relationship between NK cells and DC is the most studied. In both humans and mice, the DC compartment is heterogeneous with various subsets that each present a distinct phenotypic and functional attribute. Both human and mouse DC highly express the β-integrin $CD11c^+$, that associates with CD18 to bind fibrinogen, and iC3b complement proteins. Other cells also express $CD11c^+$, such as NK cells, B cells, Mϕ, and neutrophils, but at much lower levels.

In vivo DC are localized in the periphery where they patrol for pathogens as the key sentinels of the immune system. At this stage DC are considered immature and highly motile, with a low expression of costimulatory proteins (CD40, CD80/86) and MHC class II, and are specialized in antigen uptake and processing. As they encounter antigens that activate their extensive array of TLRs (see Table 1) they upregulate costimulatory proteins and MHC class II and migrate to lymphoid organs where they encounter other immune cells. They also secrete chemokines, and have been shown to increase ligands for NK cell activating receptors (NKp30, NKG2D, and DNAM-1 (Zamai et al., 2007). Here the activated DC secrete inflammatory chemokines and cytokines that attract and modify other immune cells, and provide the necessary priming signals for T cell and B cell maturation and differentiation. The DC fully mature as they in turn receive signals from the cells they are interacting with. As there are also tissue resident DCs, this maturation can also occur within the lymphoid tissues themselves in response to localized infection. Their activation leads to the production of many of the soluble signals that were discussed earlier (sections 1.1.4.1 and 1.1.4.2).

Table 1 TLRs common to human and mouse DC

Receptor	Ligand
TLR1	bacterial triacyl lipopeptides
TLR2	peptidoglycan, lipoprotein, zymosan, atypical LPS, lipoteichoic acid
TLR3 (endosomal)	viral dsRNA
TLR4	LPS, bacterial and viral proteins, host cell surface proteins
TLR5	bacterial flagellin
TLR6	zymosan, lipoteichoic acid
TLR7 (endosomal)	viral ssRNA
TLR8 (endosomal)	viral ssRNA
TLR9 (endosomal)	unmethylated CpG DNA (bacteria and virus)

**adapted from Akira and Takeda* (Akira, Takeda, & Kaisho, 2001). *TLR10 is not shown as its agonist/function is not yet defined and has not been described on mouse DC. TLR11 which recognizes uropathogenic bacteria is also not shown as it is not on human DC.*

DCs interact with NK cells in the periphery at various sites of infection, and anywhere that NK cells reside within or are recruited to. In the steady state or non-infected lymph node (LN) NK cells are found in the most peripheral part of the T cell zone, beneath and between B cell follicles (Walzer, Blery, Chaix, Fuseri, Chasson, Robbins, Jaeger, Andre, Gauthier, Daniel, Chemin, Morel, Dalod, Imbert, Pierres, Moretta, Romagne, & Vivier, 2007b). They are highly motile even in the absence of inflammatory signals and their motility allows them to repeatedly contact the network of LN resident DC. A recent paper by Beuneu et al. (Beuneu et al., 2009) examined the interaction of DC and NK during inflammatory conditions in vivo using two photon imaging in mice. LPS (TLR4) and poly I:C (TLR3) TLR agonists require DC help, IL-

12 in the case of LPS, and IL-15 and IFN-α(pDC) in the case of poly I:C. When these agonists were injected into mice LN NK cells were activated (increased CD69 levels) and serially engaged DCs in short lived (1-3 min) contacts. This suggested that in the LN NK cell priming by DC does not require stable interactions, and NK cells seem able to collect the necessary signals for their activation through multiple transient contacts. This is different than the longer lasting interactions observed between DC and T cells in the same tissue.

Things are different in the spleen where, during steady state conditions, NK cells are for the most part kept out of the T/B cell area (white pulp) and are mostly found in the blood rich red pulp. DCs are found near the T cell zone of the white pulp, as well as in the marginal zone. During polyI:C induced inflammation NK cells migrate out to the white-pulp of the spleen in response to inflammatory chemokines (CCXL10, CCL5), that are likely secreted by activated DCs and other TLR containing cells (Gregoire et al., 2008).

The earliest report showing that the functions of DC and NK cells are intertwined with respect to IFN-γ production and NK cell activation came in 1999. Fernandez et al. (Fernandez et al., 1999) showed that DCs were able to promote NK cell cytotoxicity against tumours in vivo, and the presence of DC was absolutely needed for NK cell IFN-γ release in vitro. In vitro studies can be misleading however as most of the DCs are not freshly isolated from tissues, but rather cultured from bone marrow progenitors using various cytokine cocktails which each promote the differentiation of a specific type of DC (Shortman & Naik, 2007). A similar need for DC in the NK cell production of IFN-γ was demonstrated by Lucas et al. (Lucas, Schachterle, Oberle, Aichele, & Diefenbach, 2007) using a transgenic mouse model that allowed for ablation of all $CD11c^{high}$ or 'conventional' DCs, while sparing the IFN-α secreting pDCs

subset. Table 2 summarizes the observed role of DCs as enablers of NK cell response to various pathogens in humans and mice.

Table 2 Role of DC and Mϕ in the NK cell response to various pathogens

Pathogen	Accessory Cell	TLR involved	Cytokines
MCMV	pDC/mDC; Mϕ	TLR9 , TLR3	IL-12, IL-15 and IFNα/β
HSV1/2	pDC/mDC; Mϕ	TLR9	I L-12, IL-18 and IFNα/β
Influenza	pDC, Mϕ	ND	IL-2, IL-12, and IFNα/β
Listeria monocytogenes	Mϕ	TLR2	IL-1β, IL-10, IL-12, IL-18, TNF
Mycobacterium tuberclosis	ND	TLR2/4 (humans)	IL-12 and IL-18 (mouse)
Toxoplasma gondii	DCs, Mϕ	TLR11 (mice)	IL-12 and IL-10
Leishmania species	DCs	ND	IL-12 and IL-18

* *adapted from Newman et al.* (Newman & Riley, 2007) *ND - not determined.*

1.1.6 Microbial products that enhance NK cell IFN-γ

In general, the NK cell response to pathogens is dependent upon the production of cytokines and other signals from activated APCs as shown above. NK cells have also been shown to respond to pathogen related products in the context of APCs. m157 is a viral gene product from MCMV that is expressed on the surface of infected host cells. A subset of NK cells directly binds this protein via Ly49H (Arase, Mocarski, Campbell, Hill, & Lanier, 2002), which itself does not contain an ITAM, but associates with the ITAM bearing DAP-12 protein to transmit its activating signal, and control viral replication and spread via the production of

IFN-γ and cytotoxicity. Similarly, the NKp46 receptors of human and mouse NK cells is capable of binding hemagglutinin derived from sendai and influenza viral strains in vitro (Sivori et al., 2004).

A more direct recognition of pathogens themselves has been suggested to occur through the TLRs found on human/mouse NK cells. In highly purified and freshly isolated human peripheral NK cells mRNAs for TLRs 2,3,5,9 were observed (Hornung et al., 2002); but the presence of mRNA does not guarantee the expression of protein, nor its functionality. Many studies therefore stimulate purified NK cells with TLR agonists as an indirect indicator of the presence of TLRs. But in order to properly ascertain whether NK cells are capable of directly responding to TLR ligands, extremely pure populations of NK are needed in order to avoid contamination with DCs/Mϕ which express TLRs. Using such highly purified NK cells from human blood Chalifour et al. (Chalifour et al., 2004) looked at IFN-γ release after stimulation with a TLR2 ligand (KpOmpA- *Klebsiella pneumonia* outer membrane protein A) and TLR5 ligand (*Escherichia coli* flagellin) and found IFN-γ in supernatants after only 8 hours of incubation. If IL-2 and IL-12 were added a 7-fold increase in NK cell IFN-γ production was noted, while IL-10 reduced the release of IFN-γ. This result suggested that during an actual infection in vivo, the concomitant release of cytokines from surrounding pathogen activated cells (DC/Mϕ) are able to control the sensitivity of NK cells to TLRs ligands. This synergy has been shown to be important for signals received through TLR9. Without IL-2, IL-12, or IL-18 NK cells do not secrete IFN-γ in response to CpG-ODNs (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006a; Lauzon, Mian, MacKenzie, & Ashkar, 2006a). The enhanced production of IFN-γ when cytokines and TLR signals converge is not that surprising as we have already seen that NK cells often require more than one signal for optimal activation. The

absolute requirement of cytokines for the TLR9 mediated activation of IFN-γ from NK cells may be due to its unique endosomal location. In DC, Mϕ, and B cells (Krieg, 2002), specialized mechanisms of uptake and delivery to TLR9 containing vesicles exist. Such mechanism might also be required for the optimal NK cell response to TLR9 agonists like CpG-ODN. The induction of IFN-γ from highly purified NK cells in response to TLR9 ligation by CpG-ODN is also observed in the presence of CD16 (FcR) stimulation after binding to mAb-coated HER2-overexpressing human breast cancer cell line or immobilized immunoglobulin G (IgG) (Roda, Parihar, & Carson, 2005); suggesting that both soluble and contact-dependent signals are able to enhance NK cell responses to TLR9 ligation.

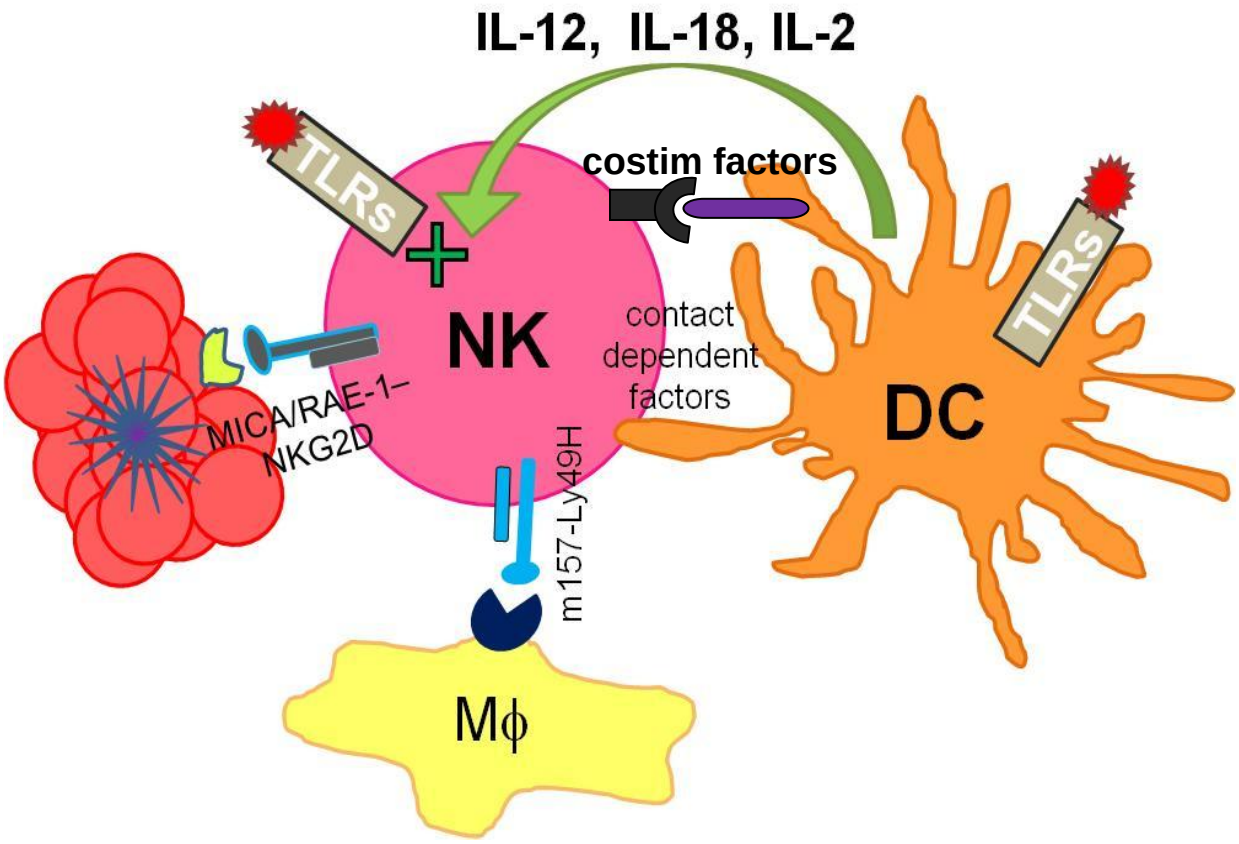

Figure 2 Summary of signals that enhance NK cell IFN-γ production

NK stimulation of IFN-γ requires the convergence of a multiple signals. Pathogens are detected by accessory cells (DC or Mϕ) via TLRs. If other costimulatory signals are received (cytokines or contact dependent) these cells release cytokines. IL-12, IL-18 and IL-2 are soluble cytokines that strongly influence inflammatory responses. The cytokines act together and/or converge with signals received through NK TLRs to induce IFN-γ from NK cells. Contact dependent signals include IL-15 trans-presentation via DC, and the secretion of IL-18 into the synaptic cleft between NK/DC, as well as the detection of upregulated stress associated ligands (MICA on human)/Rae-1 and H60 on mouse) on transformed cells via NKG2D; NK binding to MCMV proteins (m157) on infected cells (Mϕ, DC, endothelial, and epithelial cells) via Ly49H also leads to IFN-γ release.

1.2 B Cells

The adaptive arm of immunity is represented by T and B cells. Both compartments possess highly diverse clonally distributed antigen receptors that arise from the random segregation and rearrangements of their receptor genes. The B cell receptor (BCR) is made up of paired chains of immunoglobulin (Ig) proteins that can detect and bind antigen when bound on the cell surface, or when secreted as soluble antibodies. Antibody production by B cells is the basis of the humoral immune response that protects all extracellular spaces in the body against invading pathogens. B cells possess 'innate-like' functions in that they are crucial APCs to antigen-specific T cells in a process known as T/B cell collaboration; they also constitutively express TLRs and respond to various TLR agonists much like DCs and Mϕ, and release a spectrum of cytokines which have the potential to modulate the response of innate and adaptive immune cells. Thus, B cells are emerging as a functionally diverse population with the ability to act as potent immune regulators in health and disease.

1.2.1 B cell subsets

The expression of a BCR is essential to B cell survival. The very earliest BCR, the pre-BCR depends on the expression of the Ig μ-chain. Transgenic mice with a targeted mutation in the μ-gene (μMT mice or BKO) are not able to express a pre-BCR and consequently normal B cell development is completely halted (Kitamura, Roes, Kuhn, & Rajewsky, 1991). Normally in adults naive B cells emerge from the bone marrow as they successfully express surface IgM, and circulate through blood to the secondary lymphoid organs like spleen and lymph node, and also locate to the peritoneal and pleural cavities. In the spleen B cells compete to enter the follicles of

the B cell zone within the splenic white pulp (lymphocyte rich). They remain there as mature $IgM^{+}IgD^{+}$ (conventional, B2 cells) until they encounter their cognate antigen, otherwise they die off or are replaced by newly entering cells. B cells that do encounter their antigen, usually in the context of immunocomplexes presented on follicular dendritic cells (FDC), monoclonally expand to form aggregates of proliferating cells called germinal centers (GC). They then migrate through the B cell zone out towards the T cell zone where they are more likely to encounter their cognate antigen specific T cells, and receive the further signals they require to become antibody producing cells. The fate of a B cell that becomes primed with antigen but does not receive secondary signals is death. The B cells that do receive signals become either short lived plasmablasts, or longer lived plasma cells which can migrate back to bone marrow and continue releasing antibodies (Janeway, Travers, Travers, Walport, & Shlomchik, 2005).

B cells are also found within the marginal zone (MZ) of spleen, which is the interface between the white and red (blood rich) pulp and contains specialized Mϕ populations, DC, and T cells. The MZ APCs are poised to respond to any blood borne pathogens or their products as the flow of blood into the spleen is slower at this interface. MZ B cells are believed to exist in a 'pre-activated' or primed state (Pillai, Cariappa, & Moran, 2005) and are able to secrete antibodies within hours in vitro as opposed to conventional B cells which do so in days. Other specialized subsets of B cells are the B1 cells which are predominant in the fetus and in neonates. In adults these cells represent about 10% of B cells and are found in spleen and are enriched in the peritoneal/pleural cavities (Dalloul, 2009). B1 cells are specialized in responding to non-protein bacterial antigens and are considered to be innate like B cells with a capacity to self-renew.

B cells isolated from spleen are a heterogeneous population comprised of the varied subsets described. B cells can be divided into 'high' and 'low' density populations using discontinuous density gradients (Yuan, Wilder, Dang, Bennett, & Kumar, 1992). It has been suggested that the high density B cells represent resting cells, while the low density population represents an in vivo activated or primed subset as they express higher levels of CD69, exhibit spontaneous proliferation when cultured, and respond more rapidly to LPS and BCR cross-linking (Yuan, 2004). Different subsets of B cells can also be isolated based on the surface expression of various markers as shown in Table 3. In general CD19 distinguishes both human and mouse B cells. During BCR signalling it functions as a coreceptor as the phosphorylation of its cytoplasmic tail recruits Src-family kinases and activates PI-3K (Tedder, 2009). It is one of the earliest surface markers expressed on committed B cells, is stable throughout all stages of B cell development and maturation, but it is lost as B differentiate into long lived antibody producing plasma cells (Hardy & Hayakawa, 2001). CD5 expression in mice delineates a specific lineage of B1 cells (B1-b) but in humans the expression is more heterogeneous, and in can be upregulated after antigen encounter (Hardy, 2006); it is thought to mark B cells that have had repeated encounter with antigen. CD5 expressing B cells have a propensity to secrete IL-10, and the thus the potential to suppress Th1 cell development and polarized responses. CD5 is found on B1-b cells and also on $CD1d^{hi}CD5^{+}$ B10 cells (Yanaba et al., 2008). Its presence on B and T (including NKT) cells is thought to mitigate the signals received through antigen receptors. The expression of CD5 on B1 cells ensures that only very strong signals will lead to stimulation, this might be a protective mechanism considering that these B cells produce natural IgM antibodies and are considered important to the development of autoimmune reactions (Dalloul, 2009).

Table 3 B cell subsets and functions

B subset	Identification	Function
Conventional, B2	$CD19^{+}CD27^{-}CD38^{-}$	Naïve, can circulate, TD antigens
Memory B	$CD19^{+}CD27^{+}CD38^{-}CD138^{-}$	Quickly reactivated -Ab release upon antigen rechallenge
Plasma blasts	$CD19^{+}CD38^{+}CD138^{+}CD27^{hi}$	Short lived (days) Ab secreting
Plasma cell	$CD19^{-}CD38^{+}CD138^{+}CD27^{hi}$	Long lived (months) Ab secreting
B1	$CD19^{+}CD5^{+}$ (a) or $CD19^{+}CD5^{-}$ (b)	TI antigens; secrete auto-abs
Marginal zone (MZ); also B2	$CD19^{+}IgM^{high}IgD^{low}CD21^{high}CD23^{low}$	Mature , TI antigens, primed
Regulatory	$CD19^{+}CD5^{-/+}CD11b^{-}CD43^{+}CD1d^{hi}$	IL-10, Th1 suppression

** Ab- refers to antibody, TD- T cell dependent, and TI -T cell independent antigens. B1 cells in mice can secrete natural antibodies (unhypermutated polyreactive IgM) and are considered 'innate' cells*

1.2.2 B cell function

1.2.2.1 Antibody production

There are five classes of surface expressed immunoglobulin isotypes that comprise the BCR. Immature and naive B cells first express IgM^{+} as these B cells mature in the periphery, they also express IgD on their surface and when activated can secrete both IgM and IgD. After a B cell encounters its cognate antigen and begins to proliferate and has the opportunity to 'switch' to another one of the antibody classes, IgG, IgA or IgE. This process allows a B cell to retain its antigen specificity but exchange its constant region and in essence acquire a new effector function suited to the pathogen and the infection (see Table 4). This switching is

absolutely dependent upon armed $CD4^+$T cell help in the form of co-stimulatory molecules such as CD40 ligand (CD40L, CD154) and is dictated by the surrounding cytokine milieu. In mice, IFN-γ for instance is a powerful inducer of IgG2a, while IL-4 preferentially induces the IgG1 and IgE isotype switching, IL-10 promotes switching in favour of IgA (Dullaers et al., 2009), and TGF-β promotes IgG2a as well as IgA. Overall, antibodies can bind to pathogens or their toxins and neutralize their ability to enter the cell in the first place. They also effectively coat pathogens to enhance phagocytosis by macrophages or dendritic cells, a process termed opsonization, and can activate NK cell mediated ADCC. Finally, IgM and IgG bind to antigenic epitopes on the pathogen and recruit complement proteins, initiate complement cascades to allow for a specific activation of this pathway directly to the pathogen.

Table 4 Antibody class roles and triggers for Ig-class switching

Ig Class	Function	Switch triggers	Source of trigger
IgM	'fixes' complement and opsonization ; first antibody to defend against virus and bacteria	1st Ab made	IgM + B cell activation
IgG	opsonization (IgG1), ADCC by NK cells (IgG3); can cross placenta; defend against virus and bacteria; abundant in blood	IgG2a- IFN-γ; IgG1- IL-4/IL-5; IgG2b- TGF-β	Treg and Th1
IgA	protects mucosal surfaces, in secretions and breast milk; resistant to acid/enzymes of digestive tract; neutralize pathogen entry	TGF-β; IL-4 and IL-5	Th2 and Treg
IgE	parasitic defence; causes allergies (mast cell activation) and anaphylactic shock	IL-4 and IL-5	Th2

* *IgG isotypes shown are for mouse antibodies. Treg- T regulatory cell. Modified from Janeway 2005* (Janeway et al., 2005)

Activated B cells are further able to modify their antigen receptor via a process known as somatic hypermutation, or affinity maturation, which occurs in proliferating B cells that undergo point mutations in susceptible regions of the genes that code for the antigen detecting variable region of the BCR. If the resulting daughter B cell is able to bind antigen better than the original, it is selected to mature; thus with each successive round of division a B cell has the ability to increasingly enhance its affinity for the antigen. Both isotype switching and somatic hypermutation are not seen when B cells are directly stimulated by microbial antigens. Thus T-cell independent (TI) antigens like polysaccharides do not yield antibodies that are as highly

variable or as functionally versatile as those induced by antigens that require T cell interaction and help (Janeway et al., 2005).

1.2.2.2 Antigen presentation

B cells are specialized to bind soluble molecules through surface Ig, this happens independently of armed effector T cell help and often in either the peripheral blood where B cells are circulating, or in the MZ which allows them first hand access to any pathogens entering the spleen via circulation. In DC and Mϕ there is a constant sampling of the extracellular compartment via phagocytosis/pinocytosis which provides both self and foreign antigens for presentation upon surface MHC class II molecules to T cells. B cells are primarily dependent upon the BCR as a source of antigen uptake. An elegant study by Batista and colleagues (Fleire et al., 2006) has found that B cells rearrange their cytoskeletal networks after antigen recognition through their BCR. This allows the B cells to spread out over a larger distance and effectively 'grab' a larger amount of antigen than B cells that did not spread.

Once triggered by its cognate antigen, the BCR forms a signalling complex by associating with ITAM containing Igα-Igβ (CD79α/β) proteins which are required to transduce the BCR signal into the cell. The BCR-antigen complex is then internalized and targeted to endocytic compartments; here proteases degrade the antigen into fragments that are loaded onto MHC class II protein which then ferries to the B cell surface. BCR signalling has been shown to increase MHC class II production the expression of costimulatory molecules like CD80/CD86 and CD40 on the B cell. B cell APC function is most likely important only after the immune response to a pathogen has been initiated and this is highly dependent upon the early pathogen detection and priming of T cells by DC and Mϕ (D. P. Barr et al., 2007).

As APCs, human and mouse B cells have also been shown to express TLRs and respond to TLR 2, 3, 4, 5, 7, and 9 agonists in vitro (D. P. Barr et al., 2007; Pasare & Medzhitov, 2004). Indeed, LPS is a classic mitogenic stimulus for mouse B cells. Differences in the expression patterns of TLR do exist amongst B cell subsets. For instance, human peripheral blood B cells lack TLR4 but if given additional signals, like IL-12 and CD40 ligation, they upregulate TLR4 and respond to LPS (Wagner et al., 2004). Similarly, Bernasconi et al. (Bernasconi, Onai, & Lanzavecchia, 2003) found human blood memory B cells to constitutively express TLRs 6, 7, 9, and 10, while naive cells did not but could be induced to after BCR-linkage. In mice, Barr et al. (T. A. Barr, Brown, Mastroeni, & Gray, 2010) have shown that mouse splenic B cells constitutively express mRNA for TLRs1-9 but show differences in TLR protein expression levels, and differential responses to TLR agonists.

1.2.2.3 Cytokine production

In order to drive T cell differentiation, a BCR activated B cell must also provide additional signals like adhesion (ICAM-1) molecules, CD40 and CD80/86 costimulatory molecules, as well as cytokines. Naive B cells that have taken up antigen and presented it to T cells in the absence of these additional signals induce T cell tolerance, and the development of Tregs, rather than helper T subsets (Gunzer et al., 2004). B cells have been shown to be major producers of IL-10, IL-6, lymphotoxin α (LT-α), and TNF-α, as well as IL-2, IL-4, IL-12 (Lund, 2008). The production of cytokines from B cells has been observed largely with purified B cells that have been activated in vitro using a variety of means including, TLR agonists (LPS, CpG DNA, peptidoglycan), phorbol 12-myristate 13-acetate/ionomycin (PMA/Iono), IgM cross-linking, CD40 ligation with soluble CD40L, bacterial polysaccharides (TI antigens), and whole

bacteria (D. P. Barr et al., 2007; Pistoia, 1997; Schultze et al., 1999). These cytokines have the potential to feedback upon the B cells themselves and modulate their responses as B cells have been shown to constitutively express the IL-2 and IL-12 receptors (Airoldi et al., 2002; Nakanishi et al., 1992; Yoshimoto, Takeda et al., 1998). Paracrine effects of B cell cytokines include but are not limited to the positive and negative regulation of T cells during T/B co-operation via IL-2 and IL-10 respectively, IL-2 in the survival and activation of NK cells, and the effects of IFN-γ on DC/Mϕ (Pistoia, 1997).

The array of cytokines that B cells can secrete is reminiscent of the cytokine profiles of Th1/Th2 and NK1/NK2 cells. In a pivotal study, Lund and colleagues (Harris et al., 2000) showed that mouse naive B cells that initially started with the capacity to make the entire panel of B cell cytokines aforementioned could be differentiated into B effector type 1 (Be1) and B effector type 2 (Be2) which were skewed to produce IFN-γ and IL-4, respectively (Figure 3). Be1 cells depended on Th1 cells for their development and Be2 cells required the presence of Th2 cells. T and B specific antigens were also needed and provided an additional signal for optimal activation. Importantly the study showed that this differentiation of B cells into Be1 and Be2 could also be induced in vivo and during actual infection. *Toxoplasma gondii* infection in mice predominantly induces a type I cytokine response (IL-12 and IFN-γ) which produces Th1 cells, while *Heligmosomoides polygyrus* infection induces a type 2 response (IL-4 and IL-5) with Th2 cell development. Intracellular cytokine staining carried out on B cells from these mice clearly demonstrated that *T.gondii* infection resulted in B cells that produced IL-2, IL-12, IFN-γ, IL-6, and TNFα, but not IL-4 or IL-5. Conversely, *H. polygyrus* infection produced B cells positive only for IL-2, IL-4 and IL-5. Within the entire B cell compartment, the

percentages of cells positive for any of the tested cytokines were low. The question then becomes how necessary are B cells as cytokine producers during an immune response?

The protective Th1 response to *Salmonella* requires B cells as μMT mice develop only a transient protection which fails during bacterial rechallenge (Mastroeni, Simmons, Fowler, Hormaeche, & Dougan, 2000). In a very recent study Barr et al. (T. A. Barr et al., 2010) show that while the primary T cell response is BCR/B cell and MHC class II independent, it absolutely requires the MyD88-dependent secretion of cytokines by B cells. B cells which were made to either lack IFN-γ or IL-6 did not have robust or stable Th1 responses to *Salmonella*. Suggesting B cell cytokine production is just as important as their ability to present antigen later on.

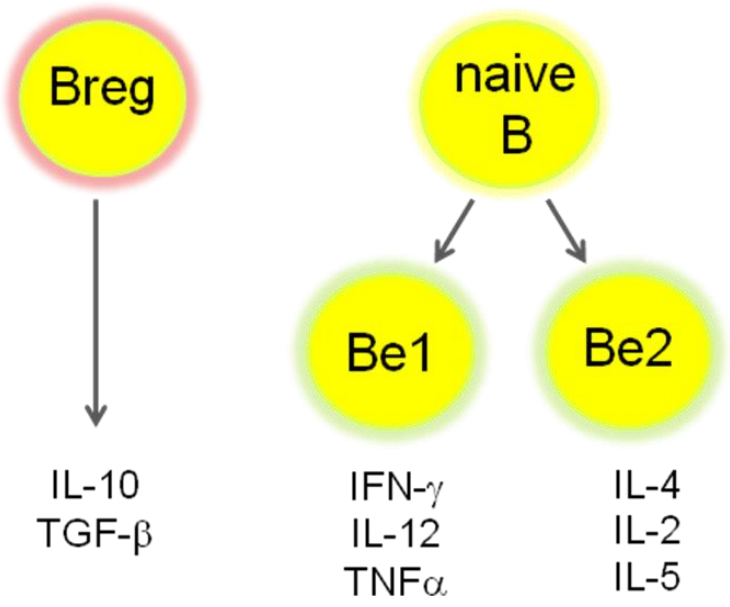

Figure 3 Cytokine secreting subsets of B cells

Naïve B cells have the capacity to secrete a wide spectrum of type 1 and type 2 cytokines. There are three specialized subsets of B cells which each secrete a different panel of cytokines. B regulatory (Breg) ($CD19^{+}CD5^{+}CD1d^{hi}$ B10 cells and the B1-B subset of CD5 expressing IL-10 producers) cells secrete IL-10 and TGF-β. Be1/Be2 cells arise from naïve cells that have been activated through BCR signaling and Th1 or Th2 cell help for Be1 and Be2 respectively. Be1 cells once activated secrete type I cytokines while Be2 cells predominantly secrete type II cytokines. The B cell origin of Breg cells is unclear (Rieger & Bar-Or, 2008)

1.2.3 B cell activation

The primary B cell immune response to TD (thymus dependent; T cell dependent) antigens can be divided into two phases: the initial encounter with armed helper T cells in the border between the B/T cell areas, and the development of the gernminal center within the B-cell follicle. DCs are very important to this process as their early and initial encounter with antigen arms antigen-specific T cells with the necessary signals to help antigen specific B cells. The interaction between the three cell types is called the primary focus and was traditionally

thought to progress as DC→T→B cell with respect to activation. There are reports that demonstrate DCs pulsed with soluble antigen (DNP-keyhole limpet hemocyanin) can also prime naive B cells in rat TD responses both in vivo and in vitro in a contact dependent manner (Wykes, Pombo, Jenkins, & MacPherson, 1998). Thus the relationship of the immune cells driving the extrafollicular phase is likely B←DC→T which would then be followed by B-T cell interaction with armed cognate antigen specific T cells stimulating the B cell through CD40L(T)-CD40 (B) interaction, and the directed release of cytokines. B cells have also been reported to form an immunological synapse with DCs during the establishment of primary humoral immune responses. Batista et al. (Batista, Iber, & Neuberger, 2001) loaded an immune complex comprised of hen-egg-lysozyme aggregated with specific IgG mAb onto the surface of FcγR-expressing myeloid cell line, and used these to look at the B cell-APC immunological synapse, and examine how it shapes the naive B cell response to antigen. The study showed that B cells rearrange the location of membrane bound receptors so that more BCR molecules are in the center of the interface between the APC and B cell, and proteins that can inhibit BCR signalling (like SHP-1 and CD22) are excluded from this region. Importantly, the entire intact antigen rather than peptide fragments is presented to the B cell. The complex of the BCR bound to intact antigen is then internalized and can be found within the B cell using microscopy. This work suggests while T cells are able to recognize degraded antigens on DC, DC are capable of presenting intact antigen to B cells, these B cells then can in turn process the internalized antigen and upregulate costimulatory markers and MHC class II and participate in B-T cell collaboration.

Activated B cells proliferate and differentiate and their progeny can become short-lived plasma cells which secrete massive amounts of Ig (Browning, 2006). The B cells then migrate

into the B-cell follicle where they aggregate into a structure of rapidly proliferating cells known as the germinal center (GC). Specialized cells in the GC called follicular dendritic cells (FDC) which bear immune complexes made of antigen/Ig proteins and help optimize affinity maturation and class switching. This is where memory B cells and plasmablasts are generated.

B cells can also secrete antibodies in the absence of any T cell- MHC class II restricted help in response to 'thymus independent', or TI antigens of which two subsets exist (TI-1 and TI-2) (Figure 4). Most pathogens express both TD and TI antigens. TI-1 and TI-2 subsets respond to polysaccharides and mitogens respectively. For TI antigens, the ancillary signals B cells require can be delivered by DCs (including pDCs), Mϕ, B cell themselves, and NK cells (Mond, Lees, & Snapper, 1995). Typically the signals are of a cytokine nature and roles for IL-2, IL-5, IFN-α, IFN-γ, IL-12, TNF-α, and GM-CSF, among others, have been described (Mond et al., 1995). IL-10 has also been shown to play a role in inhibiting the B cell response to TI but not TD antigens; while IFN-γ in the absence of other stimulatory cytokines leads to the suppression of B cell response (Mond et al., 1995; Pecanha, Snapper, Lees, Yamaguchi, & Mond, 1993). It is likely that a cytokine will have varied effects on B cell biology which could be dictated by the nature of the priming signals the B cells receives, the surrounding cytokine milieu, and which tissue type or discrete area within tissues the B cells reside. TLR signalling has been shown to lead to B cell proliferation and antibody secretion and differentiation in the absence of antigen, and when combined with TD or other TI antigens synergizes to optimize B cell function and lead to terminal differentiation (Chaturvedi, Dorward, & Pierce, 2008; Pasare & Medzhitov, 2004). TLR ligands such as LPS and CpG-containing DNA are known to act as vaccine adjuvants, enhancing the adaptive immune responses to various microbes or their components. Regardless of whether B cells are responding to TD or TI antigens, the need for co-

stimulatory signals from accessory cells helps to ensure that B cell antibody responses are provoked only against pathogens and self-immunization is avoided.

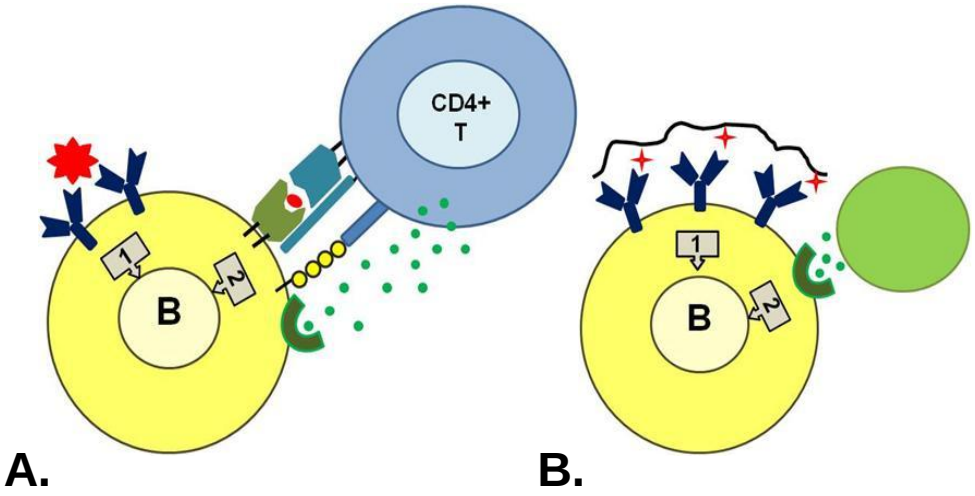

Figure 4 TD and TI antigen activation of B cells

TD (shown in A) and TI (shown in B) antigens deliver signals through BCR but other signals are needed for full B cell activation. For TD antigens T helper cells provide CD40 ligation and IL-2 and IL-4. For TI antigens BCR cross-linking due to repeating antigenic epitopes (bacterial polysaccharides or repeating surface molecules on viruses) is signal 1, and signal 2 is provided by cytokines from NK, DC/ϕ (IL-12, IFN-γ, TNF-α) and T cells. TLR signalling also constitutes an additional signal that can synergize with both TD and TI antigens (not shown) (Adapted from Janeway 2005 (Janeway et al., 2005; Janeway et al., 2005) . What is not shown here is the possibility that DC can also act as APC to the B cells in the TD antigen response as discussed in the text (section 1.2.3).

1.3 B cell and NK cell interaction

The majority of the studies that have focused on unravelling the mechanisms that regulate IFN-γ production from NK cells have concentrated on the roles of other innate cells, primarily DC (see section 1.1.5). The biology that lies behind the utility of these cells as accessories to NK cell stimulation is also found within the functional resume of a B cell. Like DC, B cells can respond to TLRs, secrete cytokines that have the potential to both stimulate and

suppress NK cell responses, and express surface molecules which can provide the co-activating signals for optimal effector function. Is it likely then that B cells may act as regulators of NK cell activity? There is evidence within the existing literature that does indeed demonstrate a distinct and dynamic relationship between NK and B cells.

The first report to highlight a potential for NK-B cell interaction demonstrated that the presence of NK cells was suppressive to B cell proliferation and IgM production in response to LPS using human cells (Nabel, Allard, & Cantor, 1982). Similar findings were soon reported with mouse NK cells and B cells stimulated with pokeweed mitogen in the presence of helper T cells (Arai, Yamamoto, Itoh, & Kumagai, 1983). The mechanisms for this observed suppression were shown in experiments with NK-B cell co-cultures and suggested to be via the induction of NK mediated cytotoxicity against conjugated B cells (Storkus & Dawson, 1986), the endogenous production of IFN-γ (Michael, Hackett, Bennett, Kumar, & Yuan, 1989) and TGF-β (Gray, Hirokawa, & Horwitz, 1994). Interestingly, both human and mouse NK cells seemed to be able to direct their suppressive effects against large and more activated B cells while sparing early/intermediate stage differentiated B cells. Michael et al. (Michael et al., 1989) showed LPS treatment could induce the proliferation of mouse spleen low density B cells (which represent in vivo activated or large B cells) and could be specifically inhibited by exogenous IFN-γ. In contrast, IFN-γ enhanced the proliferation induced by LPS in high density or small resting B cells. The downregulation of antibody production was also noted in vivo as the depletion of NK cells from adult mice and weanlings receiving pneumococcal vaccine improved the IgM response (Khater, Macai, Genyea, & Kaplan, 1986). But these findings would be contradicted by the following studies which would demonstrate that NK cells were positive regulators of human or mouse B cell responses.

The TI-2 polysaccharide α-δ-dextran (multiple anti-IgD proteins linked to high molecular weight dextran) was shown by Mond and colleagues (Pecanha et al., 1993; Snapper et al., 1993) to activate the antibody response of B cells gradient enriched from mouse spleen. While low density B cells were fully activated by α-δ-dextran, high density B cells required the presence of IL-2 or IL-5 in addition to α-δ-dextran for antibody production. If sorting was used to highly purify the resting B cell population (IgM^+) the observed response to IL-2/α-δ-dextran was lost but could be completely restored if IL-2 propagated splenic NK cells were also added to the resting B cells. This finding was in line with earlier work done by Yuan et al. (Yuan et al., 1992) which showed that highly purified mouse splenic NK cells (IL-2 propagated) could induce FACS isolated resting B cell to proliferate and secrete IgM. This stimulation was also found to depend on the addition of IL-2/IL-5, was not dependent upon cell-cell contact, and although suggested to be a soluble factor was not IFN-γ, or TNF-α, as shown by neutralization with anti-cytokine antibodies. IFN-γ production from NK cells is however at least partly necessary for an increase in the percentage of CD86 expressing B cells in mouse spleen B-NK cell co-cultures. Resting B cells cultured with IL-2 propagated NK cells express a more than five-fold increase in the percentage of cells that express CD86, going from 10% to 55%. In contrast, only 25% of B cells expressed CD86 if NK cells from IFN-γ KO mice were used, and the addition of exogenous IFN-γ could restore the 55% expression level seen in the wild-type cultures (Yuan, 2004). NK cells express the IFN-γ receptor (Kawamura et al., 1999) and when treated with IL-2 have increased T-bet expression (Gao, Jennings, & Yuan, 2008) and the potential to secrete IFN-γ (Handa, Suzuki, Matsui, Shimizu, & Kumagai, 1983). Therefore IL-2 propagated NK cells which are cultured in IL-2 for 5-7 days might already be primed, possibly

in part due to an IFN-γ dependent manner, and increase in certain surface receptors that make them more amenable to act as partners to B cell activation.

IFN-γ is known to drive Ig-class switching to lead to the polarized production of the IgG2a isoform (Snapper, Peschel, & Paul, 1988). Yet Gao et al. (Gao, Dang, & Yuan, 2001) have shown that it is not a crucial component of the NK cell driven induction of Iγ2a transcripts in mouse cells, as even IFN-γ KO NK cells were sufficient. This study, and a series of others by the same authors, did find a contact dependent feature of this productive interaction that did not include CD40-CD40L interaction, or CD28 ligation. Instead CD48 on B cells and its corresponding receptor CD2 (LFA-2) on NK cells were necessary for driving Iγ2a transcripts (Gao, Schwartzberg, Wilder, Blazar, & Yuan, 2006) that would prime the B cells for further BCR encounter or TLR ligation and result in final IgG2a release (Gao et al., 2008). Contact dependency was also shown for the ability of mouse splenic IL-2-stimulated NK cells to enhance B cell TD antigen processing and presentation to T cells (Jennings & Yuan, 2009), although the surface proteins involved in this process were not examined.

The NK cell mediated suppression of B cell function observed with mouse cells by Michael et al. (Michael et al., 1989) was shown to be dependent upon IFN-γ. While low density B cells were able to induce IFN-γ release from the IL-2 propagated NK cells, high density B cells could not. If the high density B cells were first stimulated via crosslinking of their IgM molecules for 24 h, they were able to induce IFN-γ from NK cells when placed in co-culture. The same could be seen if LPS was used as a stimulator. They also showed that NK cells directly recognized surface proteins on primed/activated B cells that were missing on resting B cells. These results clearly suggested that the B cells themselves were involved in dictating the

response and function of the NK cells they interacted with. The same group (Yuan, Koh, & Wilder, 1994) would later show that some tumour B cell lines like BCL1-11 were also inducers of NK cell IFN-γ both in vitro and in vivo (Koh & Yuan, 1997).

Considering that the production of IFN-γ is tightly regulated in NK cells and seems to require more than one signal, B cells should be able to appropriately activate NK cells by providing cytokine/contact dependent factors. Wyatt and Dawson (Wyatt & Dawson, 1991) used human autologous tonsillar NK cells cultured with various populations of tonsillar B cells and looked at their ability to form conjugates using cold target inhibitor assays. They found that low density B could form productive conjugates with NK cells led to the release of IFN-γ. They examined the surface expression of various markers on the low density B cells and found that they highly express ICAM-1 and costimulatory B7 molecules. Previous work had also shown a role for ICAM-1-LFA-1 interaction in promoting the IFN-γ response in B-NK cell (Becker, Kolanus, Lonnemann, & Schmidt, 1990).

B cells also induce other cytokines from NK cells. Gao et al. (Gao et al., 2006) found MZ, and not follicular B cells, from mouse spleen were able to induce IL-13 production from IL-2 propagated NK cells in co-cultures. This was contact dependent as transwell separation of the cell types lead to a loss of IL-13. Neutralization experiments showed neither CD28 nor CD40L were needed but CD48 neutralization in co-culture or the use of CD48KO B cells lead to a dramatic reduction in IL-13. Unlike their previous study which showed CD2 on the NK cells to be the necessary receptor for CD48, CD244 (2B4) on NK cells was key to the production of IL-13 induced by B cells, but not to IFN-γ as its production was not affected by

the loss of CD244 (via both neutralization and knock-out). Similar results were obtained if NK cells from SAP-KO mice were used as SAP is required for CD244-mediated NK cell activation.

Thus far the findings can be summarized to define IL-2 activated NK cells as regulators of B cell proliferation and/or antibody secretion and differentiation. Whether they activate or suppress B cell function is highly dependent upon the activation status of the B cell as well as the presence or absence of B cell stimulators and the nature of these stimulators. B cells are also poised to dictate the response of the NK cell that it interacts with. B cells that are more activated or at least primed are better at forming conjugates with NK cells and seem better at inducing the production of cytokines shown through the provision of coordinated contact dependent mechanisms. Therefore, this relationship might resemble that between DC-NK cells in which each cell type can influence the responses of the other through a mutual cross-talk. The need for DC derived soluble and contact dependent signals for optimal NK cell response in vitro and in vivo during actual infection has been discussed (section 1.1.5). There are reports that also show DC may either mature or be killed after interaction with NK cells. The outcome in the DC-NK cell interaction like that seen in the B-NK cell interaction depends on the differentiation status of the DC cell, but in this case it is the immature DC that are edited out by activated NK cells (Della Chiesa et al., 2003). B cells have the potential to secrete a spectrum of immune enhancing or suppressing cytokines (see section 1.2.2.3). Whether B-NK cell interaction is influenced by any of the cytokines that activated B cells likely secrete has not been shown. The need for contact does not necessarily negate the need for soluble factors as some cytokines are released into the immune interface or synapse and therefore secreted in low levels, while other cytokines require contact dependent presentation.

A potential complication associated with some of the aforementioned studies is that the NK cells utilized were propagated in IL-2 or obtained through cloning protocols involving long term cultures. An example of such a cloning protocol is the use of EBV-infected B cells to support NK cell growth and viability. This system likely has endogenous IL-12 being secreted as EBV infected B cell were the first described source of IL-12 (Kobayashi et al., 1989). IL-2 cultured NK cells are phenotypically and functionally different than resting NK cells from either human peripheral blood or mouse spleen, and are considered to be primed. Would the same interaction between B and NK cells hold if resting NK cells had been used? Similarly there is variability amongst the studies in which B cells are being used (low versus high density, spleen versus blood) and how they are activated during the course of the experiments.

It is expected that as a component of the early innate response, an NK cell (especially a primed or activated one) would be able to influence the functional activity of later acting adaptive cells like B cells. What has not been investigated in the aforementioned studies is whether B cells are able to influence the activity of resting NK cells; this is the primary focus of this thesis.

1.4 Objective of the thesis

As was earlier described, IL-12 is the primary inducer of IFN-γ and Th1 response that functions during the early innate phase of the immune response to a variety of pathogens. This has been the impetus for many experimental and clinical studies that have looked into the utility of IL-12 as an inducer of inflammation, and as a thus a potential therapeutic against pathogenic infection and tumour development/growth. NK cells are the primary source of IFN-γ during the

early phase of the immune response where IL-12 is active, but our initial results showed that IL-12 on its own did not activate IFN-γ production from highly purified and resting mouse NK cells. We initiated this project with the intent of identifying the cell types involved in mediating the IFN-γ inducing effects of IL-12.

I hypothesized that a cell type constitutively expressing the IL-12 receptor, such as DC, Mϕ, and NKT cells or B cells, would act as the primary targets for initial exogenous/endogenous IL-12 stimulation. These cells would then become activated to support the secondary stimulation of NK cells through soluble or contact-dependent factors. In Chapter 2 I tested this hypothesis by using recombinant IL-12 to stimulate cultures of murine unfractionated spleen cells depleted for various populations of interest, B cell deficient mice (BKO), or co-cultures of highly purified and resting B and NK cells, and looking at IFN-γ production from NK cells. The objective of the subsequent study presented in Chapter 3 was to examine whether B cells would be involved in regulating the NK cell cytokine response to endogenously produced IL-12. I met this objective in Chapter 3 by using the TLR9 agonist CpG ODN (type A) to stimulate the secretion of endogenous levels of IL-12 and looked at NK cell IFN-γ in unfractionated and B cell depleted-cell cultures, and B cell deficient mice.

Chapter 2 An accessory role for B cells in the IL-12 induced activation of resting mouse NK cells

A version of Chapter 2 has been published. Haddad, E., Senger, L., and Takei, F. (2009) An accessory role for B cells in IL-12 induced activation of resting mouse NK cells. J. Immunology. 183:3608-3615

2. 1 Introduction

IL-12 is a prototypic pro-inflammatory cytokine produced by dendritic cells (DCs), monocytes/macrophages and activated B cells in the early phase of an immune response to infection (Trinchieri, 2003). The importance of IL-12 in immunity has been demonstrated by the profound susceptibility of IL-12-deficient humans and mice to various pathogens (Magram, Connaughton, Warrier, Carvajal, Wu, Ferrante, Stewart, Sarmiento, Faherty, & Gately, 1996a; Mansouri et al., 2005; Rosenzweig & Holland, 2005). IL-12 is a potent inducer of IFN-γ production, which plays a pivotal role in the initiation of inflammation and the establishment of protective Th1 responses (Hsieh et al., 1993; Manetti et al., 1993). IL-12 has also been used as a therapeutic reagent against various microbial diseases (Holland, 2000; Murray & Hariprashad, 1995), and more recently has proven to have potent anti-tumor/anti-metastasis effects when administered to experimental animals and patients (Brunda et al., 1993; Colombo & Trinchieri, 2002; Heinzerling et al., 2005; Mahvi et al., 2007). The anti-cancer effects of IL-12 are primarily mediated by IFN-γ. T cells, NK cells, and NKT cells, are thought to be the primary producers of IFN-γ in response to IL-12 (Voest et al., 1995; Wigginton et al., 2001). Yet, resting human and mouse NK cells and T cells, express nil to low levels of the IL-12R, and only express appreciable levels of IL-12R after activation (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006b; Kawamura et al., 1999). Thus, the mechanisms by which resting NK cells are stimulated with IL-12 remain unclear.

IL-18 is another pro-inflammatory cytokine, initially identified as IFN-γ-inducing cytokine (Dinarello, 1999; Okamura et al., 1995). It is produced as an inactive precursor, which must be cleaved to become biologically active, and is secreted by a variety of cells including

epithelial cells, dendritic cells and macrophages (Dinarello, 1998). Like IL-12, IL-18 is a strong inducer of IFN-γ production from NK cells and T cells and is known to be involved in autoimmune and inflammatory diseases (Dinarello, 2006). While IL-18 alone is not capable of driving IFN-γ production, it has strong synergistic effects with IL-12, and the combination of these two cytokines induces large amounts of IFN-γ production (Fehniger et al., 1999). Moreover, Fantuzzi et al. (Fantuzzi, Reed, & Dinarello, 1999) have reported that both the neutralization of IL-18 and caspase 1-deficiency, which mediates the cleavage of the IL-18 precursor to active IL-18, significantly reduce the production of IFN-γ in mice receiving IL-12. These results suggest that IL-18 plays a role in the IL-12-induced production of IFN-γ . Recently, it has been reported that stimulation of ex vivo NK cells with IL-12 requires in vivo priming with IL-18 (Chaix, Tessmer, Hoebe, Fuseri, Ryffel, Dalod, Alexopoulou, Beutler, Brossay, Vivier, & Walzer, 2008b).

To elucidate the mechanisms by which resting NK cells are stimulated by IL-12, we stimulated spleen cells from normal mice with a suboptimal dose of IL-12 alone. After characterizing the cells producing IFN-γ, we tested the effects of IL-12 on various purified cell populations. We also examined mutant mice and carried out cell depletion experiments to determine the requirements for IL-12-induced IFN-γ production. Our results show that B cells are unexpectedly required for IL-12-induced IFN-γ production by NK cells.

2.2 Materials and methods

2.2.1 Mice

Wild type (WT) and TCRβ/TCRδ double knockout (KO) (TCRβδKO) C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbour, ME) and bred pathogen free in our animal facility. IL-18KO and B cell-deficient (BKO) (*Igh-6*^{tm1Cgn}) B6 mice were also purchased from the Jackson Laboratory and housed in our animal facility. Male or female 8-12 wk old pathogen free mice were used in this study. All animal use was approved by the animal care committee of the University of British Columbia, and animals were maintained and euthanized under humane conditions in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2 Antibodies, cytokines and media

Anti-CD16/CD32 FcRγ (III/II) (2.4G2) (American Type Culture Collection, Manassas, VA) was purified from hybridoma supernatant. PE, FITC, allophycocyanin, or Peridinin-chlorophyll protein (PerCP)-Cy5.5 conjugated mAbs to NK1.1, CD11c, CD3ε, CD19, B220, CD11b, F4/80, IFN-γ, and matching isotype controls were purchased from BD-Biosciences (Mississauga, ON). Mouse recombinant IL-12 was purchased from Stem Cell Technologies (Vancouver, BC). Mouse recombinant IL-18 was purchased from Biovision (Mountain View, CA). RPMI 1640 media (Stem Cell Technologies) supplemented with 10% FBS (GIBCO®, Burlington, ON), penicillin, streptomycin (Stem Cell Technologies), and 5×10^{-5} M 2-mercaptoethanol (Sigma, Aldrich, Oakville, ON) was used for cell culture.

2.2.3 Preparation of splenocytes and unfractionated cultures

Freshly isolated spleens were ground through a 70-µm nylon sieve to prepare a single cell suspension and washed with PBS. Red blood cells were lysed with ammonium chloride solution and cells were washed twice. For unfractionated cultures, cells were dispensed at a density of 1×10^6 cells/ml in 96-well plates and cultured with IL-12 (1 ng/ml) plus/minus IL-18 (10 ng/ml) for 48 h. To prepare cells for sorting, splenoyctes were treated with 2.4G2 mAb to block Fc receptors, washed, and then stained with appropriate antibodies for 30 minutes. To exclude dead cells, propidium iodide was added to a final concentration of 5 µg/ml.

2.2.4 Intracellular cytokine staining

Splenocytes were cultured at a density of 4×10^6/ml in 14 ml round bottom polypropylene tubes and treated with IL-12 (1 ng/ml) plus/minus IL-18 (10 ng/ml) for 12-48 h at 37°C. Brefeldin A was added during the final 6 h of culture. Cells were stained with appropriate mAb prior to being fixed and permeabilized with a Cytofix/Cytoperm^{TM} Plus kit (BD-Biosciences) as per the manufacturer's instructions. Permeabilized cells were then stained with allophycocyanin-conjugated anti-IFN-γ mAb. A FACSCalibur (BD-Biosciences) was used for acquisition, and FLOWJO software (BD-Biosciences) was used for analysis.

2.2.5 Cell depletion experiments

B6 splenocytes were stained for the depletion of various cell populations as follows: $CD11c^+NK1.1^-$ for DC, $CD11b^+F4/80^+CD11c^-NK1.1^-$ for macrophages, and $CD19^+$ for B cells. A FACS VantageSE (BD-Biosciences) was used to sort the DC, macrophages or B cell populations and the remaining population that was considered 'depleted' of the population of

interest. Unfractionated splenocytes stained with mAbs but not sorted were used as a control. For add-back experiments, cell populations of interest and depleted cells were mixed at the same ratio as that in the original unfractionated spleen population. For IL-12 stimulation, 2.5×10^5 cells were cultured with 1 ng/ml IL-12 in 200 μl media in 96 round bottom plates for 4 days.

2.2.6 DC isolation and co-culture

WT splenocytes were stained with PE-conjugated anti-CD11c, and $CD11c^+$ cells (DCs) were isolated using a Stem Cell Technologies EasySep® Murine PE-positive selection kit (Stem Cell Technologies). DCs (6×10^3) were cultured alone or mixed with 3×10^4 NK or $NK1.1^+$T cells in 96-well round bottom tissue culture plates (BD-Falcon) in a total volume of 200 μl. IL-12 (1ng/ml) was added at the beginning of culture and supernatants were harvested after 48 h.

2.2.7 B and NK cell co-cultures

WT and IL-18KO B6 mouse splenocytes were stained with mAbs and NK cells ($NK1.1^+CD3^-$), or B cells ($CD19^+$) were purified by two rounds of cell sorting to a purity of 95-98%. Cells were cultured in 96 well round bottom plates at varying ratios of B to NK cells with the NK cell number fixed at 3×10^4 cells in a final volume of 200 μl. Cultures were stimulated with IL-12 (1 ng/ml) for 48 h. For transwell cultures, purified B and NK cells were cultured at 4:1 or 10:1 ratios in a final volume of 600 μl in 24 well plates with cell-impermeable (0.4 μm pore-size) transwell inserts (BD-Falcon). NK cells (9×10^4 cells) in the inserts and B cells in bottom wells were stimulated with IL-12 (1 ng/ml) for 4 days, unless otherwise indicated.

2.2.8 In vivo IL-12 injection

IL-12 (1 μg) in 200 μl PBS containing 0.1% endotoxin-free bovine serum albumin (BSA) (Sigma-Aldrich) was administered i.p into each mouse daily for three consecutive days. Control mice received 3 injections of PBS-BSA. One day after the final injection, blood was collected via cardiac puncture for serum cytokine analysis.

2.2.9 IFN-γ and IL-18 ELISA

Cytokines in culture supernatants or sera were measured by ELISA for IFN-γ (eBiosciences, San Diego, CA, or BD Opteia, BD-Biosciences) and IL-18 (MBL Inc., Woburn, MA) following manufacturers' protocols. Values below 15-25 pg/ml of cytokine were not detected.

2.2.10 Statistical analysis

All statistical analyses of experimental mean values were performed using the Student's paired *t* test. Values of $p<0.05$ were considered significant.

2.3 Results

2.3.1 NK1.1$^+$ cells are the primary producers of IFN-γ among IL-12 stimulated unfractionated spleen cells

When 10^6 cells/ml unfractionated splenocytes from naïve B6 mice were stimulated with 1 ng/ml of IL-12 for 24 h, ~1.5 ng/ml of IFN-γ was detected in culture supernatants, and higher concentrations of IL-12 (10 ng/ml) did not induce more IFN-γ (Figure 5A). We therefore used 1 ng/ml of IL-12 for all further experiments. In order to identify early responders to IL-12, unfractionated spleen cells were stimulated for a total of 12 h, stained for surface markers, and analyzed for intracellular IFN-γ (Fig. 5B). The IFN-γ response was limited to the NK1.1$^+$ compartment in spleen. About 10% of NK (NK1.1$^+$CD3$^-$) and 7% of NKT (NK1.1$^+$CD3$^+$) cells were positive for intracellular IFN-γ, while no T cells (NK1.1$^-$CD3$^+$) or other spleen cells (NK1.1$^-$CD3$^-$) were IFN-γ$^+$ (Fig. 5B). Similar results were obtained with longer periods (48 and 72 h) of IL-12 stimulation (Appendix 1). IL-12 and IL-18, which are known to synergize and induce high levels of IFN-γ secretion from NK and T cells (Lauwerys, Renauld, & Houssiau, 1999), were also used to stimulate resting splenocytes. The combination of IL-12 and IL-18 induced a much higher percentage of NK cells to become IFN-γ$^+$ than stimulation with IL-12 alone (Fig. 5B). The effect on NK1.1$^+$ T cells was only marginally higher than that with IL-12 alone. The percentage of IFN-γ producing cells amongst IL-12-stimulated splenocytes was consistently about 10%. To confirm that NK1.1$^+$ cells (NK cells and NK1.1$^+$ T cells) were indeed the source of the IFN-γ detected in unfractionated spleen cultures stimulated with IL-12, NK1.1$^+$ cells were depleted from unfractionated splenocyte populations. Depletion of all NK1.1$^+$ cells from unfractionated spleen cells resulted in an almost complete loss of IFN-γ secretion (Fig. 5C). Adding NK1.1$^+$ cells back to the depleted population restored the

production of IFN-γ to a level similar to that from undepleted cells ($p>0.1$). These results show that stimulation of unfractionated spleen cells with IL-12 induces IFN-γ production by NK and $NK1.1^+$ T cells but not T cells or other spleen cells. $NK1.1^+$ cells comprise ~4% of splenocytes and only 7-10% of them produce IFN-γ upon stimulation with IL-12. Thus, about 4×10^3/ml $NK1.1^+$ cells among 10^6/ml unfractionated splenocyte cultured with IL-12 produced almost 1,500 pg/ml (~0.4 pg/cell) IFN-γ.

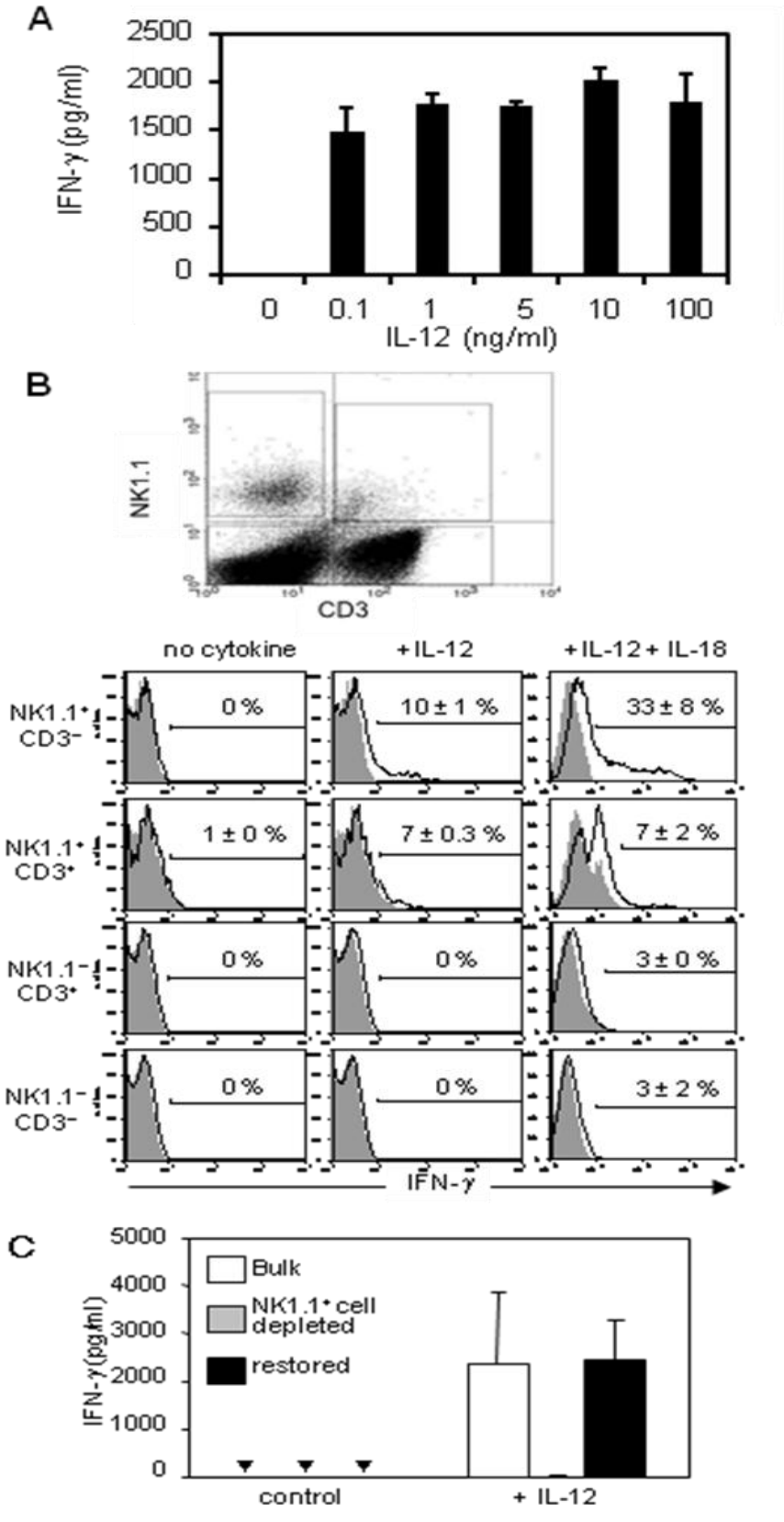
A
IFN-γ (pg/ml)
2500
2000
1500
1000
500
0
0
0.1
1
5
10
100
IL-12 (ng/ml)
B
NK1.1
CD3
no cytokine
+IL-12
+IL-12+ IL-18
NK1.1+ CD3−
NK1.1+ CD3+
NK1.1− CD3+
NK1.1− CD3−
0 %
10 ± 1 %
33 ± 8 %
1 ± 0 %
7 ± 0.3 %
7 ± 2 %
0 %
0 %
3 ± 0 %
0 %
0 %
3 ± 2 %
IFN-γ
C
IFN-γ (pg/ml)
5000
4000
3000
2000
1000
0
Bulk
NK1.1+ cell depleted
restored
control
+ IL-12

Figure 5 IL-12 stimulates IFN-γ production mainly from NK1.1$^+$ cells in unfractionated spleen

A. Unfractionated B6 splenocytes (1×10^6/ml) were cultured with varying doses of IL-12 in 96-well round bottom tissue culture plates for 48h. Cell free supernatants were assessed for IFN-γ by ELISA. Results are the mean ± SEM of three independent experiments, each with triplicate cultures. *B.* Unfractionated B6 mouse splenocytes (4×10^6/ml) were cultured with or without IL-12 (1 ng/ml) alone or with IL-18 (10 ng/ml), stained with mAbs to NK1.1 and CD3ε, fixed, permeabilized, stained for intracellular IFN-γ and analyzed by FACS. Individual populations were gated as shown in the dot plot and analyzed for intracellular IFN-γ. Filled histograms show isotype-matched control antibody staining and empty histograms show IFN-γ staining. The data are representative of at least three independent experiments, and the numbers indicate the percentages of positively stained cells (mean ± SD, n=3). *C.* NK1.1$^+$ and NK1.1$^-$ cells were FACS sorted from B6 mouse splencoytes. NK1.1$^-$ cells (2×10^5) were used as 'NK1.1$^+$ depleted' population, while 'NK1.1$^+$ restored' population was 4×10^3 NK1.1$^+$ cells combined with 1.96×10^5 NK1.1$^-$ cells. Stained unsorted splenocytes (2×10^5) were used as 'unfractionated'. Cells were stimulated as shown and cultured for 48 h. IFN-γ in the culture supernatants was analyzed by ELISA, and the results are the mean ± SEM of three independent experiments, each with triplicate cultures. (▼) denotes undetectable cytokine levels.

2.3.2 Purified NK cells are not stimulated with IL-12 alone

Although the above results showed that NK1.1$^+$ cells were the source of IL-12-induced IFN-γ, purified NK cells (3×10^5/ml) stimulated with IL-12 produced only very small amount (60-150 pg/ml) of IFN-γ (Fig. 6A, left). Purified NK1.1$^+$ T cells stimulated with IL-12 alone produced a significantly higher level (500-1000 pg/ml) of IFN-γ (Fig. 6A). Both populations produced large amounts of IFN-γ (10 - 40 ng/ml) when both IL-12 and IL-18 were added (Fig. 6A, right). These results suggested that IFN-γ produced by IL-12-stimulated unfractionated splenocytes might derive mostly from NK1.1$^+$ T cells while NK cells might secrete very little IFN-γ. Alternatively, the stimulation of NK cells with IL-12 is indirect and requires other cells. Resting NK cells do not express the IL-12R to appreciable levels (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006a), while NKT cells express the IL-12R constitutively (Kawamura et al., 1999). Therefore, IL-12 might primarily stimulate NKT cells and induce production of cytokines, which in turn stimulates NK cells. To test these possibilities, spleen

cells from TCRβδ-double KO mice, which lack all T cells including NK1.1$^+$ T cells, were tested. Unfractionated spleen cells from TCRβδ-double KO mice stimulated with IL-12 produced the same amount of IFN-γ as WT spleen cells ($p>0.1$, $n=9$) (Fig. 6B), indicating that NK cells can be stimulated with IL-12 to produce IFN-γ in the absence of NKT cells.

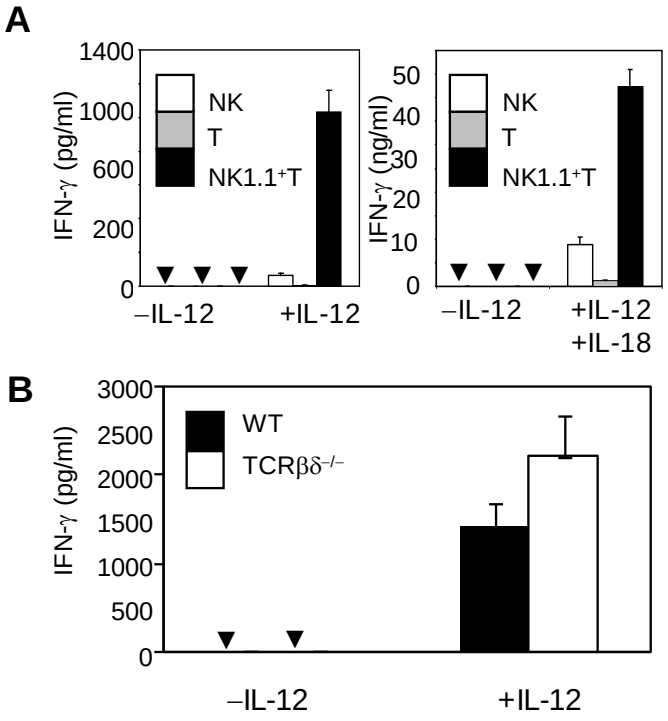

Figure 6 NK1.1$^+$ T cells are not required for IL-12 induced IFN-γ production by unfractionated splenocytes

A. NK1.1$^+$T (NK1.1$^+$CD3ε$^+$), NK (NK1.1$^+$CD3e$^-$), and T (NK1.1$^-$CD3ε$^+$) cells were purified by FACS sorting from B6 mouse splenocytes. The purified cells (3×10^4 in 200 μl media) were stimulated for 48 h as indicated. IFN-γ in the culture supernatants was analyzed by ELISA. The results are the mean ± SEM of 4 independent experiments, each with triplicate cultures. *B.* Unfractionated splenocytes from WT and TCRβδ KO mice were stimulated with 1 ng/ml IL-12 for 48 h, and IFN-γ in the culture supernatants was analyzed. The results are the mean ± SEM of 5 independent experiments, each with triplicate cultures. (▼) denotes undetectable cytokine levels.

2.3.3 DC and macrophages are not required for the stimulation of NK cells by IL-12

The above results suggested that the stimulation of NK cells with IL-12 requires the presence of other cells. Because the importance of cellular interaction with DCs for the stimulation of NK cells and NKT cells has been demonstrated (Gumperz, 2004; A. Moretta et al., 2005; Walzer et al., 2005), we first investigated whether DCs are required. Some NK cells express CD11c (C. W. Chan et al., 2006; Taieb et al., 2006), and $CD11c^+$ NK cells are preferentially stimulated by IL-12 (Appendix 2), we identified DCs by $CD11c^+NK1.1^-$ and divided unfractionated splenocytes into DCs and DC-depleted populations. DC-depleted spleen cells cultured for 4 days in the presence of IL-12 produced slightly lower level of IFN-γ than non-depleted control (Fig. 7A). Purified DCs stimulated with IL-12 did not produce a detectable level of IFN-γ, and adding back DCs to the depleted cells did not significantly increase ($p>0.1$, $n=9$) the level of IFN-γ production. Moreover, co-culturing highly purified NK cells and DCs in the presence of IL-12 for 2 days did not induce the production of a significant amount of IFN-γ, whereas DCs enhanced the production of IFN-γ by $NK1.1^+$ T cells by 2-fold (Fig. 7B). Thus, DCs promote $NK1.1^+$ T cell stimulation, but they do not seem to play a significant role in the stimulation of NK cells with IL-12. Like DCs, macrophages constitutively express the IL-12R (Watford, Moriguchi, Morinobu, & O'Shea, 2003). Therefore, we tested the effects of depletion of those cells on IL-12-induced IFN-γ production by unfractionated splenocytes. As some NK cells also express CD11b and CD11c, macrophages were identified as $CD11b^+F4/80^+CD11c^-NK1.1^-$. The depletion of macrophages had no effect ($p>0.1$, $n=9$) on the amount of IFN-γ being secreted in stimulated cultures (Fig. 7C).

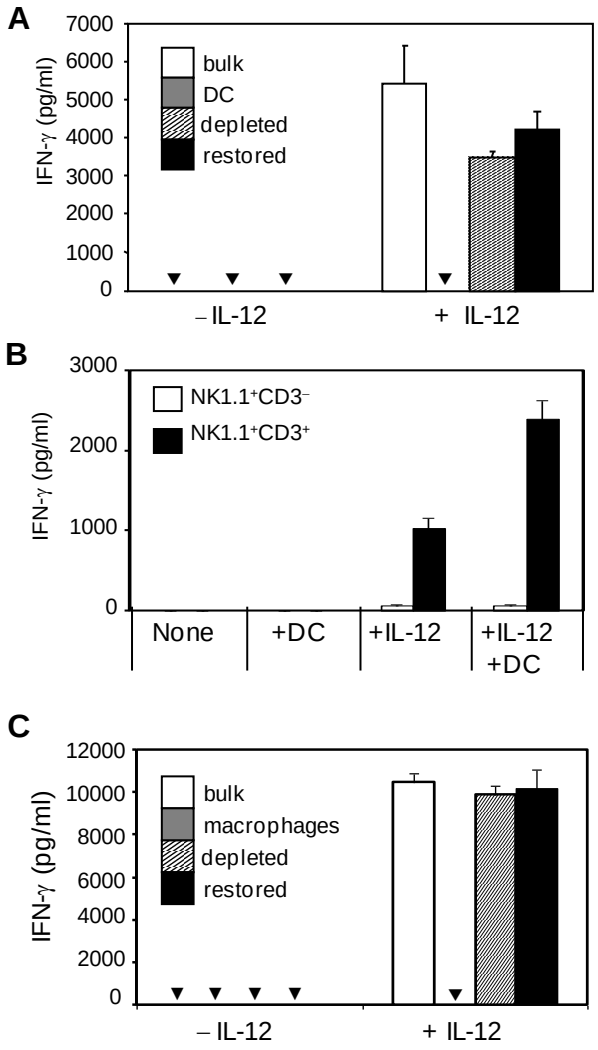

Figure 7 DC and Mϕ are not required for IL-12 induced IFN-γ production by unfractionated splenocytes

A. $CD11c^{+}NK1.1^{-}$ cells (DC) and $CD11c^{-}NK1.1^{+/-}$ cells were FACS sorted from B6 mouse splenocytes. $CD11c^{-}NK1.1^{+/-}$ cells were used as the 'depleted' population, while the 'restored' population was 2.5×10^{3} DC combined with (2.5×10^{5}) depleted cells to restore the same ratio as in the original unfractionated population. Stained unsorted splenocytes were used as 'unfractionated'. Unfractionated, depleted and restored cells and sorted DCs (2.5×10^{3}) were cultured with or without 1 ng/ml IL-12 in 200 μl media for 4 days. *B.* NK and $NK1.1^{+}$T cells were purified from B6 mouse splenocytes by FACS sorting. $CD11c^{+}$ spleen cells were purified with magnetic beads as in Materials and Methods and used as DC. NK and $NK1.1^{+}$ T cells (3×10^{5}) were cultured in the presence or absence of splenic $CD11c^{+}$ cells (6×10^{3}) with/without IL-12 for 48 h. *C.* $CD11b^{+}F4/80^{+}NK1.1^{-}CD11c^{-}$ cells (macrophages) and $CD11b^{-}F4/80^{-}NK1.1^{+/-}CD11c^{+/-}$ cells (depleted) were FACS sorted from B6 splenocytes. The 'restored' population was 3.75×10^{3} macrophages combined with 2.5×10^{5} depleted cells. All the results show the amounts of IFN-γ (mean ± SEM) in the culture supernatants of three or four independent experiments, each with triplicate cultures. (▼) denotes undetectable cytokine levels.

2.3.4 B cells are required and sufficient for IFN-γ induction from IL-12-stimulated NK cells

In contrast to the results from the DC and macrophage depletions, the removal of B cells ($CD19^+$) resulted in a striking loss of the IFN-γ production by IL-12-stimulated splenocytes, while adding B cells back to the B cell-depleted splenocytes significantly restored the production of IFN-γ (Fig. 8A). To further test the role of B cells in IL-12-mediated stimulation, splenocytes from BKO mice were stimulated with IL-12, and the amount of IFN-γ produced was compared to WT. As expected from the B cell depletion experiment, IL-12-stimulated BKO splenocytes secreted significantly less IFN-γ than WT splenocytes (Fig. 8B, left). BKO mouse spleens had slightly higher percentages of NK and NKT cells (Appendix 3), and BKO splenocytes produced larger amounts ($p<0.05$, $n=9$) of IFN-γ when stimulated with IL-12 plus IL-18 (Fig. 8B, right). Therefore, the low level of IFN-γ production by IL-12-stimulated BKO splenocytes was unlikely due to defective NK or NKT cells in BKO spleen. To determine whether B cells are sufficient for the stimulation of NK cells with IL-12, we highly purified (95-98%) NK and B cells from TCRβδ KO mice and co-cultured them with IL-12 at varying ratios of B to NK cells (2:1, 4:1, and 10:1), with the NK cell number fixed at 30,000/well (Fig.8C). The amounts of IFN-γ production increased in a dose dependent manner as the ratios of B to NK cells increased. Purified B cells without NK cells did not produce any IFN-γ with IL-12 stimulation. Additionally, we could detect low levels (37 ± 8 pg/ml, n=3) of TNF-α, another inflammatory cytokine, in the supernatants of these IL-12 stimulated B-NK co-cultures. NK cells or B cells cultured alone did not secrete any detectable TNF-α upon IL-12 stimulation. We also tested if B cells promote IL-12 induced NK cell cytoxicity. Our data show that IL-12 stimulated NK cells are poorly cytotoxic (Appendix 4). There was not a statistically significant

difference between the WT and BKO splenocytes with respect to killing ability after IL-12 stimulation (p=0.21).

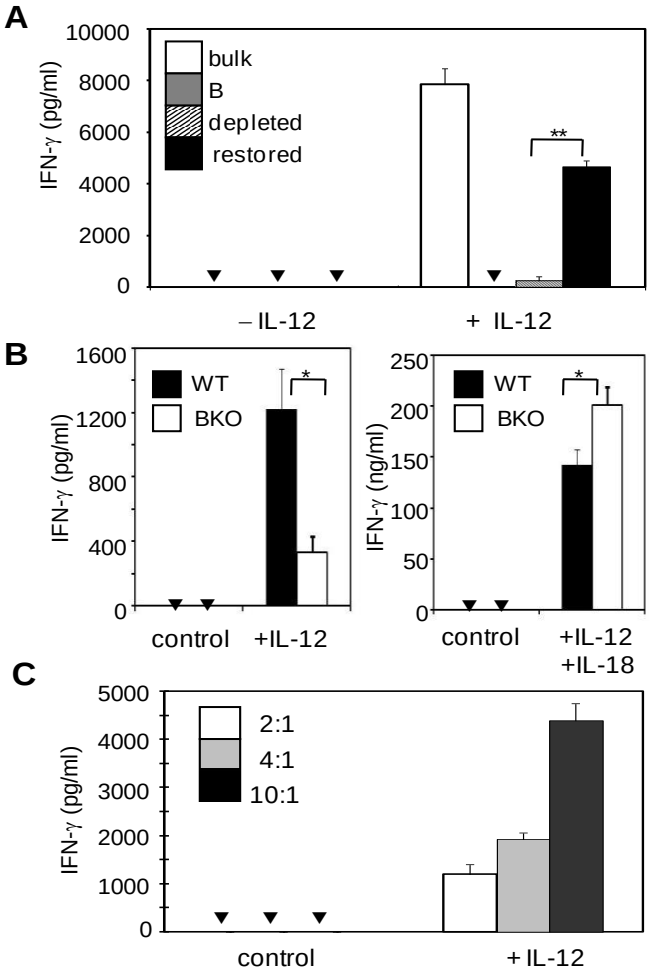

Figure 8 B cells are required for IFN-γ production from IL-12 treated unfractionated splenocytes and purified NK cells

A. $CD19^+$ cells and $CD19^-$ cells were FACS sorted from B6 splencoytes as 'B' and 'depleted' populations, respectively. For 'restored' population, 1×10^5 B cells were mixed with 1.5×10^5 depleted cells. Stained unsorted 'unfractionated' splenocytes (2.5×10^5), B cells (1×10^5), depleted cells (1.5×10^5) were cultured with 1 ng/ml IL-12 in 200 µl media for 4 days. *B.* Unfractionated splenocytes (2×10^5) from B6 and BKO mice were cultured with IL-12 plus/minus 10 ng/ml IL-18 for 48 h. *C*. B ($CD19^+$) cells and NK ($NK1.1^+CD3\varepsilon^-$) cells were purified from TCRβδ KO mouse splenocytes by FACS sorting, mixed at B:NK ratios of 2:1, 4:1, 10:1 and stimulated with 1 ng/ml IL-12 for 48 h. The results show the amounts of IFN-γ (mean ± SEM) in the culture supernatants of three independent experiments, each with triplicate cultures. (▼) denotes undetectable cytokine levels. *, P<0.05; **, P<0.01.

2.3.5 B cells provide endogenous IL-18 and cell contact in NK cell stimulation by IL-12

The above results showed that purified NK cells are not stimulated with IL-12 alone and require B cells whereas purified NK cells can be stimulated with a combination of IL-12 and IL-18 in the absence of B cells. Human tonsillar B cells constitutively express IL-18 mRNA (Airoldi et al., 2004), and mouse B cells secrete IL-18 upon Ly49D-crosslinking (Hart, Flaishon, & Shachar, 2007). Moreover, a recent study has shown that the in vivo priming of NK cells with IL-18 is important for the stimulation of NK cells with IL-12 (Chaix, Tessmer, Hoebe, Fuseri, Ryffel, Dalod, Alexopoulou, Beutler, Brossay, Vivier, & Walzer, 2008a). To clarify the role of IL-18 and B cells, we first tested unfractionated splenocytes from IL-18KO mice. IL-18KO splenocytes stimulated with IL-12 produced significantly lower levels of IFN-γ than WT splenocytes (Fig. 9A, left). This reduced response to IL-12 was not because of a functional deficiency in the IL-18KO splenocytes as they produced high levels of IFN-γ when stimulated with a combination of IL-12 and IL-18 (Fig.9A, right). To determine whether B cells play a role in the effect of IL-18 deficiency on NK cell stimulation, NK cells and B cells were purified from WT and IL-18KO splenocytes and co-cultured in various combinations in the presence of IL-12. WT B cells promoted both WT and IL-18KO NK cell stimulation with IL-12 whereas IL-18KO B cells did not have much effect on either WT or IL-18KO NK cells (Fig. 9B).

To determine whether cell contact between B cells and NK cells is also required for the stimulation of purified NK cells with IL-12, they were co-cultured in transwells. When NK cells and B cells were separated by a cell-impermeable membrane, only a small amount of IFN-γ was produced upon IL-12 stimulation, regardless of the B: NK ratios. Co-culturing NK and B cells in the same compartment, which facilitates cell-cell contact, resulted in the B cell dose dependent production of significantly higher amounts ($p<0.005$, $n=9$) of IFN-γ (Fig. 9C).

Therefore, B cell-NK cell physical contact appears important for the activation of NK cells by IL-12.

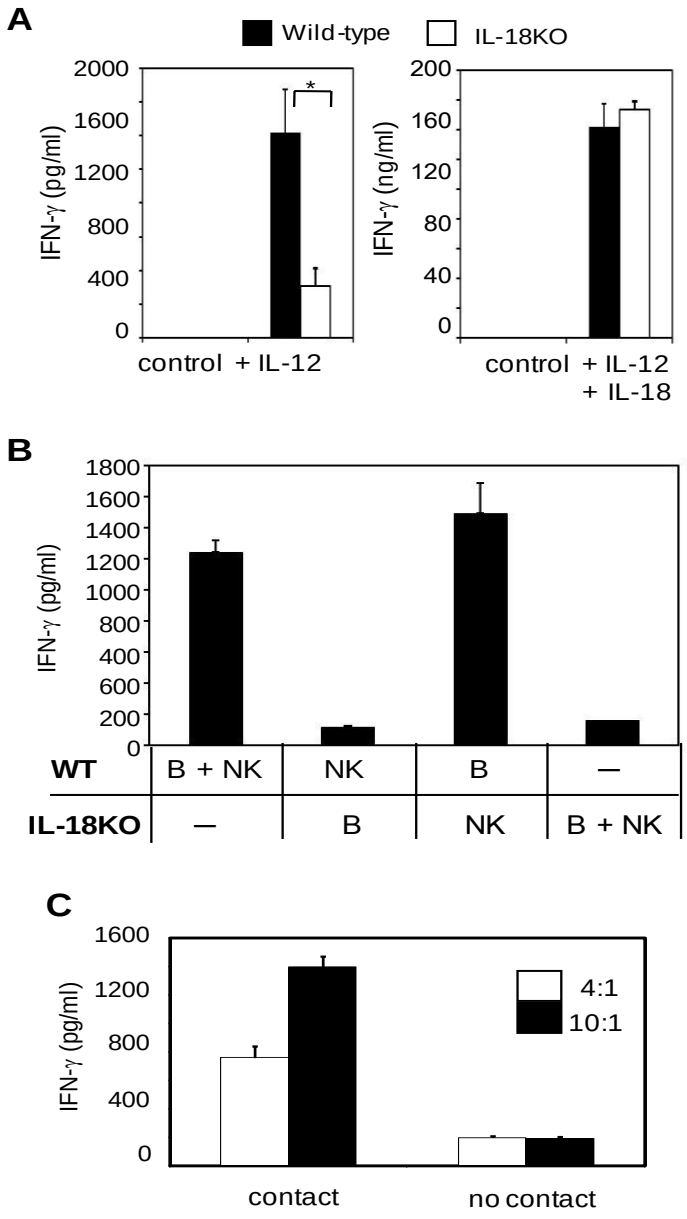

Figure 9 B cells provide IL-18 and contact dependent NK cell stimulatory signals during IL-12 activation

A. Unfractionated splenocytes (10^6/ml) from B6 and IL-18KO mice were cultured as in Fig. 2*B* for 48 h. *B*. B cells and NK cells were purified from WT and IL-18KO mouse splenocytes by FACS sorting as in Fig. 4*C*. Purified B cells (3×10^5) and NK cells (3×10^4) were combined and stimulated with IL-12 for 48 h. *C*. B cells and NK cells, purified from B6 mouse splenocytes were cultured in transwells as described in Materials and Methods. They were cultured with IL-12 in a total volume of 600 μl for 96 h. Results show the amounts of IFN-γ (mean ± SEM) in the culture supernatants from three independent experiments, each with triplicate cultures. (▼) denotes undetectable levels of cytokine. *, $P<0.05$.

2.3.6 B cell KO mice injected with IL-12 produce lower levels of IFN-γ and IL-18 than WT

The above results showed that B cells are necessary and also sufficient for the in vitro production of IFN-γ from NK cells induced with IL-12. To test whether B cells also play a role in an in vivo response to IL-12, we i.p. injected IL-12 into normal B6 and BKO mice and compared the levels of serum IFN-γ. Our preliminary studies showed that a daily injection of 1 μg per mouse for 3 consecutive days induced a significant level of IFN-γ in the serum of WT mice. Under these conditions, approximately 1,200 pg/ml of IFN-γ was detected in the sera of WT mice whereas a four-fold lower level of IFN-γ was detected in the sera of BKO mice (Fig. 10A). No IFN-γ was detected in the sera from vehicle (PBS plus 0.1% BSA)-injected WT or BKO mice. Since our in vitro results also suggested that endogenous IL-18 is important for IL-12-induced IFN-γ production, we also measured serum IL-18. Unlike IFN-γ, IL-18 was detected in the sera of vehicle-injected WT and BKO mice. Whereas the level of serum IL-18 increased significantly ($p<0.05$) following IL-12 injection in both WT and BKO mice, it was significantly lower ($p<0.01$, $n=4$) in BKO mice than WT mice (Fig. 10B).

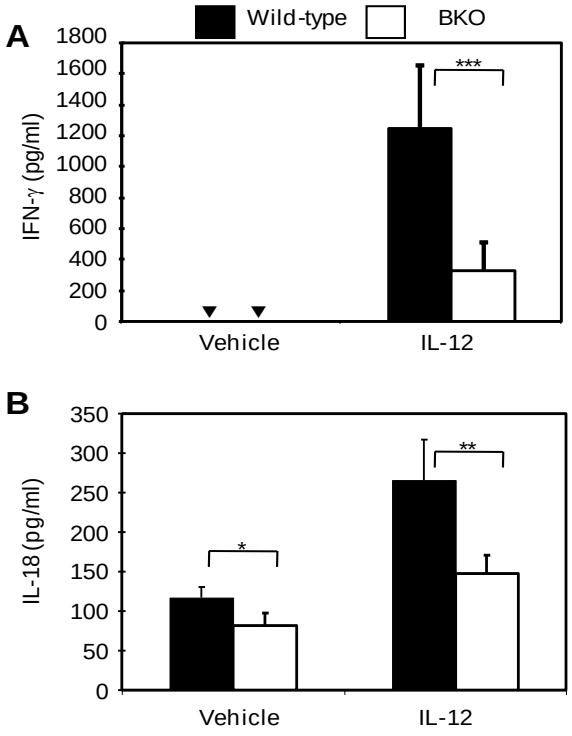

Figure 10 B cells are required for optimal IFN-γ and IL-18 production in vivo induced by IL-12 injection

B6 and BKO mice were i.p. injected with 1,000 ng of IL-12 or PBS as vehicle, for three consecutive days. Blood was collected 24 h after the last injection and serum levels of IFN-γ (*A).* and IL-18 (*B).* were analyzed by ELISA. The results are mean ± SD of 4 mice in each group. (▼) denotes undetectable levels of cytokine. *, P<0.05; **, P<0.01; ***, P<0.005

2.4 Discussion

The data presented above have revealed a novel role for B cells in the stimulation of mouse NK cells with IL-12 in vitro and in vivo. IL-12 is considered to be a potent stimulator of NK cells and stimulation of unfractionated spleen cells with a suboptimal dose of IL-12 alone induces IFN-γ production by NK cells and $NK1.1^+$ T cells. However, purified NK cells are not stimulated by IL-12 alone, whereas in the presence of B cells purified NK cells are stimulated

by IL-12 and secrete significant amounts of IFN-γ. B cell depletion from normal spleen inhibits IL-12-induced IFN-γ production, and spleen cells from B cell-deficient mice produce much lower levels of IFN-γ than WT spleen cells. Moreover purified B cells but not DCs support IL-12-induced IFN-γ production by purified NK cells. Therefore, B cells are necessary and also sufficient for NK cell stimulation with IL-12. Importantly, the critical role for B cells in IL-12-induced IFN-γ production is not restricted to in vitro cultures. Injection of IL-12 into B cell-deficient mice results in significantly lower levels of IFN-γ than those in WT mice. It has been proposed that this difference might be due to IFN-γ production by B cells (Thibodeaux et al., 1999). However, we do not observe IFN-γ secretion from B cells. It should also be noted that B cell-deficient spleen cells stimulated by IL-12 do produce some IFN-γ, albeit significantly less than WT spleen cells do. This is most probably due to a B cell-independent stimulation of $NK1.1^+$ T cells, as purified $NK1.1^+$ T cells are stimulated by IL-12 alone and produce IFN-γ. Similarly, the reduced IFN-γ production in B cell-deficient mice injected with IL-12 may also be due to $NK1.1^+$ T cell stimulation. Nevertheless, spleen cells from TCRβδ double KO mice, which lack all T cells including $NK1.1^+$ T cells, produce no less IFN-γ than WT spleen cells, indicating that NK cells are an important primary source of IFN-γ upon IL-12 stimulation.

The precise mechanisms by which B cells help NK cell stimulation with IL-12 are still unclear. As reported by Chaix et al. (Chaix, Tessmer, Hoebe, Fuseri, Ryffel, Dalod, Alexopoulou, Beutler, Brossay, Vivier, & Walzer, 2008b), spleen cells from IL-18-deficient mice stimulated by IL-12 produce significantly less IFN-γ than WT spleen cells. This has been suggested to be due to an in vivo priming of NK cells by IL-18. In our current study, a very

small amount of IFN-γ is produced by purified WT NK cells stimulated by IL-12 alone. Furthermore, NK cells from IL-18KO mice can be stimulated by IL-12 in the presence of WT B cells whereas WT NK cells are not stimulated by IL-12 in the presence of IL-18KO B cells. These results suggest that the production of IL-18 by B cells, rather than the in vivo priming of NK cells by IL-18, is critical. However, we have been unable to detect IL-18 in the cultures of purified NK cells and B cells stimulated with IL-12. It should be noted that purified NK cells treated with IL-12 require as little as 10 pg/ml of recombinant mouse IL-18 to produce the level (~1,500 pg/ml) of IFN-γ commonly seen in our cultures (Appendix 5). It is then possible that the level of IL-18 secreted by B cells in our cultures while sufficient for synergizing with IL-12 for IFN-γ is too low to be detected by ELISA. At the same time, our results with transwell cultures indicate that physical contact between the B cells and NK cells is required. Therefore, IL-18 secretion may not be the only mechanism by which B cells support IL-12-mediated NK cell stimulation. It is possible that for IL-18 production IL-12-stimulated B cells require contact-dependent co-stimulatory signals from the NK cells to produce IL-18. To examine this possibility, we have tested spleen cells from CD40-KO and CD80/86-double KO mice. These cultures produced only slightly lower levels of IFN-γ than WT cells upon IL-12 stimulation. An alternative possibility is that B cells may not only produce IL-18 but also present it to NK cells, perhaps via the IL-18R. The IL-18R consists of two chains, the α-chain that binds IL-18 and the β-chain that does not efficiently bind IL-18 but associates with the complex of IL-18/IL-18Rα and transduces signals (Kato et al., 2003). To test whether B cell-derived IL-18 is bound to IL-18Rα on B cells and presented to NK cells expressing IL-18Rα, we have tested IL-18Rα-KO mouse spleen cells and found that IL-18Rα-deficiency does not have a significant effect on B cell-NK cell cooperation in IL-12 stimulation (Appendix 6). Therefore, it is unlikely that B cells trans-present IL-18 to NK cells. Recently, human DCs have been shown to make contact with

NK cells and secrete IL-18 into the cleft at the contact site (Semino et al., 2005). Whether B cells also form similar contact with NK cells remains to be determined.

In 1991, Wyatt and Dawson (Wyatt & Dawson, 1991) showed that human B cells could form conjugates with NK cells, which resulted in the release of increased levels of IFN-γ. Yuan et al. (Michael et al., 1989; Yuan, 2004) also found that in vivo activated splenic B cells induce IFN-γ production by IL-2-stimulated NK cells, and this was dependent upon direct physical contact between the B and NK cells. However, it should be noted that the combination of IL-2 and IL-12 or IL-18 strongly stimulates NK cells and induces IFN-γ production without B cells. In our current study, resting NK cells and B cells cooperate in NK cell stimulation induced by IL-12.

It is unexpected that B cells, rather than DCs, are critical for the IL-12-mediated stimulation of NK cells. It has been shown that DCs promote NK cell stimulation by trans-presenting IL-15 (Lucas et al., 2007). IL-12 also stimulates IL-15 production by DCs (Ohteki et al., 2006). However, in our study with IL-12, DCs show no effect on IL-12-mediated NK cell stimulation. It should be noted that DCs used for most studies on NK-DC interaction are generated in vitro whereas DCs in our current study are freshly isolated $CD11c^+$ cells. DCs thus purified from normal spleen do not seem to be stimulated by IL-12 as they do not support NK cell stimulation with IL-12. Even stimulation with IL-12 plus IL-18, which strongly stimulates NK cells and $NK1.1^+$ T cells, does not induce much IFN-γ production by DCs. Thus, although DCs are known to be the major sources of IL-12 and IL-18 (D. P. Barr et al., 2007; Stober, Schirmbeck, & Reimann, 2001) they do not seem to produce IL-18 when stimulated with IL-12

alone. Monocytes and macrophages have also been shown to be important partners to NK cell IFN-γ production. In response to TLR ligands (Carson, Ross et al., 1995; Kloss et al., 2008; Yoshimoto et al., 1998) or pathogens (Baratin et al., 2005; Lapaque, Walzer, Meresse, Vivier, & Trowsdale, 2009), monocytes and macrophages can upregulate NK cell stimulatory factors like NKG2D ligands, IL-15, IL-12 and/or IL-18 and activate NK cells. However, our depletion of monocytes/macrophages had no effect on the IL-12-induced IFN-γ production by NK cells; most likely because monocytes/macrophages are not stimulated with IL-12 alone.

IL-12 is often used to enhance immune responses against infections and cancer. The adjuvant effect of IL-12 is thought to be primarily mediated by IFN-γ. NK cells, NKT cells and T cells have been reported to produce IFN-γ upon stimulation by IL-12 (Nakahira, Ahn, Park, Gao, Tomura, Park, Hamaoka, Ohta, Kurimoto, & Fujiwara, 2002b; Ortaldo et al., 2006; Smyth et al., 2002; Takeda et al., 2000)(Nakahira, Ahn, Park, Gao, Tomura, Park, Hamaoka, Ohta, Kurimoto, & Fujiwara, 2002a; Ortaldo et al., 2006)(Nakahira, Ahn, Park, Gao, Tomura, Park, Hamaoka, Ohta, Kurimoto, & Fujiwara, 2002a; Ortaldo et al., 2006), but our results have clearly shown that NK cells and NK1.1^{+} T cells are the only significant IFN-γ producers upon IL-12 stimulation. Furthermore, our finding that B cells are critical for IFN-γ production by NK cells in vitro as well as in vivo have important implications to the clinical use of IL-12.

Chapter 3 IL-10 secreting B cells suppress CpG ODN induced NK cell activation

3.1 Introduction

NK cells are innate lymphocytes that have the ability to kill transformed or infected cells and secrete immunomodulating cytokines. Activated NK cells rapidly secrete high levels of IFN-γ, a cytokine that is critical to establishing an inflammatory immune environment that controls pathogens and tumor spread. Current experimental and clinical regimens that aim to augment IFN-γ levels often use adjuvants that activate NK cells either directly or indirectly. Bacterial DNA is a potent inducer of NK cell IFN-γ (Cowdery, Chace, Yi, & Krieg, 1996), and short single-stranded oligodeoxynucleotides containing cytosine-guanine dinucleotide motifs (CpG ODNs), are synthetic mimics of bacterial unmethylated DNA that have also been used to induce IFN-γ from NK cells (Roda et al., 2005). CpG ODNs induce a potent Th1 polarized response which is dependent on the production of IL-12 (Krieg, Love-Homan, Yi, & Harty, 1998). CpG ODNs bind to TLR 9 located in the endosome of B cells and plasmacytoid DC (pDC) in humans. In mice TLR9 is expressed on a broader array of immune cells, including B cells, macrophages, myeloid DC and pDC (Akira et al., 2001; Gururajan, Jacob, & Pulendran, 2007). Synthetic CpG ODNs are one of three types, A, B or C, which vary in their structural attributes, as well as in their stimulatory targets and biological effects. A-type CpG-ODNs have a central palindromic region of unmethylated CpG motifs on a phosphodiester backbone, capped at each end with a phosphorothioate poly-G string. These CpG-ODNs preferentially stimulate plasmacytoid DCs (pDCs) to secrete IFN-α (in humans and mice), and IL-12 from macrophages (in mice only). B-type CpG-ODNs have only the nuclease resistant phosphorothioate backbone with one or more CpG dinucleotides. While they are strong activators of B cell proliferation, maturation, and IgM and IL-6 secretion, B-type CpGs are weak stimulators of IFN-α from pDCs (Krieg, 2002). C-type CpG-ODNs have been recently described (Hartmann et al., 2003;

Vollmer et al., 2004) and are a structural and functional hybrid between the A- and B-types. Whether the differences in activity of the three types of CpG-ODNs is due to variation in cellular uptake, TLR9 binding, and/or signaling, is still unclear. In general, CpG ODNs have immune enhancing effects and as such have been investigated experimentally and clinically as adjuvants for treating microbial infection, allergy, asthma, and cancer (Klinman, 2004; Vollmer & Krieg, 2009; Weiner, 2009).

NK cells, via their production of IFN-γ, are thought to play an important role in the immune enhancing effects of CpG ODNs (Cowdery et al., 1996). Although it is still unclear whether NK cells directly respond to CpG ODN (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006b; Lauzon, Mian, MacKenzie, & Ashkar, 2006b), NK cell activation with CpG ODN has been shown to depend on IFN-α and IL-12 secretion by pDCs and macrophages (Chace, Hooker, Mildenstein, Krieg, & Cowdery, 1997; Marshall, Heeke, Abbate, Yee, & Van Nest, 2006).

IL-12, a proinflammatory cytokine that induces the production of IFN-γ, has been shown to have anti-tumor and anti-metastatic properties when used as a single therapeutic or in combination with other immune stimulatory compounds including IL-2, IL-15, and IL-18. IL-12 is thought to mediate its therapeutic effects directly through the activation of NK, T and NKT cells and their subsequent release of IFN-γ. We have shown that splenic NK cells are not activated by exogenously added IL-12 alone and require accessory help from B cells in the form of secreted IL-18 (Haddad, Senger, & Takei, 2009). We have now extended these studies and examined whether B cells also act as positive regulators of NK cells in response to CpG ODN stimulation. Unexpectedly, we have found that a subset of B cells produces IL-10 in response to

CpG ODN stimulation and suppresses rather than activates NK cell IFN-γ during CpG ODN stimulation.

3.2 Materials and methods

3.2.1 Mice

C57BL/6 (WT), TCRβ/TCRδ double knock out (KO) (βδKO) (B6. 129P-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J), Rag KO (B6.129S7-*Rag1*^{tm1Mom}/J) and B cell deficient (BKO) (B6. *Igh-6*^{tm1Cgn}), and IL-12KO (B6.129S1-*Il12a*^{tm1Jm}/J) mice were all purchased from The Jackson Laboratory and bred or housed pathogen free in our animal facility. Male or female 8-12 wk old mice were used in this study. All animal use was approved by the animal care committee of the University of British Columbia, and animals were maintained and euthanized under humane conditions in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.2 Antibodies and reagents

Anti-CD16/CD32 FcRγ (III/II) (2.4G2) (American Type Culture Collection, Manassas, VA) was purified from hybridoma supernatant. PE, FITC, Allophycocyanin, or Peridinin-Chlorophyll Protein (PerCP)-Cy5.5 conjugated mAbs to NK1.1, CD3ε, CD19, CD5, and IFN-γ and matching isotype controls were purchased from BD-Biosciences. RPMI 1640 media (Stem Cell Technologies) supplemented with 10% FBS (Life Technologies), penicillin, streptomycin (Stem Cell Technologies), and 5×10^{-5} M 2-mercaptoethanol was used for cell culture (Sigma-Aldrich). Class A 2216 CpG ODN (CpG-2216) and Class B 1826 CpG ODN (CpG-1826) were purchased from InvivoGen, prepared according to manufacturer's instructions and used in cultures at a final concentration of 2 μg/ml. Neutralizing antibody to mouse TGF-β was

purchased from R&D Systems and used at a final concentration of 2μg/ml. IL-10 neutralizing antibody (JES5-2A5) (eBioscience) was optimized and used at a final concentration of 2 μg/ml. NS-398 and SC-58125, cyclooxygenase-2 (COX-2) inhibitors, were purchased from Cayman Chemical, and 1-methyltryptophan (1-MT) which was used to inhibit IDO was purchased from Sigma-Aldrich. IL-12 (R&D Systems) and IL-18 neutralizing antibodies (MBL International) were optimized and used at 0.5 and 1.0 μg/ml, respectively. Recombinant mouse IL-12 (Stem Cell Technologies), IL-18 (MBL International), and IL-10 (eBioscience) were titrated and used at optimal concentrations.

3.2.3 CpG ODN stimulation of unfractionated splenocyte

Single cell suspensions of freshly isolated spleens were prepared as previously described (Haddad et al., 2009) dispensed at 4×10^6 cells/ml in 96-well plates and cultured with 2 μg/ml of CpG ODN for 24 h. For intracellular staining, splenocytes were cultured at a density of 4×10^6/ml in 14 ml round bottom polypropylene tubes and treated with CpG ODNs (2 μg/ml) for a total of 24 h. Brefeldin A was added during the final 5 h of culture. Cells were stained with appropriate mAbs prior to being fixed and permeabilized with a Cytofix/Cytoperm^{TM} Plus kit (BD-Biosciences) as per the manufacturer's instructions. Permeabilized cells were then stained with allophycocyanin-conjugated anti-IFN-γ mAb. A FACSCalibur (BD-Biosciences) was used for acquisition, and FLOWJO software (BD-Biosciences) was used for analysis.

3.2.4 B cell isolation and depletion

For cell sorting, splenoyctes were treated with 2.4G2 mAb to block Fc receptors, washed, and then stained with appropriate antibodies for 30 minutes. To exclude dead cells, propidium

iodide was added to a final concentration of 5μg/ml. For B cell depletion and add-back experiments to BKO splenocytes, WT splenocytes were prepared as above and stained with CD19 mAb. B cells were depleted as $CD19^+$ cells using BD Influx or FACs Aria cell sorters (BD-Biosciences). Restored cultures consisted of 4×10^5 each of sorted B cells and B cell depleted cells. Unfractionated splenocytes stained with mAbs but not sorted were used as a control. For add-back experiments, 4×10^5 sorted WT B cells were added to 4×10^5 unfractionated unsorted, 2.4G2 treated, BKO splenocytes in a total volume of 200 μl. Cultures were either left untreated or stimulated with CpG ODNs for 24 h. Sorted B cells (purity > 98%) were cultured at a density of 4×10^6 cells/ml in 96 well round-bottom tissue culture plates and stimulated with CpG ODNs for 24 h.

3.2.5 RNA extraction, reverse transcription and quantitative PCR

Unfractionated WT splenocytes were cultured at 4×10^6 cells/ml in 14 ml round bottom tubes and stimulated with CpG ODNs for 6 h. Cell pellets were blocked with 2.4G2 and stained with mAbs against CD19 and CD5 in order to isolate CD5 positive and negative B cells. RNA was extracted using the RNeasy Plus mini kit (QIAgen), treated with Turbo DNA free (Ambion) according to the manufacturer's instructions, and 0.1-1 μg RNA was used as a template for reverse transcription with random primers using Superscript III (Invitrogen) according to the manufacturer's instructions. Control reactions were performed with no reverse transcriptase to ensure there was no DNA contamination. The levels of *Il10* transcripts were detected by quantitative PCR. cDNA template was diluted 10-fold for *Il10* and 100-fold for *Gapdh* amplification and 3 μl was used in each 10 μl reaction containing 1 x FAST SYBR green mix (Applied Biosystems) and 0.2 μM of each primer. Reactions were run on an Applied Biosystems 7500 Fast real-time PCR machine with 20 s initial denaturation at 95ºC followed by

40 cycles of 3 s at 95ºC and 30 s at 60ºC. Agarose gel electrophoresis and dissociation analysis were performed to ensure each primer pair only produced one PCR product. Data were analyzed using the ΔC(T) method of relative quantitation. The primers used were: *Il10* forward 5´-CCAGTTTTACCTGGTAGAAGTGATG-3´, and *Il10* reverse 5´-TGTCTAGGTCCTGGAGTCCAGCAGACTC-3´, and *Gapdh* forward 5´-GACTTCAACAGCAACTCCCAC-3´, and *Gapdh* reverse 5´-TCCACCACCCTGTTGCTGTA-3´.

3.2.6 NK cell purification

NK cells were purified from spleen cells either by using the EasySep® Mouse NK1.1 Negative Selection Kit (StemCell Technologies) or by FACs sorting of the $NK1.1^{+}CD3\varepsilon^{-}$ population. The purity of the NK cells isolated by both methods was between 95-98%.

3.2.7 Cytokine ELISA

Cytokines in culture supernatants were measured by ELISA for IFN-γ, IL-12 (eBiosciences), IL-10 (BD Opteia, BD-Biosciences) and IL-18 (MBL Inc.) following manufacturers protocols. Values below 15-25 pg/ml of cytokine were not detected.

3.2.8 Statistical analysis

All statistical analyses of experimental mean values were performed using the Student's *t* test. Values of $p<0.05$ were considered significant.

3.3 Results

3.3.1 NK1.1$^+$ cells are the primary source of IFN-γ in CpG ODN stimulated unfractionated splenocytes

Unfractionated WT splenocytes were stimulated with either CpG-2216 (A-type CpG ODN) or CpG-1826 (B type CpG ODN), and cell-free supernatants were assessed for IFN-γ production after 24 h. CpG-1826 induced very low levels (<120 pg/ml) of IFN-γ at all concentrations tested (1-10μg/ml) (Fig. 11A). In contrast, CPG-2216 induced significantly higher levels of IFN-γ (>1ng/ml, with a range from 500-1700 pg/ml) in a dose dependent manner (Fig. 11A). At 2 μg/ml (310 nM) CpG-2216 induced about 1,500 pg/ml of IFN-γ. Although higher levels of IFN-γ (2,200 pg/ml) could be induced with 5 μg/ml (770 nM) of CpG-2216, high concentrations of ODN, exceeding 330 nM, are thought to induce CpG-independent stimulation (Brummel & Lenert, 2005; Haas, Poe, Steeber, & Tedder, 2005; Vollmer & Krieg, 2009). Therefore, 2 μg/ml (310 nM) of CpG-2216 was used for all the subsequent experiments as a sub-optimal concentration for in vitro stimulation. To identify the primary source of the CpG-2216-induced IFN-γ, unfractionated spleen cells were stimulated with CpG-2216 for 20 h, stained for surface markers and then various populations were examined for intracellular levels of IFN-γ. As seen in Fig. 11B, 14% of NK cells (NK1.1$^+$CD3$^-$) and 5% of NKT (NK1.1$^+$CD3$^+$) cells were positive for intracellular IFN-γ, while the T cell (NK1.1$^-$CD3$^+$), and the remaining cell (NK1.1$^-$CD3$^-$) populations were negative. B cells (CD19$^+$) and DC (CD11c$^+$NK1.1$^-$CD3$^-$) were also negative (results not shown).

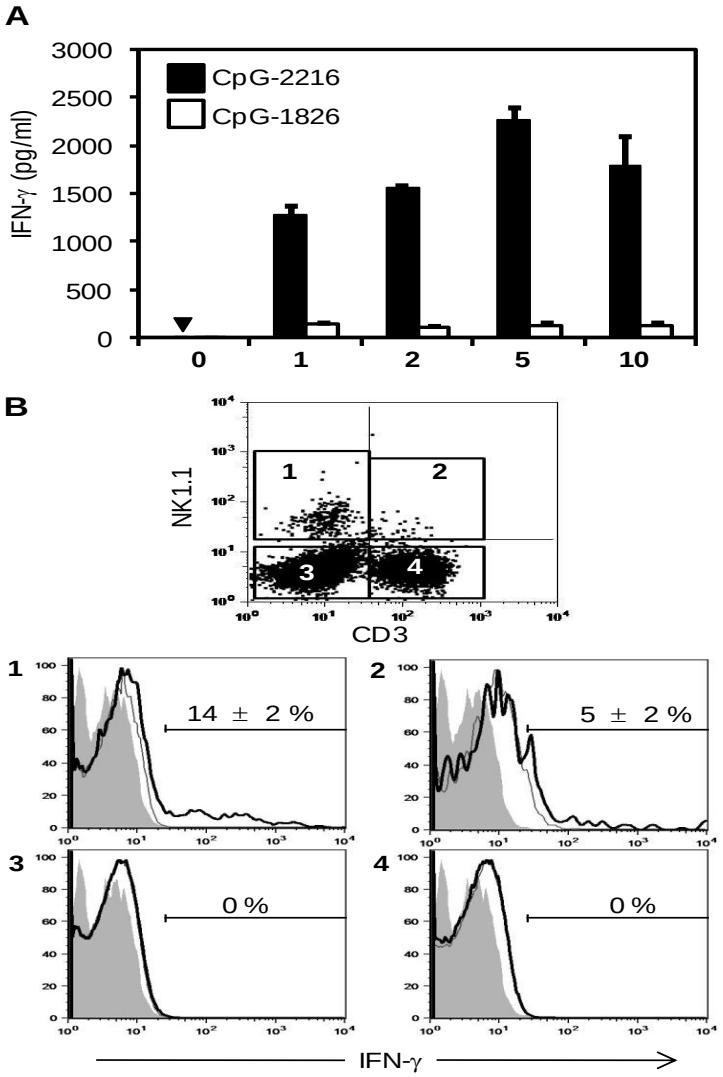

Figure 11 CpG ODN induces IFN-γ primarily from NK1.1$^+$ cells in unfractionated spleen

A. Unfractionated splenocytes (8×10^5/200 μl) were treated with CpG-2216 or CpG-1826 for 24h and supernatants were assayed for IFN-γ. Mean ± SEM of three independent experiments, each with triplicate cultures. (▼ undetectable cytokine). *B.* Unfractionated splenocytes (4×10^6/ml) were cultured with or without CpG-2216 (2 μg/ml) for 24 h, stained with mAbs to NK1.1 and CD3ε, fixed, permeabilized, stained for intracellular IFN-γ and analyzed by flow cytometer. NK cells (NK1.1+CD3ε$^-$), NKT cells (NK1.1$^+$CD3ε$^+$), double negative (NK1.1$^-$ CD3ε$^-$), and T (NK1.1$^-$CD3ε$^+$) cells were gated as shown by numbers in dot plots, and the staining of intracellular IFN-γ in each population is shown in histograms. Filled histograms show staining with isotype-matched control Ab, open grey and black histograms show IFN-γ staining for unstimulated and stimulated cultures, respectively. The data are representative of at least four independent experiments and the numbers indicate the percentages of positively stained cells (mean ± SD, n=4). (▼) denotes undetectable cytokine levels.

3.3.2 CpG-2216 stimulation of B cell deficient spleen cells induces more IFN-γ^+ NK cells and higher levels of IFN-γ secretion

The stimulation of spleen cells with CpG-2216 has been shown to induce IL-12 secretion (Takeshita & Klinman, 2000), and we have previously shown that B cells are required for the stimulation of NK cells by IL-12 (Haddad et al., 2009). Therefore, we tested whether CpG-2216-mediated stimulation of NK cells in unfractionated splenocyte populations also required B cells. Unexpectedly, intracellular IFN-γ staining of B cell deficient (BKO) mouse spleen cells stimulated with CpG-2216 showed a significantly higher percentage of IFN-γ^+ NK cells than those of WT spleen cells (Fig. 12A). In contrast, CpG-2216-stimulated T cell deficient βδKO mouse spleen cells showed the same percentage of IFN-γ^+ NK cells as those of WT spleen cells (Fig. 12A). In all cases, NK cells were the majority of intracellular IFN-γ^+ cells after CpG-2216 stimulation. To confirm that higher percentages of IFN-γ^+ NK cells resulted in more IFN-γ secretion, unfractionated spleen cells from WT, BKO and βδKO mice were stimulated with CpG-2216 overnight and the amounts of IFN-γ secreted in the media were measured. Consistent with the intracellular IFN-γ staining, stimulation with CpG-2216 resulted in significantly higher ($p< 0.005$, n=9) amounts of IFN-γ secretion by BKO mouse spleen cells than WT and βδKO mouse spleen cells (Fig. 12B). Thus, B cell deficiency enhances the stimulation of NK cells by CpG-2216.

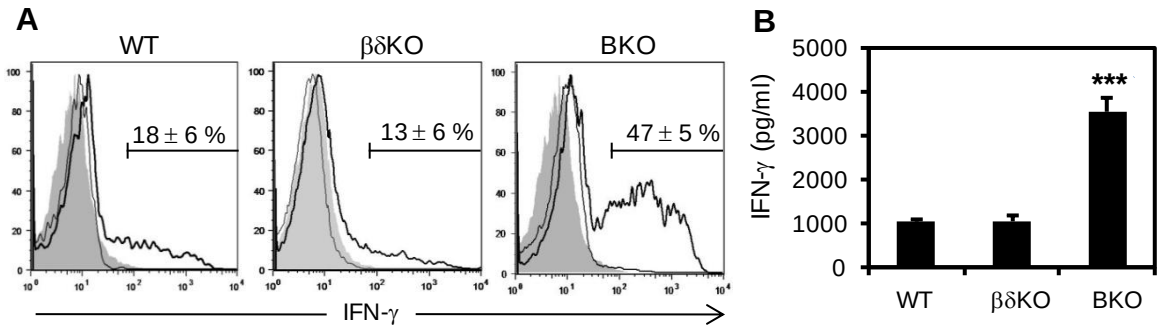

Figure 12 CpG ODN induces more IFN-γ production by B cell deficient splenocytes than WT splenocytes

A. Unfractionated splenocytes (4×10^6/ml) from WT, βδKO and BKO mice were cultured with or without CpG-2216 (2 μg/ml) for 24 h, and intracellular IFN-γ in NK cells ($CD3^-NK1.1^+$) was analyzed as in Fig. 1. Filled histograms show isotype-matched control Ab staining, open grey and black histograms show IFN-γ for unstimulated and stimulated cultures, respectively. The data are representative of five independent experiments and the numbers indicate the percentages of positively stained cells (mean ± SD, n=5). *B.* Cell free supernatants from the above cultures *(A)* were assessed for IFN-γ. The results shown are the mean ± SEM of five independent experiments, each with triplicate cultures. ***, $p < 0.005$.

3.3.3 B cells suppress NK cell production of IFNγ induced by CpG-2216

To test whether B cells suppress IFN-γ production by NK cells during CpG-2216 stimulation, we first examined the effects of B cell depletion on IFN-γ production in unfractionated splenocytes. WT mouse spleen cells were sorted into $CD19^+$ and $CD19^-$ populations to high purity (98-100%), stimulated with CpG-2216 for 24 h, and IFN-γ secreted into the media was measured. B cell depleted ($CD19^-$) spleen cells secreted more than two fold higher amount of IFN-γ than non-depleted unfractionated spleen cells, whereas purified $CD19^+$ B cells did not secrete appreciable amounts of IFN-γ. Moreover, adding back purified $CD19^+$ cells to the B cell depleted population reduced the amount of IFN-γ secretion to that of the

unfractionated spleen cells (Fig. 13A). To further test the inhibitory effects of B cells, BKO mouse spleen cells were mixed with purified B cells from WT spleen cells at a ratio of 1:1 and stimulated with CpG-2216. The addition of purified B cells reduced the amount of IFN-γ secretion by more than 3-fold (Fig. 13B). These results suggested that B cells indeed suppress the CPG-2216-induced IFN-γ production by NK cells.

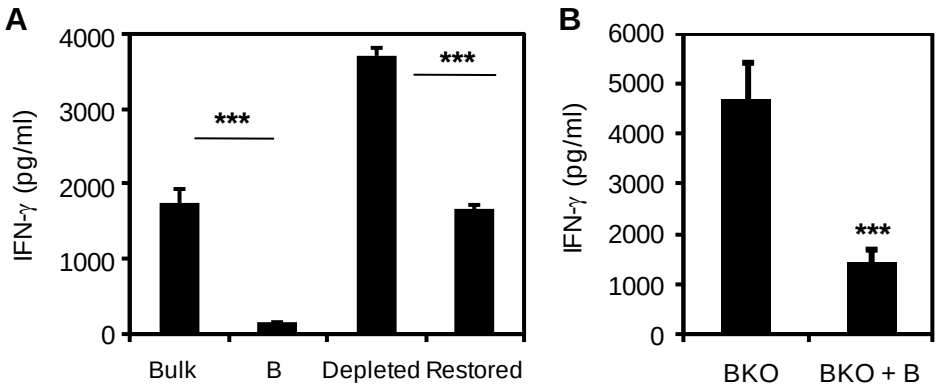

Figure 13 B cells supress CpG ODN induced NK cell production of IFN-γ

A. $CD19^+$ cells and $CD19^-$ cells were FACs sorted from WT splenocytes as B cells and depleted populations, respectively. For the restored population, 4×10^5 B cells were mixed with 4×10^5 depleted cells. All cells (8×10^5 in 200 μl) were stimulated with 2 μg/ml of CpG-2216 24 h. *B.* B cells ($CD19^+$) were FACs sorted from WT splenocytes. BKO cells were either cultured in unfractionated (8×10^5) or co-cultured with sorted B cells (4×10^5 of each cell type) and stimulated with 2 μg/ml of CpG-2216 for 24 h. All results show the amounts of IFN-γ in the culture supernatants (mean ± SEM) of three independent experiments, each with triplicate cultures. ***, $p < 0.005$.

3.3.4 IL-10 inhibits CpG-2216 induced IFN-γ production

To gain insight into the mechanism by which B cells suppress CpG-2216-induced IFN-γ production, we tested the effects of neutralizing antibodies to IL-10 and TGF-β, the archetypical inhibitors of immune inflammatory response (Letterio & Roberts, 1998; Moore et al., 2001; T. R.

Mosmann & Moore, 1991). The neutralization of IL-10, but not TGF-β, significantly increased the amount of IFN-γ secreted by CpG-2216-stimulated unfractionated spleen cells (Fig. 14A). Although cyclo-oxygenase-2 (COX-2) and IDO have been shown to suppress NK cell activation (Chen, Perussia, & Campbell, 2007; Spaggiari et al., 2008), neither the COX-2 inhibitor NS-398 nor the IDO inhibitor 1-methyl tryptophan had significant effects on CpG-2216 induced IFN-γ production at any of the doses tested (Fig. 14B). These results suggest that IL-10 is produced by CpG-2216 stimulated spleen cells and inhibits IFN-γ production, whereas other potential suppressor mechanisms including TGF-β, COX-2, and IDO, do not seem to play a significant role in NK cell stimulation with CpG-2216. As we observed that CpG-1826 induced very low levels of IFN-γ, we tested whether this was due to an overproduction of IL-10. Although IL-10 neutralization increased the level of IFN-γ by 3-fold, the overall level of IFN-γ was similar to that seen in untreated CpG-2216 stimulated cultures (Appendix 7).

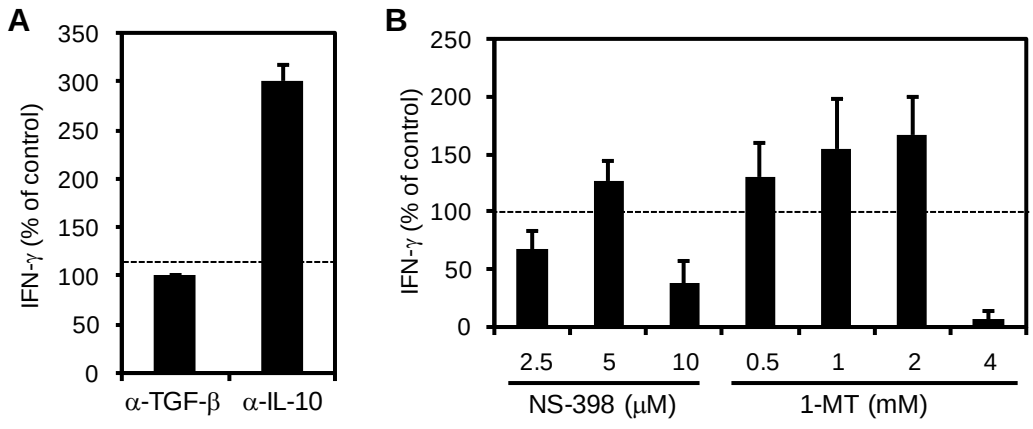

Figure 14 IL-10 is involved in the suppression of CpG ODN induced IFN-γ production

Unfractionated WT splenocytes (8×10^5 / 200μl) were stimulated and treated as indicated and after 24 h supernatants were assessed for IFN-γ production. *A*. 2 μg/ml of neutralizing antibodies to TGF-β (α-TGF-β), or IL-10 (α-IL-10) cytokines were added to CpG-2216 stimulated cultures. Fold increases in IFN-γ production are shown and were calculated as a percentage of IFN-γ production from treated/stimulated cultures divided by IFN-γ production from stimulated cultures. *B.* Inhibitors to COX-2 (NS-398) and IDO (1-MT) enzymes were added at various doses. NS-398 at 2.5, 5, and 10 μM, and 1-MT at 0.5,1, 2, and 4 mM were added to CpG-2216 stimulated cultures. The results shown are the mean ± SEM of three independent experiments, each with triplicate cultures.

3.3.5 $CD5^+$B cells produce IL-10 in response to CpG-2216 stimulation

To test whether a specific subset of B cells produce IL-10 during stimulation of unfractionated spleen cells with CpG-2216, all B ($CD19^+$) cells as well the $CD5^+$ and $CD5^-$ subsets of B cells were purified from WT spleen cells and stimulated with CpG-2216 for 24 h, and the amounts of IL-10 secreted in the media were determined. Unfractionated B cells were directly stimulated with CpG-2216 and secreted IL-10 (Fig. 15A), and only the $CD5^+$, but not $CD5^-$, subset secreted IL-10. The relatively modest amounts of IL-10 produced by purified $CD5^+$ B cells, as compared to unfractionated spleen cells, suggested that other cells might

enhance CpG-2216-induced IL-10 production. Therefore, we stimulated WT unfractionated spleen cells with CpG-2216 and analyzed IL-10 mRNA levels in B cell subsets by quantitative RT-PCR. As IL-10 transcripts in unfractionated spleen cells stimulated with CpG-2216 peaked at 3-6 h, with a significant increase as early as 30 minutes post stimulation and a decline after 24 h (data not shown), we analyzed *Il10* mRNA levels from purified B cell subsets sorted from unfractionated splenocyte cultures 6 h post-CpG-2216 stimulation. $CD5^+$ B cells were found to express a much higher level of *Il10* mRNA than $CD5^-$ B cells, whereas non-B cells ($CD19^-$) expressed a very low level of *Il10* mRNA (Fig. 15B).

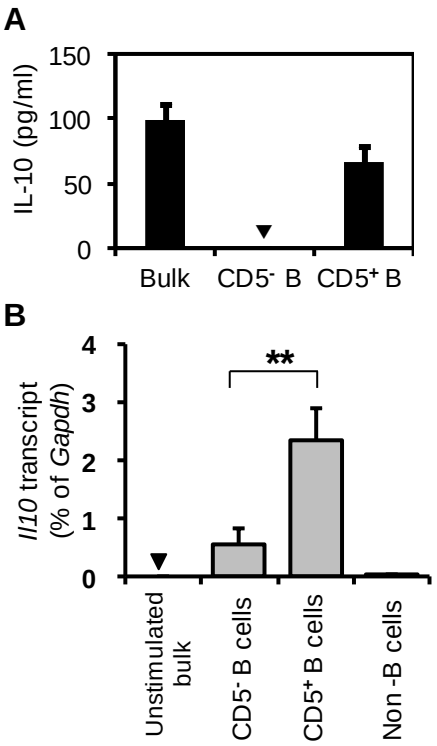

Figure 15 B cells are a source of CpG ODN induced IL-10

A. Unfractionated WT spleen cells, FACS sorted $CD5^-$ and $CD5^+$ B cells were cultured at 1×10^5 /200μl in 96-well round bottom tissue culture plates. Cells were either left unstimulated or treated with 2 μg/ml of CpG-2216 for 24 h, supernatants were then assayed for IL-10. The data shown are the mean ± SEM of three independent experiments, each with triplicate cultures. ▼, undetectable levels of cytokine *B.* Unfractionated WT splenocyte (4×10^6/ml) were stimulated with CpG-2216 (2 μg/ml) for 6 h, and then CD5 negative and positive B ($CD19^+$) cells as well as non-B cells ($CD19^-$) were purified from the stimulated spleen cell culture. Unfractionated spleen cells that were not sorted and unstimulated were used as control. The levels of IL-10 mRNA were assessed by Quantitative PCR as described in material and methods. Data were analyzed by the ΔC(T) method of relative quantitation, and are shown a percentage of IL-10 gene expression normalized to GAPDH expression. The data are the mean ± SD of three independent experiments (n=3). ***, $p < 0.05$.

3.3.6 IL-10 directly and indirectly inhibits IFN-γ production by NK cells

To elucidate the mechanisms by which CpG-2216-mediated stimulation of NK cells is inhibited by IL-10, we first investigated how CpG-2216 stimulates NK cells in the

unfractionated spleen cell population. Similar to the previously reported results with CpG-1826 (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006a), purified NK cells did not secrete IFN-γ in response to CpG-2216 stimulation (Fig 16A, control) unless IL-12 or IL-18 was also added (Fig. 16A). As NK cell stimulation by CpG ODNs is thought to be dependent on APC-derived IL-12, we tested the effects of the neutralization of IL-12 and IL-18, which are known to stimulate NK cells (Lauwerys et al., 1999). IFN-γ production by unfractionated spleen cells stimulated with CpG-2216 was almost completely inhibited by anti-IL-12 mAb (Fig. 16B). Anti-IL-18 mAb also partially inhibited the production of IFN-γ. To further test the role of IL-12 in the CpG ODN stimulation of NK cells, we stimulated spleen cells from WT and IL-12KO mice with CpG-2216 and compared the levels of IFN-γ production. No IFN-γ was detected in the IL-12KO cultures (Fig. 16C, control), whereas the addition of exogenous IL-12 (1 ng/ml) restored the production of IFN-γ to the levels similar to the WT cultures (Fig. 16C). Therefore, CpG-2216-mediated stimulation of NK cells in the unfractionated spleen cell population seems to be absolutely dependent on the production of IL-12. Indeed, CpG-2216 stimulation of WT unfractionated spleen cells induced a significant amount of IL-12 production, and BKO spleen cells produced an almost 4-fold higher amount of IL-12 compared to WT spleen cells (Fig. 16D). Furthermore, anti-IL-10 mAb increased by almost 6-fold the amount of IL-12 produced by WT unfractionated spleen cells stimulated with CpG-2216 (Fig. 16E). These results suggest that IL-10 inhibits the production of IL-12, which is required for NK cell stimulation with CpG-2216.

As resting NK cells are known to express a functional receptor for IL-10 (Carson et al., 1995), we tested whether IL-10 can directly inhibit NK cells. As shown above, NK cells were not stimulated by CpG-2216 alone. Therefore, we used IL-12 (1ng/ml) and IL-18 (1 and 10ng/ml), a potent combination of cytokines for NK cell IFN-γ production, to stimulate NK

cells purified from RagKO mouse spleen cells, and tested the effects of recombinant mouse IL-10 (250 - 1000 pg/ml). At 250 pg/ml, IL-10 dramatically reduced IFN-γ production, while higher doses completely inhibited the production of IFN-γ from purified NK cells (Fig. 16F).

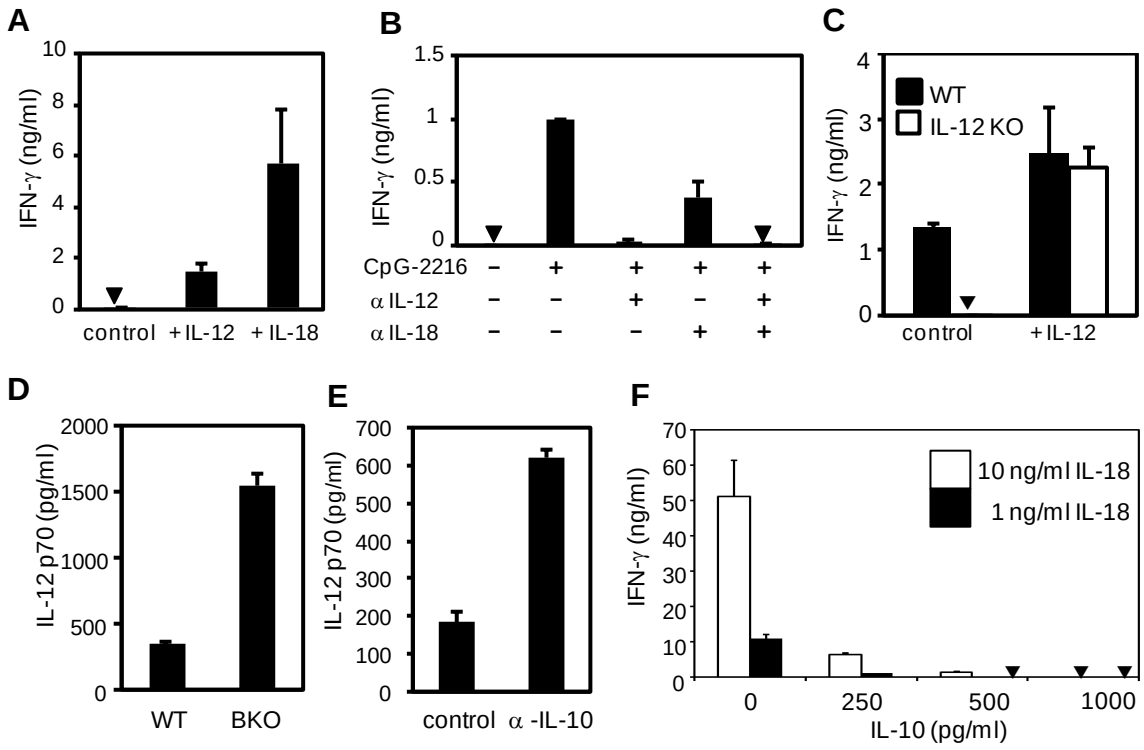

Figure 16 IL-10 suppresses NK cell production of IFN-γ in a two pronged manner

A. Purified NK cells (3×10^4/200 μl) in 96 well round bottom plates were stimulated with CpG-2216 alone or in combination with either 1 ng/ml of IL-12 or 10 ng/ml of IL-18. Supernatants were assessed for IFN-γ after 48 h. The results shown (mean ± SD) are representative of at least three independent experiments, each with triplicate cultures. *B.* Unfractionated WT splenocytes (8×10^5/200 μl) were stimulated with 2 μg/ml of CpG-2216. IL-12 neutralizing antibody was used at 0.5 μg/ml, and the IL-18 neutralizing antibody was used at 1 μg/ml. After 24 h supernatants were analyzed for IFN-γ levels. The results shown represent one of three independent experiments, each with triplicate cultures (mean ± SD). *C.* Unfractionated WT or IL-12KO splenocytes (8×10^5/200 μl) were cultured in the absence (control) or presence of 2 μg/ml of CpG-2216. After 24h supernatants were assessed for IFN-γ. Results are the mean ± SD of one of three independent experiments, each with triplicate cultures. *D.* Unfractionated WT or BKO splenocytes were cultured (8×10^5/200μl) were stimulated with 2 μg/ml of CpG-2216 as above. After 24 h cell-free supernatants were collected and assessed for IL-12 production by ELISA. Results are the mean ± SEM of three independent experiments, each with triplicate cultures. *E*. Unfractionated WT splenocytes (8×10^5/200μl) were cultured with CpG-2216 (2 μg/ml) and neutralizing antibody to IL-10 (2 μg/ml). The levels of IL-12 were assessed 24 h post-stimulation and are shown as the mean ± SEM of three independent experiments each with triplicate cultures. *F*. Purified NK cells (3×10^4/200 μl) were left untreated or stimulated with 1 ng/ml of IL-12 and either 1 ng/ml or 10 ng/ml of IL-18. IL-10 was also added as indicated. Cell-free supernatants were collected after 24 h and assessed for IFN-γ by ELISA. The results shown (mean ± SD) are representative of at least three independent experiments, each with triplicate cultures. ▼, undetectable levels of cytokine.

3.4 Discussion

Our current study has shown that CpG-2216, an A-type CpG ODN, stimulates unfractionated splenocytes and induces IFN-γ production by NK cells. In response to CpG-2216, NK cells in B cell-deficient and B cell-depleted spleen cells produce significantly more IFN-γ than NK cells in B cell-sufficient spleen cells. Adding B cells back to B cell-deficient spleen cells effectively inhibits CpG-2216-induced IFN-γ production. Thus, B cells seem to suppress the stimulation of NK cells by CpG-2216. The suppressive activity of B cells is likely mediated, at least in part, by IL-10, as the neutralization of IL-10 with mAb greatly enhances CpG-2216-induced IFN-γ production, and CpG-2216-stimulated B cells produce IL-10. Furthermore, among CpG-2216-stimulated spleen cells, IL-10 mRNA is mostly detected in $CD5^+$ B cells whereas it is undetectable in non-B cells. Thus, IL-10 production by $CD5^+$ B cells is likely a critical regulatory mechanism controlling CpG-2216-mediated NK cell stimulation. Our results also suggest that B cell-derived IL-10 may inhibit NK cell stimulation by two mechanisms. First, IL-10 may inhibit the production of IL-12, which is required for the stimulation of NK cells by CpG-2216. Second, it may directly inhibit NK cells. As shown with other CpG ODNs (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006a; Lauzon, Mian, MacKenzie, & Ashkar, 2006b), CpG-2216 alone does not directly stimulate purified NK cells and requires IL-12 or IL-18. Neutralization of IL-12 in unfractionated spleen cell cultures completely inhibits CpG-2216-induced IFN-γ production, whereas the neutralization of IL-18 is only partially inhibitory. As CpG-2216 induces more IL-12 production by B cell-deficient spleen cells than wild type spleen cells, and the neutralization of IL-10 significantly increases the amount of IL-12 production, B cell-derived IL-10 likely inhibits IL-12 production, which in turn inhibits NK cell-mediated IFN-γ production. At the same time, exogenously added IL-10 effectively inhibits

stimulation of purified NK cells with a combination of IL-12 and IL-18, indicating that IL-10 also has a direct inhibitory effect on NK cells.

Our study is not the first to demonstrate that $CD5^+$ B cells are the primary source of IL-10 in response to CpG ODN A stimulation, or that B cells are the major source of IL-10 in WT (C57Bl/6) mice in response to CpG ODN classes. Sun et al. (Sun, Deriaud, Leclerc, & Lo-Man, 2005) previously showed that murine splenic $CD5^+$ but not $CD5^-$ B cells in neonatal mice secreted IL-10 in response to all CpG ODN classes (A, B and C) and that this IL-10 was able to limit the production of IL-12 from DC, as well as their differentiation and maturation, thus lead to the development of a Th2 rather than a Th1 response. CD5 expressing B cells represent almost 50% of all splenic B cells in neonatal (7 day old) mice but only 5-10% in adult mice (8-10 weeks), whether the IL-10 in adult mice was restricted to the CD5 subset was not examined. Here we show that CD5 B cells in adult mice are the primary source of IL-10 in response to CpG ODN A stimulation. Our results showing that IL-12p70 levels are compromised in the presence of B cell sourced IL-10 supports that seen by Sun et al. (Sun et al., 2005) in neonates. Our study shows that this IL-10 is also capable of directly suppressing NK cell IFN-γ in addition to limiting the availability of IL-12.

Multiple cell types, including regulatory T cells, DCs, macrophages, NK cells, and regulatory B cells, have been shown to produce IL-10 (Saraiva & O'Garra, 2010). In our current study, the stimulation of unfractionated spleen cells with CpG-2216 rapidly induces IL-10 gene expression, which peaks at 6 h and then declines. Whereas B cell-deficient spleen cells stimulated by CpG-2216 also produce IL-10 (Appendix 8), IL-10 mRNA is undetectable in non-

B cells in WT spleen cells. The neutralization of IL-10 also significantly increased the amount of IFN-γ produced by CpG-2216- stimulated BKO and RagKO spleen cells. Similar results were seen by Sun et al. (Sun et al., 2005) who found that while B cells were the primary source of IL-10 in WT neonate spleen, significant (but lower when compared to WT) levels of IL-10 were also found in neonate BKO spleen cells. It is likely that other cells types are more vigorously stimulated by CpG-2216 and produce IL-10 in order to control the CpG ODN immunostimulatory response when B cells are absent.

Both stimulatory and inhibitory effects of IL-10 on NK cell cytotoxicity have been reported (Mocellin, Panelli, Wang, Nagorsen, & Marincola, 2003; Mocellin et al., 2004). However, many of these studies have used IL-10 in combination with other factors or used IL-2 activated NK cells which are functionally very different from the resting NK cells used in this work. To our knowledge this is the first time that IL-10 has been shown to act alone and directly suppress IFN-γ production by NK cells. We also found that IL-10 inhibits the proliferation of IL-2-stimulated NK cells (data not shown). IL-10 either alone or in combination with LPS has been shown to upregulate the expression and function of the suppressor of cytokine signaling 3 (SOCS3) in macrophage/monocytes (Dalpke, Opper, Zimmermann, & Heeg, 2001; Qasimi et al., 2006). Once activated, SOCS3 inhibits LPS driven signal transduction and blunts the inflammatory responses of macrophages in a STAT3 dependent manner. Porcine NK cells express mRNA for SOCS3, and if stimulated with TRL7/8 ligands show an upregulation of its expression (Toka, Nfon, Dawson, & Golde, 2009). IL-10 has also been shown to increase the mRNA and protein level of Tristetrapolin (TTP), an RNA destabilizing factor. The action of TTP in activated murine macrophages leads to the reduced stability and decay of mRNA for

TNF-α and IL-1α, and ultimately results in the dramatic loss of production of these cytokines (Schaljo et al., 2009). In activated mouse T cells, the action of TTP is associated with IFN-γ mRNA decay (Ogilvie et al., 2009). Whether TTP plays a role in controlling NK cell IFN-γ has not yet been addressed.

Regulatory/suppressor B cells that secrete IL-10 and curtail the progress of immune mediated inflammatory responses have been described in both humans and mice (Lund & Randall, 2010). Unlike conventional B cells that secrete a variety of cytokines (Lund, 2008), suppressor B cells are skewed towards IL-10 production. Tedder and colleagues have shown that IL-10 secreting B cells can be identified as $B220^{+}CD19^{hi}CD1d^{hi}CD5^{+}$ cells which represent 1-3% of adult mouse splenocytes (Yanaba, Bouaziz, Matsushita, Tsubata, & Tedder, 2009). Termed B10 cells, these cells secrete IL-10, but not IL-12, upon stimulation with LPS or IgM/CD40-ligation, and can suppress T cell mediated inflammation during contact hypersensitivity responses (CHS) (Yanaba et al., 2008). The $CD5^{+}$ B cells that we describe herein also display an IL-10 bias upon CpG-2216 stimulation as IL-12 was not detected in culture supernatants (not shown). Using an IL-10 transcriptional reporter mouse, Madan et al. (Madan et al., 2009) similarly found $B220^{+/-}CD19^{+}CD5^{+}$ cells to be the dominant cell type among IL-10 expressing cells in the spleen of naïve and virally challenged mice, and also in *ex vivo* cells and mice treated with LPS or CpG-1826. However, unlike B10 cells, this group did not find $CD1d^{hi}$ expressing cells to be a major source of IL-10. $CD5^{-}$ B cells that comprise the B-1b and the conventional B-2 B cell subsets have been shown to be a source of IL-10 secretion upon CD40 ligation (Fillatreau, Gray, & Anderton, 2008; Wagner et al., 2004). CD40 ligation in

the absence of IgM cross-linking can lead to the production of IL-10 from all human and mouse B cells regardless of their developmental or functional status (O'Garra et al., 1990).

The CpG-2216 and CpG-1826 that were used in this study are of the A-type and B-type CpG-ODNs, respectively. They differ not only in the stimulation of NK cells but also in B cell stimulation. Whereas CpG-1826 induces a very low level of IFN-γ production by NK cells, it is a potent stimulator of IL-10 production from purified B cells (Appendix 9). The poor stimulation of NK cells by CpG-1826 seems in part due to IL-10 over-production, because IL-10 neutralization increases the level of IFN-γ production induced by CpG-1826. However, it does not reach the level induced by CpG-2216, indicating that other factors, yet to be determined, make CpG-1826 a poor stimulator of NK cells as compared to CpG-2216.

A role for B cells as suppressors of anti-tumour immunity has been demonstrated by others. Inoue et al. (Inoue, Leitner, Golding, & Scott, 2006) found that mouse tumour cells lines which expressed CD40 ligand (CD40L, or CD154) were able to induce IL-10 secretion from sorted splenic B cells. This IL-10 was found to suppress CTL and NK cell IFN-γ in vitro, and diminished tumour regression and clearance in vivo. Conversely, the regression of tumours was seen in the mice that lacked B cells. Subsets of B cells have also been described regulate to autoimmunity primarily through IL-10 (Martin & Chan, 2006; Rafei et al., 2009). Mice deficient for CD19 or BKO develop a severe non-remitting form of EAE, a model of human MS (Matsushita et al., 2006), and B cells that are stimulated with GIFT15, a chimeric novel cytokine composed of IL-15 fused to GM-CSF, are able to reverse EAE in mice, largely via the production of IL-10 (Rafei et al., 2009). Regulatory B cells may soon be shown to also play a role in the prevention or control of rheumatoid arthritis and allergy, as IL-10 has been found to

be important to the recovery of both diseases (Chung, 2001). Lenert and colleagues (Lenert, Brummel, Field, & Ashman, 2005) have demonstrated that marginal zone B cells from lupus prone mice, but not normal mice, respond to CpG-2216 and upregulate the expression of CD86 and secretion of IgM and IL-10. CD5 is not found on normal splenic marginal zone B cells but has been found on circulating regulatory B cells found in normal human peripheral blood (Blair et al., 2010). CD5 expression is also upregulated in human marginal zone B cell lymphomas (Baseggio et al., 2010), and human chronic lymphocytic leukemia B cells, phenotypically identified as $CD19^{+}CD5^{+}$, and shown to produce IL-10 (Fayad et al., 2001; Jahrsdorfer et al., 2005).

IFN-γ is a powerful driver of Th1-type inflammation that while imparting immune protection, if left unregulated, can lead to severe pathology. NK cells are primary producers of the protective levels of IFN-γ that are required not only for the prevention of disease and infection, but also key to the success of current cancer therapies and vaccination strategies. We have uncovered a regulatory pathway of B cells in suppressing NK cell IFN-γ production during the early stages of an immune response to CpG ODN. Our work may have important implications for the use of CpG ODNs as adjuvants and therapeutics.

Chapter 4 General summary and discussion

The immune system by nature must be aggressive. Its job is to mount an assault against anything that is deemed foreign and therefore potentially of danger to the health and viability of the host. Its response must be therefore strictly regulated in order to avoid the aggression being turned back on the host; this is the reason why multiple signals are needed for the induction of a functional response, and perhaps is the underlying reason for the great complexity that exists within the immune cellular network, with so many cells presenting overlapping and often redundant functions, and many new functional subsets emerging. IFN-γ is a powerful inflammatory cytokine that is necessary for the resolution of various pathogenic infections and an important inhibitor of tumour growth and metastasis. IFN-γ overexpression is associated with adverse outcome and may lead to pathology. Its levels are controlled during the course of an immune response by suppressive cytokines like TGF-β and IL-10, which also play a role in terminating the inflammatory response.

The work presented in this thesis has shown that NK cells in resting unfractionated spleen cultures are a primary source of IFN-γ in response to IL-12, whether it is exogenously administered (as a recombinant protein in Chapter 2), or endogenously produced (after CpG ODN stimulation in Chapter 3). My work supports the idea that unprimed NK cells require more than one signal to become fully activated, as highly purified and resting NK cells do not release IFN-γ after either IL-12 treatment or CpG ODN stimulation. Importantly, I have found that B cells are intimate regulators of resting NK cell cytokine production, acting as both enhancers and suppressors of IFN-γ production.

4.1 Summary

In Chapter 2, I focused on elucidating the cell type and cellular factors that provide the co-stimulatory signals required to induce IFN-γ from NK cells after exogenous IL-12 treatment. I show that B cells but not DC, NKT, or macrophages function as important accessory cells to IFN-γ production from resting NK cells. I went on to show that B cells produce IL-18 which synergizes with the added IL-12 to optimize NK cell cytokine release. I also determined that B cells provide undefined contact /proximity dependent signals that aid in the induction of IFN-γ by IL-12. Finally, I showed that B cells are required for de novo IFN-γ production and increased IL-18 release following administration of IL-12 in vivo.

In Chapter 3, I investigated the ability of B cells to enhance IFN-γ production from resting mouse NK cells in the presence of the endogenously produced IL-12 induced by CpG ODN (type A) stimulation. In direct opposition to the results with exogenous IL-12, the presence of B cells suppressed the production of IFN-γ from NK cells in unfractionated spleen cultures. Depleting B cells from spleen cultures or spleen cell suspensions from B cell deficient mice resulted in significantly higher NK cell IFN-γ release. This was driven in part by IL-10 which blunted the levels of endogenously produced IL-12 in CpG ODN stimulated cultures, as well as directly acted on the NK cells themselves to impair IFN-γ release. I went on to show that not all B cells suppress the NK cell cytokine response, and only a small subset of splenic B cells that express CD5 were activated by the CpG ODN to produce IL-10.

4.2 Significance of the work

The regulatory mechanisms controlling NK cell cytokine production have only been studied in the last fifteen years or so. It is now clear that NK cell IFN-γ production is under as strict a regulation as their cytotoxicity is. What has also become clear is that NK cells are dependent upon the actions of other cell types, typically DC or Mϕ, which provide diverse stimulatory signals. Various groups have looked at the interaction between B and NK cells, but apart from a few studies (Michael et al., 1989), the majority of the work has concentrated on the effects of NK cell activity upon B cell antibody production.

My work which demonstrates that the B cell compartment can act to enhance or suppress IFN-γ production from NK cells, adds yet another layer of regulation to the function of NK cells. The ability of the B cells to respond directly to IL-12 and to CpG ODN and secrete IL-18 to enhance, or IL-10 to suppress, NK cell cytokine production occurs within 24 hours of stimulation. Thus my work also supports a more innate like role for B cells that is distinct from their adaptive phase production of antibodies, or antigen presentation to T cells.

4.3 Strength and limitations

The immune response is a complicated network of cells and cellular factors. In order to cleanly tease out the cellular players involved in mediating the IFN-γ inducing effects of IL-12 on NK cells only resting tissues and cells from healthy animals were used; cells were not derived from cultured progenitors, or subjected to any pre-culture conditions as these likely prime or preactivate immune cells. In addition I primarily utilized FACS for all cell isolation or

depletion from unfractionated spleen tissue in order to avoid contamination with unwanted cell populations.

The major limitation of this research is the use of the BKO mice. The embryonic loss of B cells in mice has been shown to lead to disorganized splenic architecture. These mice lose their distinct splenic T cell and MZ regions (Dingjan et al., 1998; Ngo, Cornall, & Cyster, 2001). A transient regarrangement in splenic architecture has been noted after viral and bacterial infection (Beattie, Engwerda, Wykes, & Good, 2006; Benedict et al., 2006); yet once the infection is cleared the splenic architecture is re-established. In mice the embryonic and permanent loss of the B cell compartment has been shown to lead to the loss of MZ Mϕ (marginal zone macrophages), a population of Mϕ that are specialized at polysaccharide uptake and are closely associated to MZ B cells (Pillai et al., 2005) as well as FDCs (Endres et al., 1999). Thus results obtained from using BKO mice for in vivo experiments, or in vitro unfractionated spleen cultures, might be complicated by a loss of other potentially important cell types. A possible remedy to this would be to run parallel in vivo/in vitro experiments in which anti-CD19 antibodies are used to temporarily deplete the B cell population. We somewhat addressed the issue of KO mice in the in vitro experiments which used FACS to deplete all CD19 expressing cells from otherwise normal (WT) spleen. Further to this, the addition of highly purified B cells to highly purified NK cells in the presence of IL-12 clearly demonstrated that B cells were able to support NK cell activation and IFN-γ release (Chapter 2), similar experiments were not run for the CpG ODN study (Chapter 3) as its stimulation of IFN-γ from NK cells requires further stimulatory signals that B cells do not provide.

With regards to CpG ODN, there are three synthetic classes currently available (Class A, B, and C), and while all generally resemble the unmethylated DNA found in bacteria, each class seems to be specialized to activate different cell types (Krieg, 2002). Whether similar classes of CpG motif containing pathogenic DNA exist is not yet clear. Thus our results may not parallel what is seen in an actual pathogenic infection but what might occur in the use of these specific CpG ODN classes as therapeutics. This is true for IL-12 as well, as it is unlikely that IL-12 is found in the absence of other cytokines after a physiologically relevant infection. However our results showing that B cells respond to IL-12 to enhance NK cell IFN-γ might be more applicable to therapeutic strategies which have employed the use of IL-12 to combat cancer or prime anti-pathogen responses.

Overall, the research presented herein may find potential application in therapeutic strategies that strive to either shut off or turn on Th1 polarized inflammatory responses. This might be achieved by harnessing the ability of the B cell compartment to influence the response of NK cells, the primary source of IFN-γ.

4.4 Future directions

A natural continuation of the research presented here would be to examine the B-NK cell relationship when the NK cell IFN-γ response is needed for the resistance or resolution of actual infection or disease. But some aspects of the B-NK cell relationship would need to be clarified first.

4.4.1 B cell subsets

While I show that the splenic subset of CD5 expressing B cells were the primary responders to CpG ODN in terms of IL-10 secretion (Chapter 3), we did not examine whether a subset of B cells was responsible for the IL-12 induction of IFN-γ from purified NK cells (Chapter 2). The existence of a subset is suggested in the results that show a dose-dependency in the B-NK cell co-cultures with a greater IFN-γ release obtained as the B:NK cell ratio was increased. Is IL-12 alone enough to stimulate the production of IL-18 from B cells? If so, it might only be true for a subset of already primed or in vivo activated splenic B cells, as suggested by studies by the Yuan group (Yuan, 2004). Possible candidates would be the $CD27^{+}$ memory type B cells, or the MZ and B1 subsets. It would also be interesting to determine if the CD5 B cell production of IL-10 and suppression of NK cell IFN-γ is restricted to TLR9 triggering or is noted after any TLR signaling in these cells.

4.4.2 B-NK cell contact

As mentioned it is possible that IL-12 alone is not enough to drive B cell activation and may serve only as an initial priming signal. The B cell induction of IFN-γ from purified NK cells in response to IL-12 was shown to be contact, or at least close proximity, dependent. This might mean that contact between the NK and B cell might provide the B cell with the second stimulatory signal it needs to secrete IL-18. While resting NK cells are not stimulated by IL-12 alone to secrete IFN-γ, they might be activated to upregulate surface proteins, or release other cytokines, that might provide help for B cell activation. It would also be interesting to decipher whether all NK cells would have this possibility as only a small subset of NK cells (the NKDC subset) were positive for IFN-γ after IL-12 stimulation of unfractionated spleen. A possible

candidate for the reciprocal B-NK cell activation might be the CD2-CD48 interaction which other groups have previously noted (Yuan, 2004).

The interaction between DC and NK cells in secondary lymphoid organs after the initiation of an inflammatory response has been investigated (Della Chiesa, Sivori, Castriconi, Marcenaro, & Moretta, 2005; L. Moretta et al., 2006). NK cells and B cells generally occupy different compartments of the spleen but might interact under stimulatory conditions. It would be interesting to observe the splenic distribution of NK cells and B cells in steady state and after either IL-12 or CpG ODN stimulation using immune-histochemical techniques or intravital staining.

4.4.3 NK cell activation and suppression

In Chapter 2 the NKDC subset of splenic NK cells was shown to be the primary responder to IL-12 stimulation. It is possible that these NK cells are either stimulated by the IL-18 secreted by B cells directly and represent a key B cell interacting subset of NK cells, or are affected by the function of other subsets of NK cells that are stimulated by B cells. Other groups have shown that the NKDC subset is also the primary source of IFN-γ in response to CpG ODN 1826 type B stimulation when combined with IL-18 (Chaudhry, Kingham, Plitas, Katz, Raab, & DeMatteo, 2006a). However we did not detect significant production of IFN-γ in response to CpG ODN 1826 and only observed NK cell IFN-γ in response to CpG ODN 2216, a type A ODN. It would be interesting to see if NKDC are the primary producers of IFN-γ in response to ODN 2216 stimulation and thus are the primary targets of B cell suppression as well. In Chapter 3, I show for the first time that NK cells can be directly suppressed by IL-10. It is likely that IL-

10 is not the only way that B cells or other cells are able to suppress the NK cell response during CpG ODN stimulation. Mesenchymal cells that express IDO have been shown to suppress NK cell function (Spaggiari et al., 2008). In terms of immune cells, B cells, DC, and NK cells have all been shown to express functional IDO enzyme. While we were not able to identify IDO positive cells using flow cytometry, western blotting, and qPCR techniques, the use of IDO inhibitor (1-MT) demonstrated that IDO activity was present in CpG ODN stimulated cultures. We found (Appendix 10) that in WT mice inhibitors of IDO combined with IL-10 inhibitors led to increases in IFN-γ from NK cells but were not effective on their own. Conversely, in B cell deficient mice (RagKO and BKO) IDO inhibitor on its own led to increases in IFN-γ equivalent to that of IL-10 inhibitors, and further increases were noted when both inhibitors were used. It is possible that in the absence of B cell regulation, there is potential for over abundant IFN-γ in response to inflammatory stimulators like CpG ODN. Increased IDO activity might allow for more vigilant control of these responses in the absence of B cells.

4.4.4 A possible role for B cell regulation of NK cells during actual pathogenic infection

NK cells are required for the effective clearance of viral infections especially those of the Herpesviridae family including Cytomegalovirus, Varicella Zoster, Epstein Barr virus (EBV), and Herpes Simplex virus -1 and -2. In both humans and mice, HSV-1 infects epidermal or epithelial cells before establishing latent infection in sensory neurons. The control of viral dissemination is controlled by a collaborative effort between the innate and adaptive arms of immunity, with important roles for pDC, conventional DC, NK cells, and $CD4^+/CD8^+$ T cells being reported in the current literature. NK cells are thought to be important to the very early control of HSV spread, and their activation and function during HSV infection has been shown to be dependent upon $CD11c^+$ cells (Kassim, Rajasagi, Zhao, Chervenak, & Jennings, 2006),

IFN-α (Hochrein et al., 2004), IL-18 (Reading et al., 2007), and the synergistic action of TLR2 (responding to unidentified viral component) and TLR9 (viral genomic DNA) signalling (Sorensen et al., 2008). Kastrukoff et al. (Kastrukoff et al., 2010) have recently shown that resistance to lethal encephalitis in mice orally infected with HSV-1 is mediated by the concerted action of NK/NKT and $CD8^+$ T cells. B cells may also play a role in regulating HSV-1 infection. While agammaglobulinemic patients show a normal response to HSV cutaneous infection with respect to herpetic lesions, there have been reports to suggest mice with B cell deficiencies have a greater susceptibility to HSV infection when compared to normal mice (Deshpande, Kumaraguru, & Rouse, 2000). This has been attributed to a lack of IgM antibodies that might neutralize the virus and prevent its spread.

Whether B cells affect the course of HSV-1 infection through their secretion of cytokines and potential regulation of NK cell function has not been investigated. Our preliminary results from a collaborative study with Dr. Lorne Kastrukoff at the University of British Columbia are presented in Appendix 1K. The strain differences between WT mice and BKO mice were examined with respect to the response to HSV-1 mucosal infection (oral route). While the cervical draining LN from resting WT or BKO mouse were not different with respect to size or the total number of lymphocytes, after 3 days post-infection there was a clear increase in both the overall size of WT LNs when compared to BKO (Appendix 11, A). This difference was also paralleled in the number of lymphocytes obtained from WT versus BKO superficial cervical LN, with no increases over uninfected control BKO mice observed after 2, 3, or 5 days after infection while WT mice LN had a doubling over control after 2 days of infection and that seemed to continue up to 5 days post-infection (Appendix 11, B). There was no change in the proportions of relevant immune cells such as NK, NKT, T, B, and DCs (not shown). The most

altered surface expression marker of those tested was CD69 expressed on NK and T cells isolated from the superficial cervical LNs (Appendix 11, C). NK cells from WT mice started out with a low level of CD69 expression that was much more highly expressed in terms of intensity and the number of NK cells expressing it after 2 and 5 days post-infection. In contrast, BKO NK cells started out with a somewhat higher expression of CD69 even when resting which did not increase dramatically after HSV-infection and did not reach the intensity levels seen in WT NK cells day 5 post-infection. CD69 was also somewhat increased in the T cells from the LNs in WT mice and was only slightly higher in the T cells from BKO mice. These preliminary results suggest that the deficiencies in B cells may have detrimental effects upon early NK cell activation during HSV-1 in mice and warrant more study.

It is possible that the B cells are providing stimulatory help to NK cell activation at the point of infection (the lip), or to the activation of other cell types such as DCs which would be important to the effective priming of T cell responses that are also important to the control of HSV-1 spread.

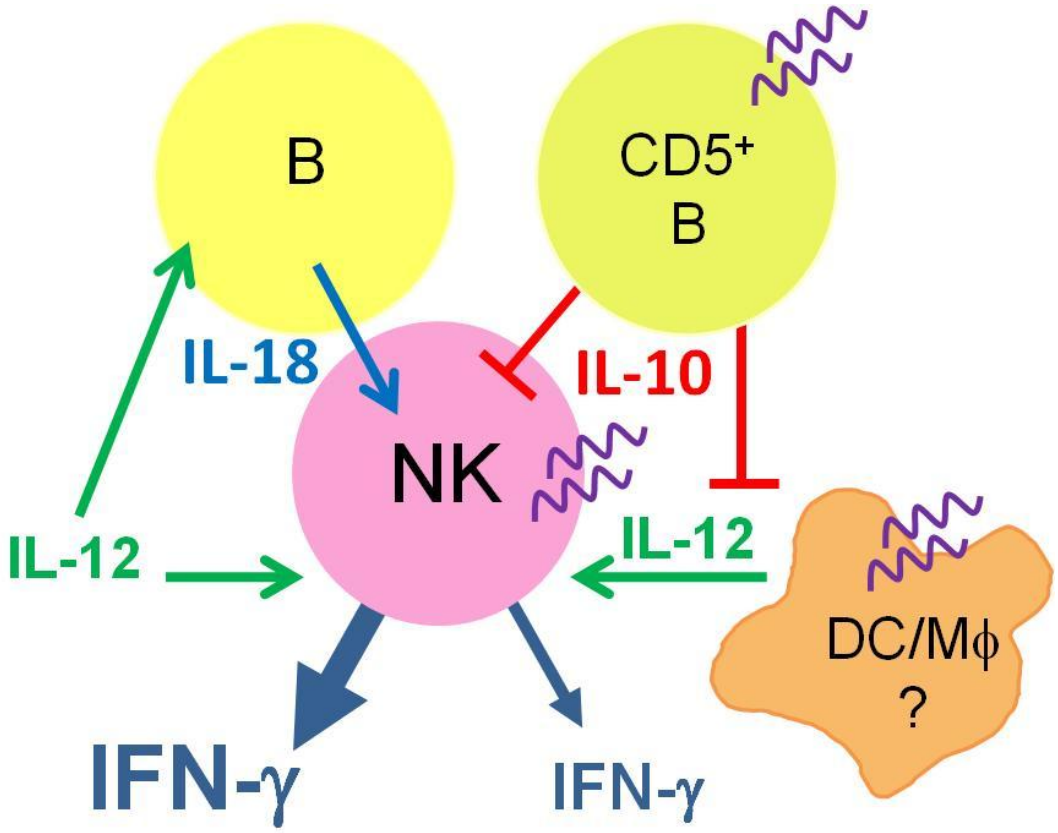

Figure 17 B cells act as enhancers or suppressors of NK cell IFN-γ in response to IL-12

Resting mouse NK cells are induced to secrete IFN-γ in response to IL-12 through a mechanism that first requires the stimulation of IL-18 production from resting B cells. While IL-12 alone is not sufficient in driving NK cell activation which might be due to a limited number of IL-12R sites found on resting NK cells. The production of IL-18 from B cells synergizes with IL-12 to induce optimal IFN-γ production. There appears to be a need for contact between the B cell and NK cell, while it does not depend on CD40 or CD80/86 ligation, the nature of this contact has yet to be tested. CpG ODN (represented as a purple wavy line) is not able to stimulate NK cell activation alone. It may mediate its pro-IFN-γ effects through the induction of IL-12 from cells like DC or Mϕ, or other TLR9 containing cell types. It is possible that the IL-12 or IL-18 secreted from CpG ODN activated cells are able to combine with CpG ODN to directly activate NK cell cytokine release. B cells on the whole do not seem to be involved in the stimulation of NK cells in response to CpG ODN but instead a subset of B cell that expresses CD5 secretes IL-10 which inhibits the production of IL-12 from activated DC/ Mϕ or another cell type and also was shown to act directly upon the NK cell itself. The production of IL-10 does not lead to a complete loss of IFN-γ but instead blunts the response so that the overall CpG ODN induction of IFN-γ is restricted and potentially controlled in order to avoid pathology. In the absence of B cells it is possible that other cell types step up and secrete IL-10 or utilize other means of suppressing IFN-γ production (like IDO) to maintain overall control and prevent over exuberant IFN-γ in response to CpG ODN.

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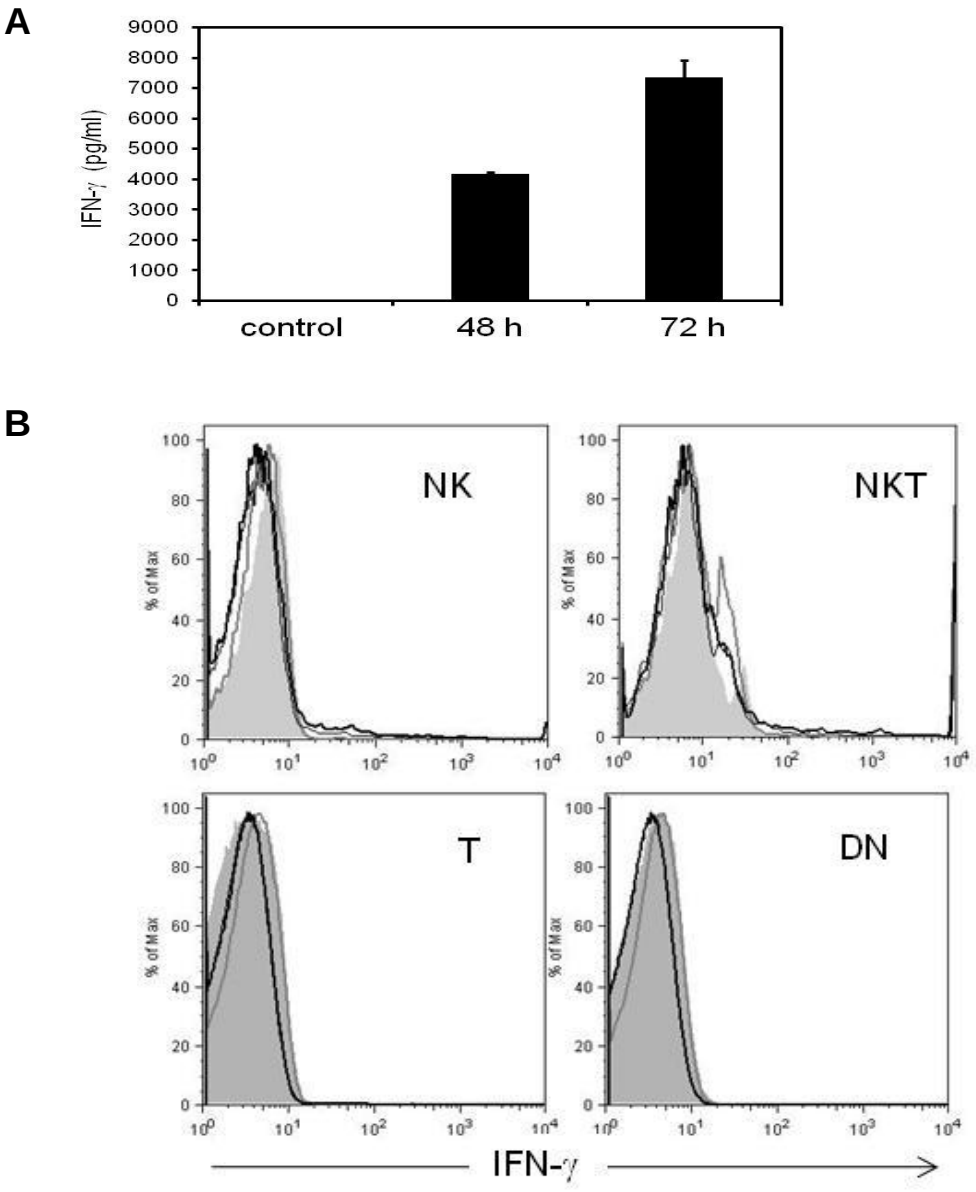
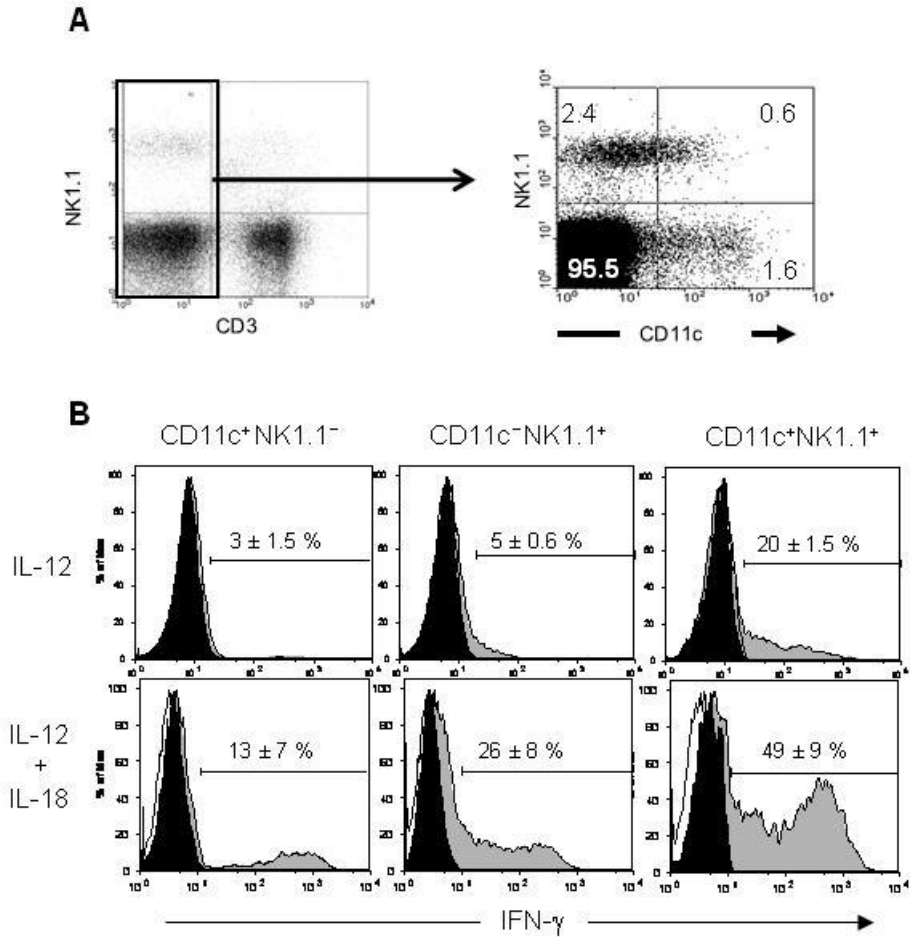
Appendices



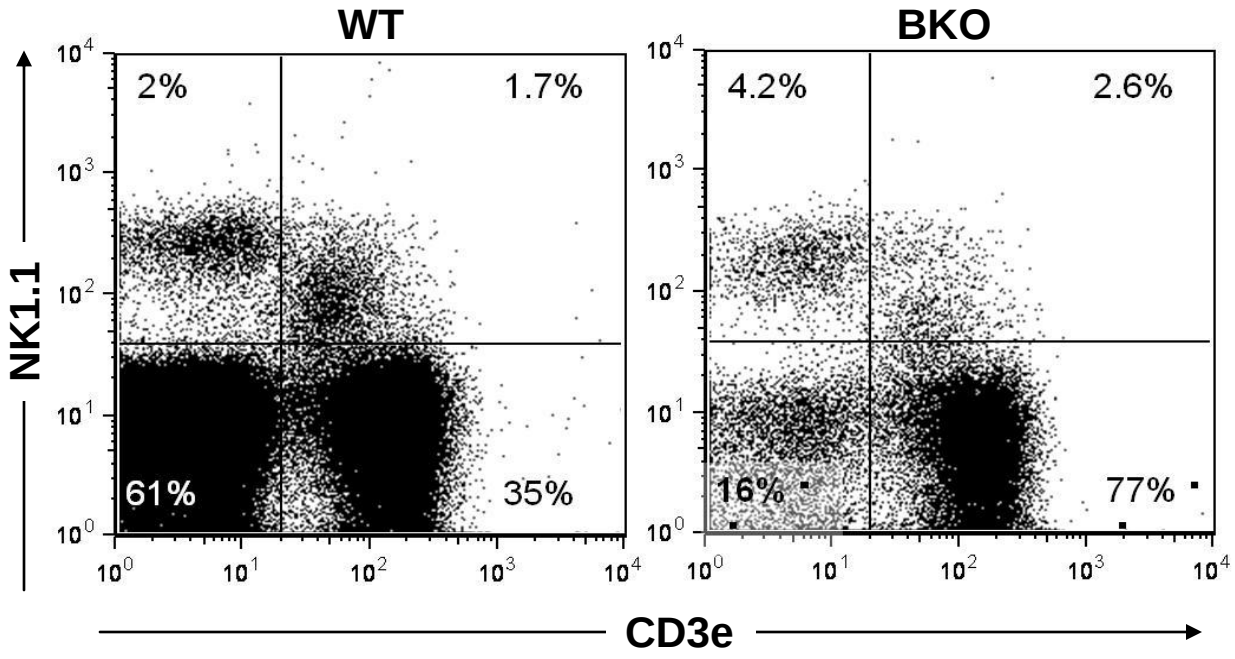
Appendix 1 Populations within spleen cells that are IFN-γ^+ after 48 or 72 h of stimulation with IL-12

A. WT splenocytes were cultured at 4×10^6 cells/ml and stimulated with 1ng/ml of IL-12 for either 48h or 72h at 37ºC. Cell free supernatants were assessed for levels of IFN-γ by ELISA. Data is the mean ± SD from one experiment (n=1) per time point with triplicate cultures. B. Cultured cells were stained with NK1.1 and CD3 mAbs and then fixed, permeabilized, and stained for intracellular IFN-γ. Results are shown as percentage of cells positive for intracellular IFN-γ in NK ($NK1.1^+CD3^-$), NKT ($NK1.1^+CD3^+$), T ($NK1.1^-CD3^+$) and double negative (DN) ($NK1.1^-CD3^-$) populations.



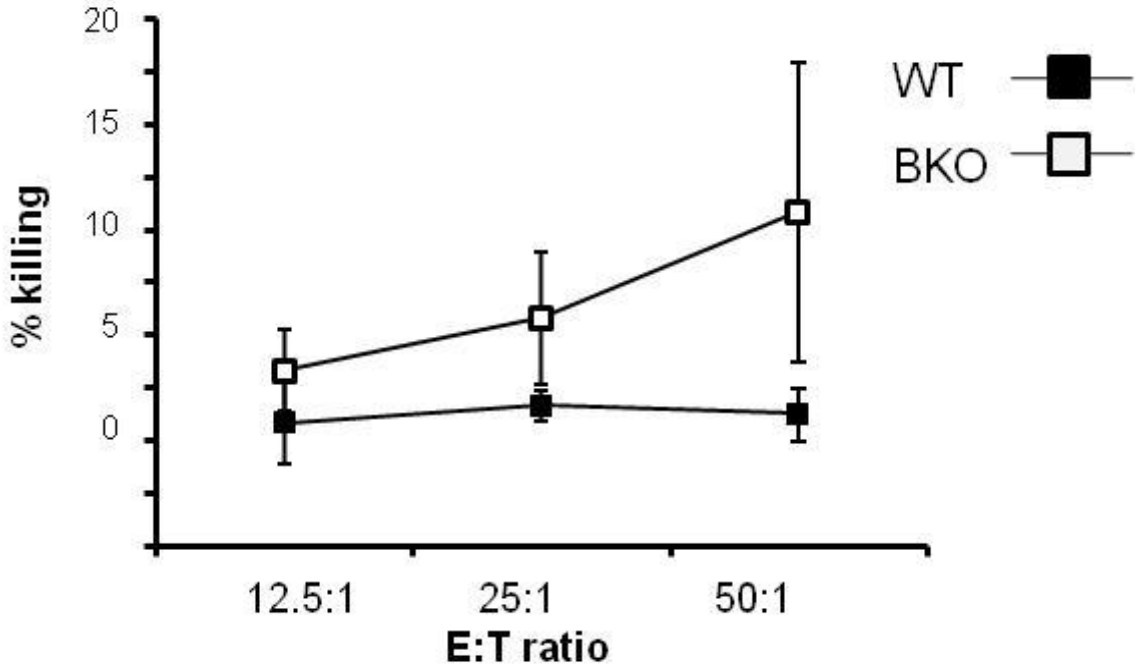
Appendix 2 CD11c^{+} NK cells are the main producers of IFN-γ among IL-12 stimulated NK cells

A. WT mouse splenocytes were stained with mAbs to NK1.1, CD3 and CD11c and analyzed by FACS. NK1.1$^{+/-}$ CD3^{-} were gated and analyzed for the expression of CD11c. The numbers show percentages of cells in each quadrant. *B.* WT spleen cells were cultured with or without 1 ng/ml IL-12 or IL-12 and1 ng/ml IL-18 overnight, stained for CD3, NK1.1, CD11c and intracellular IFN-γ. Cell populations identified by NK1.1 and CD11c expression as in (A) were gated and analyzed for intracelluar IFN-γ. Black histograms show staining with isotype matched control antibody, grey histograms show IFN-γ. The data are respresentative of three independent experiments, mean % ± SD shown.



Appendix 3 Percentage of NK cells found in WT and BKO non-infected mice

Freshly isolated and resting spleen cells from either WT or BKO mice were stained with NK1.1 and CD3 mAbs in order to show percentages NK ($NK1.1^{+}CD3^{-}$), NKT ($NK1.1^{+}CD3^{+}$), T ($NK1.1^{-}CD3^{+}$) and double negative (DN) ($NK1.1^{-}CD3^{-}$) populations. The results presented are representative from one experiment.



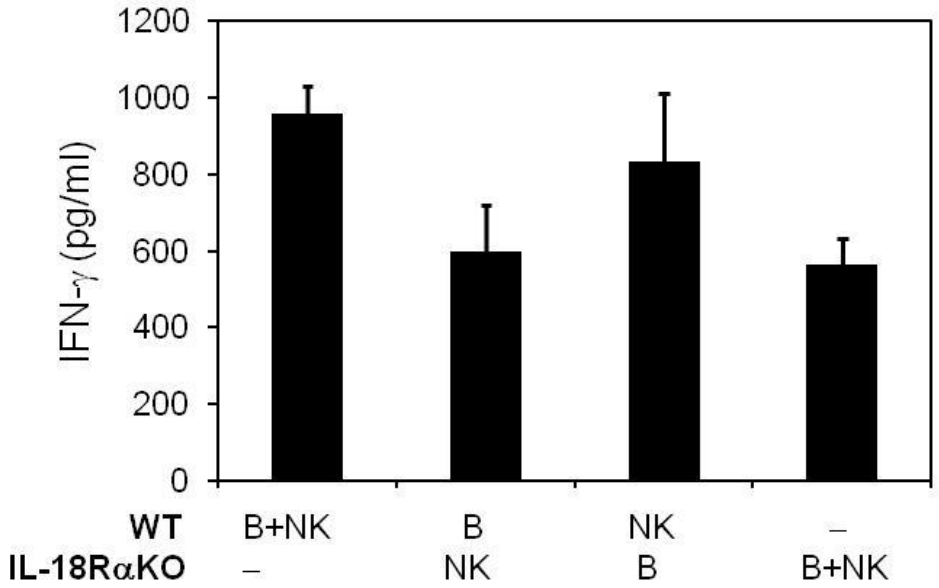
Appendix 4 B cells do not promote NK cell cytotoxicity against YAC-1 cells in IL-12 stimulated splenocytes

WT or BKO splenocytes were cultured at 4×10^6 cells/ml and stimulated with 1ng/ml of IL-12 overnight. These cells were used as effector cells and cultured with CFSE (carboxyfluorescein diacetate succinimidyl ester, Invitrogen, Carlsbad, CA) labeled YAC-1 cells as targets at E:T (effector:target) ratios of 12.5:1, 25:1, and 50:1. After a 4 h incubation, samples were stained with 7-AAD (Invitrogen) and analyzed on a flow cytometer. Killed targets were identified as $CFSE^+$ and $7\text{-}AAD^+$. There is no significant difference between WT or BKO killing ability ($p=0.21$, ANOVA).

IL-18 addition (pg/ml)	IFN-γ (pg/ml) detected in supernatants
10,000	> 2000
1000	> 2000
100	> 2000
*10	1550
*1	45

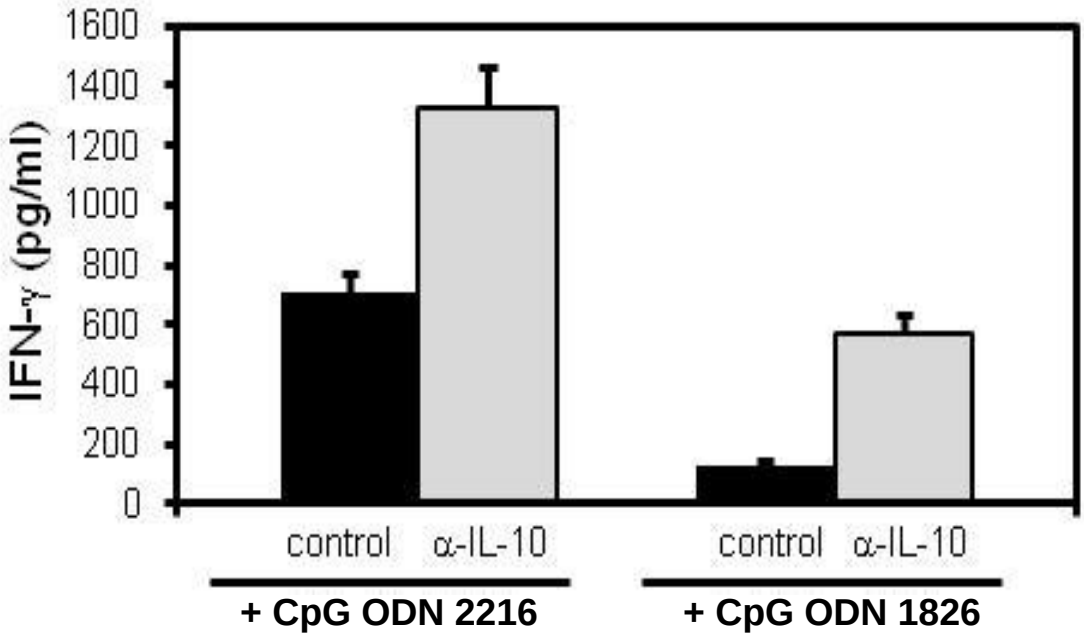
Appendix 5 The concentration of IL-18 required to stimulate IFN-γ from IL-12 activated NK cells is below that of ELISA detection limits of IL-18 in supernatants

NK ($NK1.1^{+}CD3^{-}$) cells were FACS sorted from WT spleen and cultured at 3×10^{4} per well in 200μL of media in a 96 round bottom tissue culture plate. Cells were stimulated with 1ng/ml of IL-12 with varying concentrations of IL-18 (pg/ml). Cell free supernatants were collected after 48 h of culture and assessed for IFN-γ levels by ELISA. Values of IFN-γ above 2000 pg/ml are shown as > 2000. (*) denotes that the amount of IL-18 added is undetectable in the standard IL-18 ELISA kit (10 and 1pg/ml). Results are from triplicate wells of one experiment.



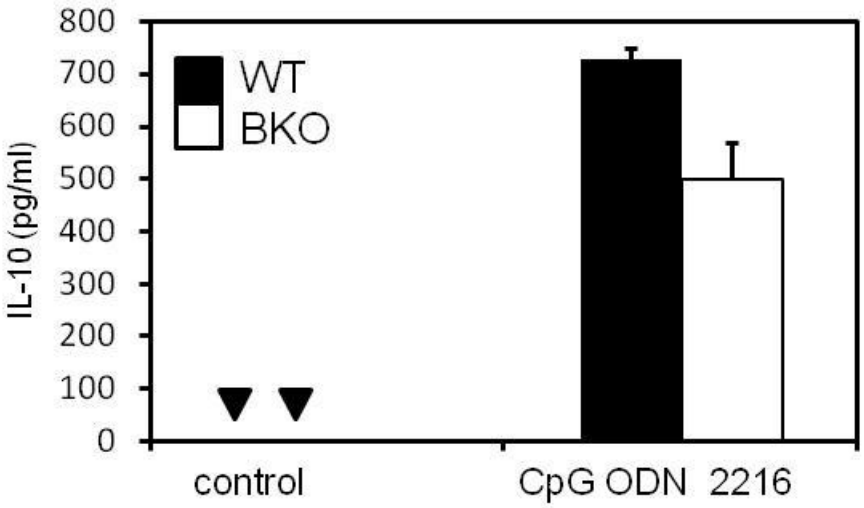
Appendix 6 IL-18Rα is not needed for NK cell stimulation by B cells during IL-12 activation

Purified B ($CD19^+$) cells (3×10^5) and NK ($NK1.1^+CD3^-$) cells (3×10^4) from WT and IL-18RαKO mice were combined and stimulated with IL-12 for 48 h. The results show the amounts of IFN-γ (mean ± SEM) in the culture supernatants of three independent experiments, each with triplicate cultures ($p>0.05$ and $p>0.5$).



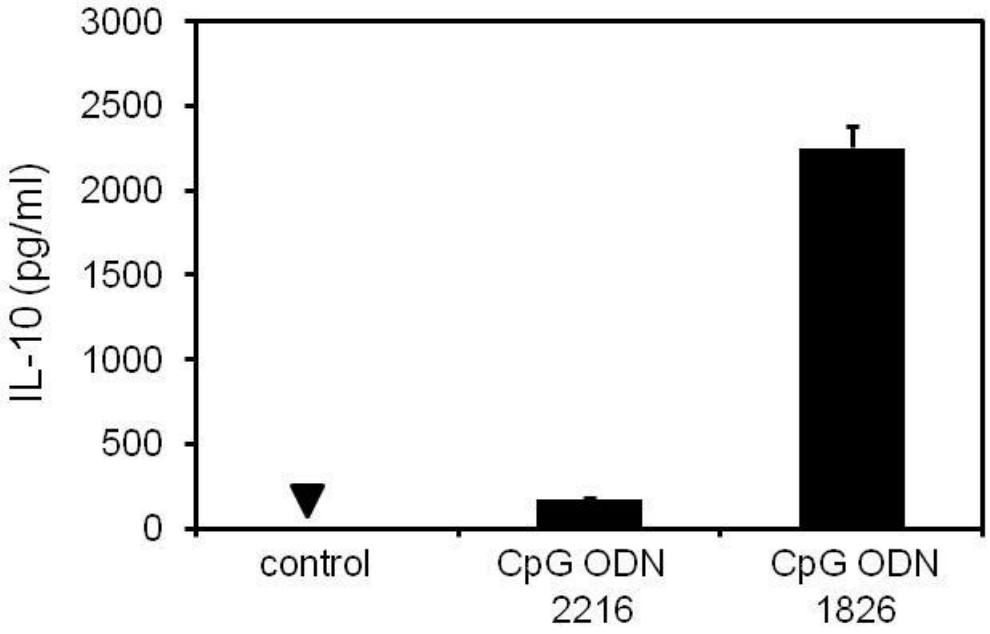
Appendix 7 CpG ODN 1826 is a poor stimulator of WT IFN-γ even in the presence of IL-10 neutralization

Unfractionated WT splenocyte cultures (8×10^5/200μl) in 96-well round bottom tissue culture plates were stimulated with 2μg/ml of CpG ODN 2216 (4×10^6/ml)) or CpG ODN 1826 (2μg/ml) in the presence or absence of IL-10 neutralizing antibody (αIL-10, at 2μg/ml). Results are the mean ± SEM of three independent experiments, each with triplicate cultures.



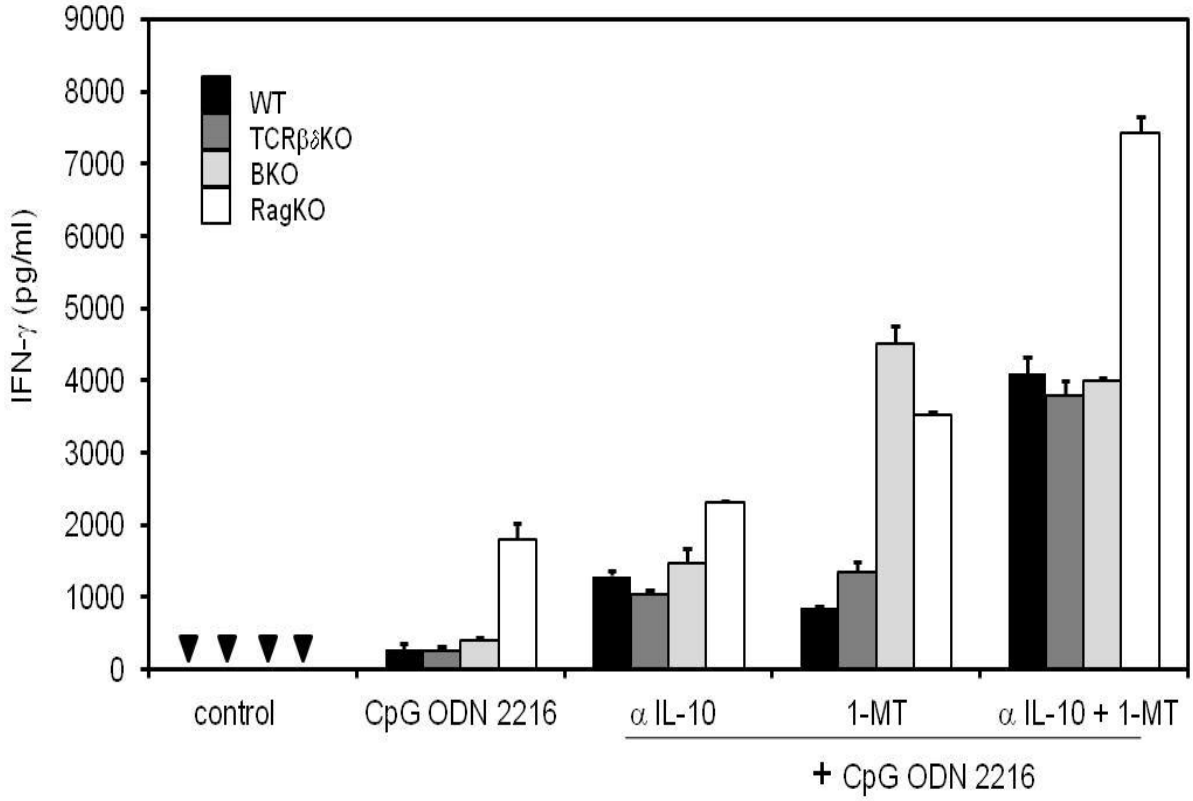
Appendix 8 CpG ODN 2216 induces the production of IL-10 from WT and BKO spleen cells

Unfractionated WT or BKO splenocytes were cultured (8×10^5/200μl) in 96-well round bottom tissue culture plates and stimulated with 2μg/ml of CpG ODN 2216. After 24h cell-free supernatants were collected and assessed for IL-10 production by ELISA. Results are the mean ± SEM of three independent experiments, each with triplicate cultures. ▼, undetectable levels of cytokine.



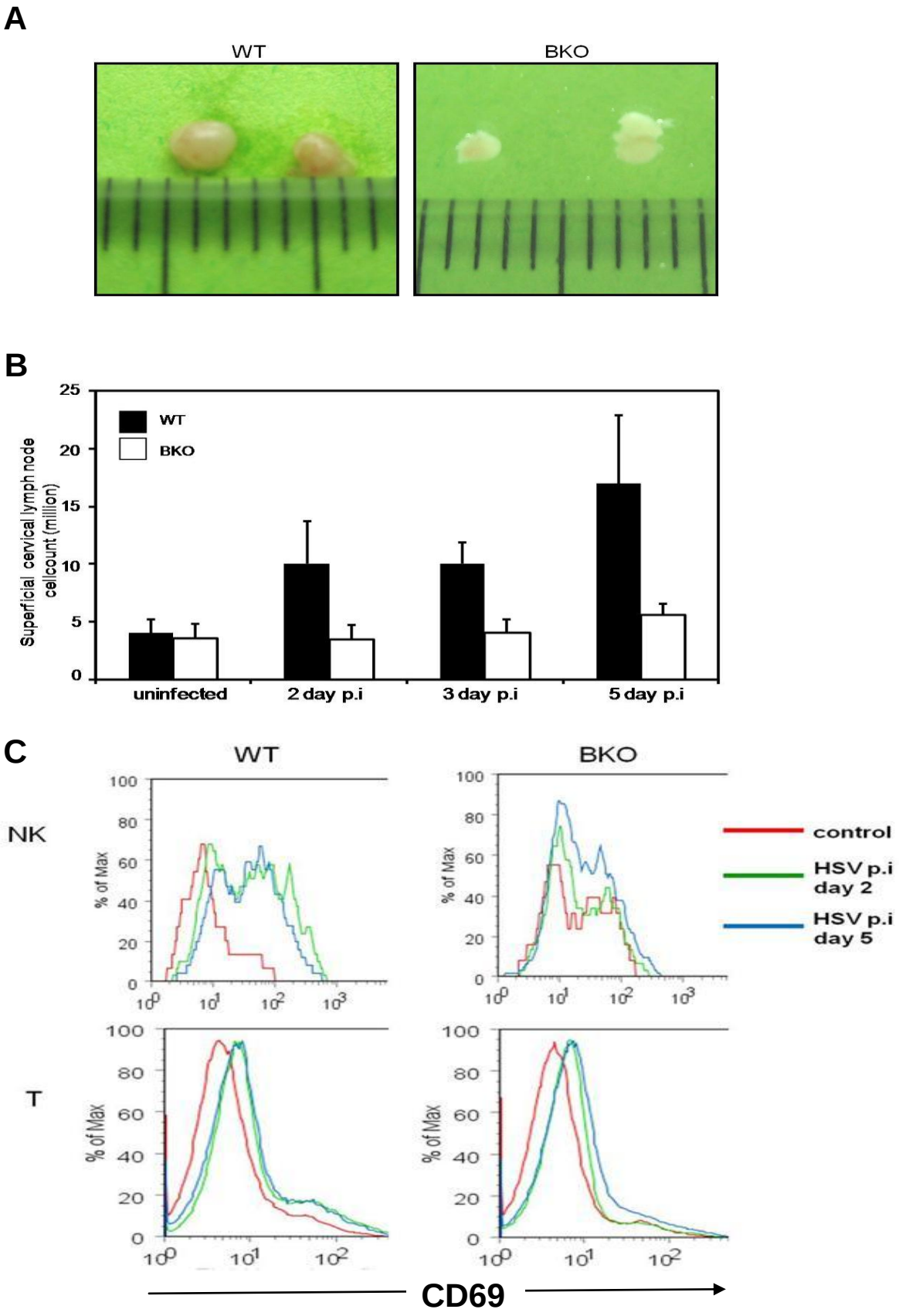
Appendix 9 B cells secrete IL-10 in response to CpG ODN 2216 and 1826

FACS purified B cells ($CD19^+$) from WT splenocytes were cultured (8×10^5/200μl) in 96-well round bottom tissue culture plates and stimulated with 2μg/ml of CpG ODN 2216 or CpG ODN 1826. After 24h cell-free supernatants were collected and assessed for IL-10 production by ELISA. Results are the mean ± SEM of three independent experiments, each with triplicate cultures. ▼, undetectable levels of cytokine.



Appendix 10 Strain comparisons of IFN-γ production after CpG ODN 2216 in the absence of IL-10 and/or IDO activity

WT, TCRβδKO,BKO, and RagKO splenocytes were cultured (8×10^5/200μl) in 96-well round bottom tissue culture plates and stimulated with 2μg/ml of CpG ODN 2216. Cultures were also treated with IL-10 neutralizing antibody (αIL-10) at 2μg/ml, or the IDO inhibitor 1-methyl-tryptophan (1-MT) at 2mM, or a combination of both. After 24h cell-free supernatants were collected and assessed for IFN-γ production by ELISA. Results are the mean ± SEM of three independent experiments, each with triplicate cultures. ▼, undetectable levels of cytokine


A
WT
BKO
B
Superficial cervical lymph node cellcount (million)
WT
BKO
uninfected
2 day p.i
3 day p.i
5 day p.i
C
WT
BKO
NK
T
% of Max
control
HSV p.i day 2
HSV p.i day 5
CD69

Appendix 11 WT and BKO mice differ in the response to HSV-1 infection with respect to draining lymph node lymphocyte numbers and NK and T cell activation status

WT and BKO mice 10 week female mice were infected with a sublethal dose of virus via the oral mucosa. Draining cervical lymph nodes (CLN) were excised from euthanized animals at days 2, 3 or 5 post-infection (p.i). A. Demonstrates differences in general size of CLN at day 3 p.i in WT and BKO mice. B. Shows the number of lymphocytes in CLN removed from non-infected and infected WT or BKO mice at varying days after viral infection. Results represent the mean ± SD of total lymphocytes obtained from the CLNs from 4 mice for each time point and condition. C. CLN lymphocytes were stained for various cell populations (NK, NKT, T and DN) and examined for the expression of various markers. CD69, which is an early marker of activation was the only marker that was differentially expressed between the non-infected and infected WT and BKO strains.