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Regulation of B Cell Function by Human Invariant Natural Killer T Cells in Health and Autoimmune Disease

A dissertation submitted to Trinity College Dublin as requirement for the degree of Doctor of Philosophy (PhD)

2011

BY

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List of abbreviations used

Abbreviation	Full name
α-GC	α-galactosylceramide
ACAID	Anterior chamber associated immune deviation
AHR	Allergen-induced airway hyperreactivity
ANA	Anti-nuclear antibodies
APC	Allophycocyanin
APLS	Anti-phospholipid syndrome
BAFF	B cell-activating factor
BCA	Bicinchoninic acid
BCR	B cell receptor
Breg	Regulatory B cells
BSA	Bovine serum albumin
CBA	Cytometric bead array
CD	Cluster of differentiation
CIA	Chronic collagen-induced arthritis
CBP	CREB-binding protein (coactivator of CREB)
CREB	cAMP-response element-binding protein
CRP	C reactive protein
cRPMI	Complete RPMI
Cy5/7	CyChrome5/7
DC	Dendritic cells
DN	Double negative
dsDNA	Double-stranded deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EB/AO	Ethidium bromide/acridine orange
ENA	Extractable nuclear antigens
ESR	Erythrocyte sedimentation rate
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FMO	"Fluorochrome minus one" control
FSC	Forward scatter channel
HMB-PP	(E)-4-hydroxy-3-methyl-but-2enyl pyrophosphate
HRP	Horse radish peroxidase
HTLV-1	Human lymphotropic virus 1
Ig	Immunoglobulin

Abbreviation	Full name
IL	Interleukin
iNKT	Invariant natural killer T cells
JNK	c-Jun NH_2-terminal protein kinase
LPS	Lipopolysaccharide
MAIT cells	Mucosal-associated invariant T cells
MAPK	Mitogen-activated protein kinase
MSK	Mitogen- and stress-activated protein kinase
mAb	Monoclonal antibody
NEAA	Non-essential amino acids
NOD	Non-obese diabetic (mice)
NZB/W F_1	(New Zealand black x New Zealand white) F_1 hybrid murine model
PBA	Phosphate-buffered saline containing BSA and azide
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein complex
PHA	Phytohemagglutinin
PKB	Serine/Threonine protein kinase B (also known as Akt)
PMA	Phorbol-12-myristate-13-acetate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
RNP	Ribonucleoprotein
RSV	Respiratory syncytial virus
RT	Room temperature
SEB	Staphylococcal enterotoxin B
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
snRNP	Small nuclear ribonucleoprotein
SSC	Side scatter channel
T2-MZP	Transitional 2-marginal zone precursor (B cells)
TCM	T cell medium
TCR	T cell receptor
TLR	Toll-like receptor
TMB	3,3,5,5-tetramethylbenzidine
TNF	Tumour necrosis factor

Abbreviation	Full name
Tr1	IL-10-producing $Foxp3^{-}CD4^{+}$ regulatory T cells
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cells
MFI	Mean fluorescence intensity
ANOVA	Analysis of variance
PPD	Purified protein derivative
SLE	Systemic lupus erythematosus
SLEDAI	Systemic lupus erythematosus disease activity index
Th1/Th2	T helper 1/T helper 2
NA	Not applicable
ns	Not significant

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1 Abstract

Invariant natural killer T (iNKT) cells are a subset of innate T lymphocytes that express a semi-invariant T cell receptor that recognises glycolipids presented on CD1d by antigen-presenting cells. They have the unique ability to stimulate both humoral and cell-mediated immune responses by direct cytotoxicity, rapid cytokine release and inducing maturation of antigen-presenting cells (APC). iNKT cells are gaining a reputation as immunoregulatory cells and have been shown to interact with B cells, the main effector of humoral immune responses. Activation of iNKT cells in mice enhances antibody responses to co-administered antigen but little is known about the phenotypic and other functional changes on B cells that occur in response to iNKT help and whether iNKT cells regulate various B cell subsets differentially, such as regulatory B cells and memory B cells. Thus the effect of co-culturing human iNKT cells on B cell maturation and antibody production *in vitro* was investigated, with a closer look at the effect of iNKT cells on various regulatory and memory B cell subsets. The effect of iNKT cells on B cell function was investigated in both healthy controls and SLE patients.

It was observed that iNKT cell help to B cells induced upregulation of B cell expression of CD40, CD69, CD58, CD86, CD80, CD83 and CD95, but reduced their ability to present antigens to naive T cells. iNKT cell help to B cells also enhanced antibody secretion but had little effect on cytokine production. The iNKT-induced antibody production was contact-dependent whereas iNKT-induced B cell activation was not. We found that the interaction between iNKT cells and B cells was dependent on CD1d, the CD40-CD154 co-stimulatory signal, IL-13 and the delta isoform of phosphoinositide 3-kinase but not IL-4. $CD4^+$ iNKT cells were most efficient at activating B cells compared to $CD8^+$ and double negative (DN) iNKT cells but did not display superiority in induction of antibody production. In addition, co-culture of B cells with $CD4^+$ iNKT cells resulted in the expansion of a B cell population that expressed high levels of CD5 and CD1d, and produced IL-10, a phenotype that defines regulatory B cells in mice. Phenotyping of iNKT and B cell subsets from the peripheral blood of systemic lupus erythematosus (SLE) patients elucidated that their $CD4^+$ iNKT cells and naïve B cells were expanded and activated, whereas memory B cells and regulatory B cells were decreased in frequencies but activated. iNKT cells expanded from SLE patients appeared to be more activated than iNKT cells expanded from healthy individuals. They had greater stimulatory activity on B cells, causing greater B cell activation and cytokine production, which occurred without the need for α-galactosylceramide. Although iNKT cells from SLE patients induced less antibody production overall from B cells compared to healthy

iNKT cells, they induced greater production of IgG_2 antibodies, which are observed in Th1 responses, Th1 predominance being characteristic of lupus nephritis.

Our results suggest that human iNKT cells are important immunoregulators of B cell function and they appear to be dysfunctional in SLE, leading to dysregulation of B cell activation and antibody production. iNKT cells could potentially be targeted to alter the magnitude or isotype of antibody production, allowing the use of iNKT cell agonists as adjuvants for vaccines or in immunotherapy for autoreactive antibody-mediated autoimmune diseases.

2 Introduction

2.1 The Immune System

2.1.1 Innate and Adaptive Immunity

The immune system is classically divided into two categories: the innate immune system and the adaptive immune system. The innate immune system is often described as the first line of defense against microbial challenge. It is characterised by its immediate non-specific response towards the pathogen and lack of immunological memory. Conversely, the adaptive immune system is characterised as a highly-specific response. There is a lag time between the initial appearance of the pathogen and the mounting of the response against it, and the memory cells that develop remain in circulation for a long time after the primary immune response. (Janeway et al., 2005)

For effective elimination of pathogens during an immune challenge, crosstalk between innate and adaptive immunity is vital. Cells of the innate immune system, such as dendritic cells, are known to act directly as antigen-presenting cells to activate cells of the adaptive immune system, such as T cells and B cells (Bousso, 2008; von Andrian and Mempel, 2003), or indirectly by secreting soluble factors such as cytokines (Bell, 2002). Innate cells can also interact with each other to optimise immune responses: for example, dendritic cells can enhance natural killer cell activation, cytotoxicity and IFN-γ secretion (Hamerman, Ogasawara, and Lanier, 2005). In recent years the view of the scientific community on the role of the adaptive immune system has evolved. Research has shown that some cells of the adaptive immune system have innate functions too (Hu et al., 2007; Nakanishi, 2001), and as a result subsets of innate T cells have emerged.

2.2 Cells of the Immune System

The cells that constitute the immune system originate from hemopoietic stem cells in the bone marrow, giving rise to two major lineages of cells – lymphoid progenitor cells and myeloid progenitor cells. Lymphocytes are generated from lymphoid progenitor cells and consist of T cells, B cells and natural killer (NK) cells. Myeloid cells are generated from myeloid progenitor cells and consist of monocytes, macrophages, dendritic cells and granulocytes. (Janeway et al., 2001)

2.2.1 T cell development and cellular immunity.

T cells are generated in the thymus (Miller and Osoba, 1967) from lymphoid progenitor cells which have trafficked through blood from the bone marrow to settle in the thymus (Schwarz and Bhandoola, 2006). Once these progenitors reach the thymus they are referred to as "thymocytes" lacking expression of CD4, CD8 and a functional T cell receptor ($CD4^-CD8^-$ double negative, DN). T cell receptor (TCR) rearrangement of the γ, δ, and β loci occurs during development from the DN1 to DN3 stage, which is regulated by Notch signalling and IL-7 (Guido, 2006). Successful TCRβ rearrangement (Born et al., 1985) allows DN3 thymocytes to assemble the pre-TCR complex for 'β-selection' whereby thymocytes with gross defects in the TCRβ arrangement are eliminated (Guido, 2006). Surviving DN3 thymocytes proliferate and generate a large pool of $CD4^+CD8^+$ double positive (DP) thymocytes which then proceed on to TCRα rearrangement to form a completely assembled TCR (Krangel, 2009). The majority of thymocytes develop into α:β T cells while a minority develop into γ:δ T cells. Thymocytes undergo positive selection to select for those whose TCR can recognise self-peptide:self-MHC complexes with weak affinity, followed by loss of the CD4 and/or CD8 co-receptor molecules. Finally they undergo negative selection to eliminate those with high affinity to self-peptide:self-MHC complexes to form a mature (but antigen-naïve) T cell (Janeway et al., 2005).

T cell-mediated immunity is also known as cellular or cell-mediated immunity. During infection, naïve T cell activation occurs in response to foreign antigen presented on antigen-presenting cells such as macrophages and dendritic cells, resulting in their proliferation and differentiation into effector T cells (Lanzavecchia, 1998). Conventional dogma states that these effector T cells form either $CD4^+$ T helper cells that promote activation of humoral and innate immunity (Stevens et al., 1988; Zhu, Yamane, and Paul, 2009) or $CD8^+$ cytotoxic T cells that mediate cell killing by releasing perforin, granzymes and granulysin which disrupt the integrity of the cell membrane. However, recent research has shown that $CD4^+$ (Martorelli et al., 2010) and $CD4^-CD8^-$ DN T cells also have cytotoxic functions (Stenger et al., 1997). $CD4^+$ T cells can be further subdivided into different subsets based on their cytokine secretion profile, namely Th1 (IFN-γ, TNF-α), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17, TNF-α, IL-6, IL-21, IL-22), Treg (IL-10, TGF-β), Th9 (IL-9), Th22 (IL-22) and follicular T helper cells (ThFH) (IL-10, IL-4, CXCL13, IL-21, IL-2) (Beissert, Schwarz, and Schwarz, 2006; Glimcher and Murphy, 2000; King, 2009; Locksley, 2009; Trifari et al., 2009; Zhou, Chong, and Littman, 2009). These subsets are shown in Figure 2.2.1.

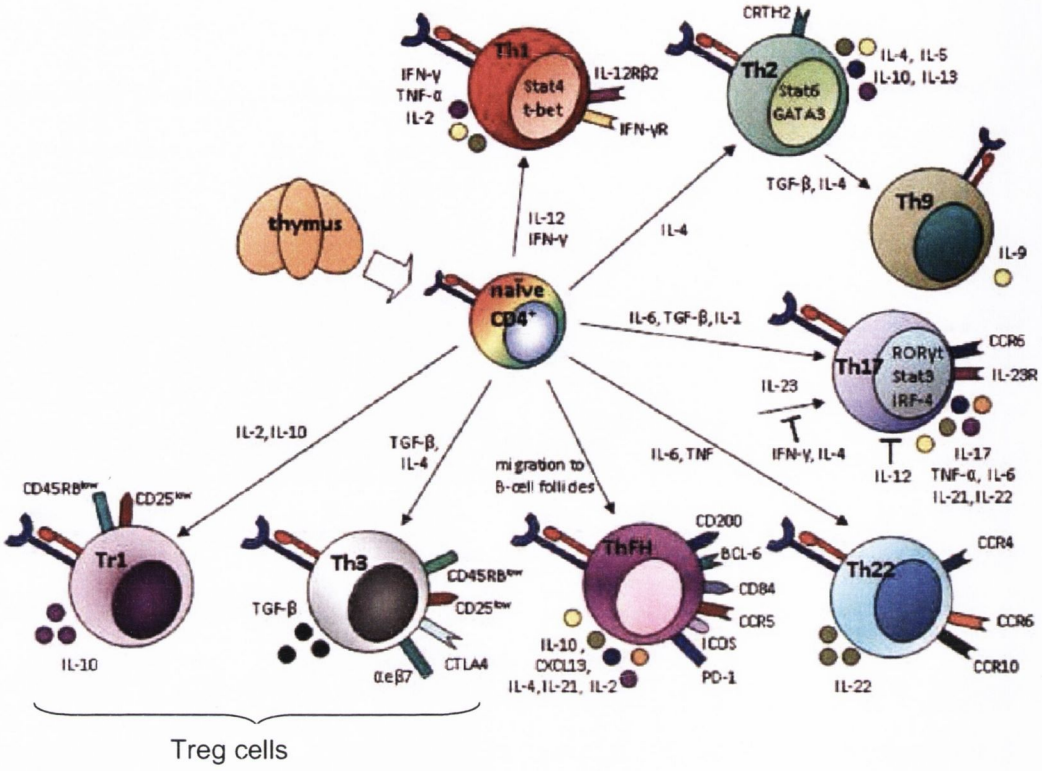

Figure 2.2.1. Currently known subsets of CD4$^+$ T cells. Adapted from (Martorelli et al., 2010). Naïve CD4$^+$ T helper cells are able to differentiate into many different helper subsets expressing different surface receptors, transcription factors that selectively induce their differentiation and cytokine production profiles.

2.2.1.1 Innate T cells.

Innate T cells belong to a subset of T lymphocytes that possess both innate and adaptive properties. Two of the most studied innate T cells are the γδ T cells (which express the γδ TCR instead of the conventional αβ TCR) and the natural killer T cells. Innate T cells are thought of as immunoregulatory cells that can enhance or suppress the immune system depending on the stimuli present in the immune microenvironment, although what causes the shift in balance remains to be elucidated (Kawakami, 2004).

2.2.2 B cell development and humoral immunity.

B cell development occurs in the bone marrow with the aid of bone marrow stromal cells which provide soluble factors facilitating B lymphocyte proliferation and differentiation as

well as cell-cell interaction. The stages of B cell development are defined by sequential rearrangement and expression of heavy and light immunoglobulin chains on its surface. Progenitors that are committed to B cell lineage are known as pro-B cells, whereby rearrangement of the heavy chain occurs. At the pre-B cell stage, the heavy chain (or μ chain) is expressed intracellularly and transiently on the surface in combination with a surrogate light chain to form a pre-B cell receptor (Guelpa-Fonlupt et al., 1994). Expression of the pre-B cell receptor signals the cell to halt rearrangement of the heavy chain and to initiate light chain rearrangement. Completion of light chain assembly and expression of IgM on the cell surface result in the formation of an immature B cell. Immature B cells undergo selection for self-tolerance and survival ability and migrate to the periphery, undergoing further differentiation to form naïve B cells with the addition of IgD expression on their surface. (Ghia et al., 1996; Janeway et al., 2005)

B cell-mediated immunity is known as humoral immunity, or antibody-mediated immunity. Activation of naïve B cells occur when they are exposed to antigen (T cell-independent activation) or activated helper T cells (T cell-dependent activation) that recognise the antigen that has been intracellularly processed and presented on MHC II by the B cells to the helper T cells (Lanzavecchia, 1990). T cell-independent activation is less common and two categories exist. Type I T cell-independent activation occurs when antigens such as the RNA phage Qβ coats cross-link the IgM B cell receptor (BCR) (Fehr et al., 1998), whereas type II T cell-independent activation occurs when macrophages/dendritic cells present the antigen to the B cells, causing cross-linking of the BCR (Craxton et al., 2003). In T cell-dependent activation, co-stimulatory signalling (e.g. CD40-CD154) and cytokine production (IL-4, IL-5 and IL-6) by the helper T cells aids in activation of the B cells, driving proliferation and differentiation of the B cells into antibody-secreting plasma cells or memory B cells. Antibodies produced by the B cells opsonize pathogens for phagocytosis by macrophages, activate the complement system and/or natural killer cells to kill the pathogen, and neutralize the pathogens so that they are unable to bind to cells and infect them. (Janeway et al., 2005)

2.2.3 Monocytes, macrophages and dendritic cells

Monocytes are bone marrow-derived myeloid cells that circulate in the blood and populate tissues as macrophages during homeostasis and inflammation (van Furth and Cohn, 1968). Circulating blood monocytes constitutively differentiate into macrophages

and dendritic cells in peripheral tissues during steady state, and can also participate directly in immune defense against microbial pathogens (Serbina et al., 2008).

Macrophages are tissue-resident phagocytic cells that are well-equipped with an expansive range of pattern recognition receptors that are able to recognise both exogenous and endogenous ligands, suggesting that they have a dual role in normal tissue function and host defense (Gordon, 2002). They are also professional antigen-presenting cells that facilitate B cell and T cell activation during immune challenge (Martinez-Pomares and Gordon, 2007). Under steady-state conditions, a subset of IL-10-producing macrophages mediate clearance of apoptotic cells (Xu et al., 2006).

There are two types of dendritic cells (DC) – the classical myeloid DC, which originate from the myeloid lineage, and plasmacytoid DC (pDC), which originate from a lymphoid lineage (Corcoran et al., 2003). Myeloid DC are highly phagocytic when immature and phagocytosis of foreign pathogens causes them to mature into professional antigen-presenting cells that migrate into lymphoid organs, express lymphocyte co-stimulatory receptors and secrete cytokines to initiate immune responses by B and T cells (Banchereau and Steinman, 1998). pDC are relatively long-lived compared to classical DC and they are specialized in anti-viral defenses through the secretion of type I interferons (Colonna, Trinchieri, and Liu, 2004).

2.2.4 Granulocytes

Granulocytes are characterised by the presence of granules in their cytoplasm and are also termed polymorphonuclear leukocytes. They consist of eosinophils, basophils and neutrophils which protect against infection by degranulation. Neutrophils have phagocytic properties in addition to their ability to release cationic proteins, defensins, proteolytic enzymes, cathepsin G, lysozyme and myeloperoxidase (Mayer, 2006). Eosinophils are important in host defenses against parasites (Rothenberg and Hogan, 2006), acting as professional antigen-presenting cells and immunomodulators of other cells such as B cells, $CD4^+$ T cells, dendritic cells, mast cells, neutrophils and basophils (Akuthota et al., 2008). Basophils are also professional antigen-presenting cells and are critical for induction of Th2 cell differentiation and inflammatory responses to helminth parasites or allergens (Siracusa et al., 2010). Injured basophils release histamine and prostaglandin to increase vascular permeability and recruitment of other effector immune cells such as phagocytes (Mak and Saunders, 2011). Recently, a novel

population termed "nuocytes", which expand *in vivo* in response to IL-25 and IL-33, has been identified to be critical for the early IL-13 response against helminths (Neill et al., 2010).

2.3 Innate Lymphocytes

2.3.1 Natural killer (NK) cells

NK cells are distinguished by the expression of NK1.1 on $CD3^-$ lymphocytes in mice and $CD56^+NKp46^+$ on $CD3^-$ lymphocytes in humans (Walzer et al., 2007). NK cells can secrete cytokines and chemokines that influence the immune response and kill infected or transformed cells by perforin/granzyme expression or Fas-receptor mediated apoptosis (Kim et al., 2000; Smyth et al., 2005). NK cells most typically secrete IFN-γ, which promotes a Th1 immune response, causing antigen-presenting cells to upregulate expression of MHC I (Wallach, Fellous, and Revel, 1982), activate macrophage killing of intracellular pathogens and inhibit proliferation of viral- and malignant-transformed cells (Maher et al., 2007). They can also secrete IL-10 and Th2 cytokines (Viganò et al., 2001, Feistritzer et al., 2005). NK cells are major cytokine producers during bacterial sepsis, priming macrophages to clear the infection by phagocytosing the bacteria, and activation of bacteria killing and recruitment of other immune cells through the release of nitric oxide and IL-6 (Scott et al., 2003). They are also important players in anti-viral and anti-tumour innate immunity, being able to kill infected or transformed cells via perforin/granzyme or death receptor-related pathways such as Fas/FasL (Smyth et al., 2005). NK cells are regulated by opposing signals delivered through activating and inhibitory receptors, whereby activation, blastogenesis and effector functions are initiated by stimulating activating receptors but limited or terminated by inhibitory receptors and cytokines. There are three distinct receptor families in involved in NK cell recognition of MHC I – Ly49, CD94/NKG2 and KIR, which contain both activating and inhibitory receptors (Lanier, 1998).

2.3.2 CD1-restricted T cells

CD1-restricted T cells recognise self and microbial lipid antigens presented on CD1, a family of non-polymorphic MHC I-like molecules specialized in lipid/glycolipid presentation (Brigl and Brenner, 2004). The 5 isoforms of CD1 are categorised into 3

groups: group 1 consists of CD1a, CD1b and CD1c, group 2 consists of CD1d and group 3 consists of CD1e (Porcelli and Modlin, 1999). Group 1 CD1-restricted T cells do not express an invariant TCR like group 2 CD1-restricted T cells (better known as natural killer T cells) and display full activation when exposed to CD1-expressing antigen-presenting cells in the absence of foreign lipid (Porcelli et al., 1989). They are also able to recognise endogenous glycosphingolipids (De Libero, Collmann, and Mori, 2009). In human adult peripheral blood and neonatal cord blood, group 1 CD1-restricted T cells were found in high frequencies in $CD4^+$ and DN T cells, expressing a diverse polyclonal TCR repertoire. The group 1 CD1-restricted cells display a naïve phenotype at birth but increase in memory phenotype with age (De Lalla et al., 2011; Young and Gapin, 2011). In contrast, group 2 CD1-restricted T cells acquire an activated memory phenotype prior to birth (van der Vliet et al., 2000).

2.3.3 γδ T cells

γδ T cells are T lymphocytes that bear the γδ TCR instead of the αβ TCR found on majority of conventional T cells. They are present in low frequencies in blood and lymphoid organs and are predominantly found in epithelial tissues (Girardi et al., 2001). They are thought to be immunoregulatory because of pathological immune dysregulation observed in their absence in murine models (Hayday and Tigelaar, 2003). γδ T cells surprisingly have antigen-presentation capabilities similar in potency and efficacy to dendritic cells (Moser and Brandes, 2006). However their antigen- and ligand-specificities are not well-understood, although recent reports show that there are subsets that can recognise CD1d (Huber, 2010; Liu and Huber, 2011) and CD1c (Spada et al., 2000). The most well-characterised subset of γδ T cells are the Vγ9Vδ2 T cells, which make up the majority of γδ T cells in human peripheral blood and are reactive to pyrophosphate intermediates of the isoprenoid synthesis pathway, such as isopentenyl pyrophosphate and (E)-4-hydroxy-3-methyl-but-2enyl pyrophosphate (HMB-PP) (Morita et al., 2007). Activated Vγ9Vδ2 T cells contribute to early defense against bacteria, viruses and tumours, displaying rapid cytotoxicity and releasing chemokines and cytokines that result in neutrophil recruitment and activation (Agrati et al., 2009), monocyte differentiation (Eberl et al., 2009), dendritic cell maturation into antigen-presenting cells (Devilder et al., 2006, Dunne et al., 2010) and B cell differentiation into antibody-producing plasma cells (Caccamo et al., 2006). Upon activation γδ T cells rapidly produce cytokines such as IFN-γ and IL-17 and their characteristic ability to lyse target cells has identified them as potential targets in anti-tumour immunotherapy (Hao

et al., 2010). Recognition of tumour cells can occur by ligation of NKG2D or the Vγ9Vδ2 TCR (Wrobel et al., 2007).

2.3.4 $CD56^+$ T cells

$CD56^+$ T cells are a subset of T lymphocytes that express the CD56 receptor typically found on natural killer cells (Schmidt et al., 1986). Their distinguishing traits are their cytolytic activity and their ability to rapidly produce large quantitities of Th1 and Th2 cytokines upon activation without need for priming or clonal expansion (Kelly-Rogers et al., 2006). $CD56^+$ T cells are abundant in the liver and are critical in mediating clearance of hepatitis C viral infection (Ye et al., 2009). Natural killer T cells are often included in this category as they can also express CD56 on their surface.

2.3.5 Mucosal-associated invariant T (MAIT) cells

MAIT cells express a semi-invariant TCR encoded by Vα7.2-Jα33 in humans and Vα19-Jα33 in mice (Porcelli et al., 1993; Tilloy et al., 1999). MAIT cells are preferentially located in the gut lamina propria in both mice and humans and their TCR are restricted by the MHC class I-related molecule 1 (MR1) (Treiner et al., 2003). Accumulation of MAIT cells in the gut requires MR1-expressing B cells and commensal flora, and like CD1-restricted T cells in humans, they display a naïve phenotype at birth but progressively differentiate into a memory phenotype after birth (Martin et al., 2009). Human MAIT cells produce IFN-γ, granzyme B and large amounts of IL-17 upon stimulation with PMA and ionomycin (Dusseaux et al., 2011). Their antigen-specificities are currently unknown (Gapin, 2009; Huang et al., 2009).

2.3.6 Natural killer T (NKT) cells

NKT cells are broadly classified into two groups. Type I NKT cells are known as invariant NKT (iNKT) cells whereas type II NKT cells are known as non-invariant NKT cells (Godfrey et al., 2000). The most well-characterised group of CD1-restricted T cells are the iNKT cells, which are CD1d-restricted, express a semi-invariant TCR and display an effector/memory phenotype (Bendelac et al., 1997; Dellabona et al., 1994).

Type II NKT cells express variable TCR chains and have been identified in both humans and mice (Behar et al., 1999). Type II NKT cells are also CD1d-restricted, however, unlike type I iNKT cells, type II NKT cells do not require endosomal targeting of CD1d for recognition (Chiu et al., 1999). Type I and type II NKT cells are thought to exert opposite regulatory effects on the immune response during infection with helminths (Mallevaey et al., 2007). Type II NKT cells have also been shown to regulate type I iNKT cell activity, inducing anergy in iNKT cells via interaction with hepatic DCs (Halder et al., 2007).

2.4 Invariant Natural Killer T (iNKT) Cells

2.4.1 Phenotype and Function

iNKT cells belong to an innate-like subset of T lymphocytes and they share receptors also found on natural killer cells, such as NK1.1 in mice and CD161 in humans (Brigl and Brenner, 2004; Porcelli and Modlin, 1999). They are phenotypically and functionally diverse, and can be subdivided into several subpopulations with the $CD4^+$ iNKT and $CD4^-CD8^-$ double negative (DN) populations being most common in mice, whereas the $CD4^+$, DN and $CD8^+$ iNKT (consisting of $CD8\alpha^+\beta^-$ or $CD4^-CD8\alpha^+\beta^+$) subsets exist in humans (Montoya et al., 2007, O'Reilly et al., 2011). They are present in very low numbers in human peripheral blood, constituting only 0.01 – 1% of T cells in healthy adults (Porcelli and Modlin, 1999; Porcelli et al., 1993), but are enriched in liver (Kenna et al., 2003) and omental tissue (Lynch et al., 2009).

iNKT cells are distinguished by their surface expression of the invariant T cell receptor Vα24-Jα18/Vβ11 in humans and they recognise glycolipids presented by CD1d, a non-polymorphic, non-classical major histocompatibility complex (MHC) I – like molecule found on antigen-presenting cells (Bendelac et al., 1997; Brigl and Brenner, 2004; Porcelli and Modlin, 1999). Unlike conventional T cells, iNKT cells do not require co-stimulatory signals through the CD4 or CD8 co-receptors for antigenic recognition and TCR signalling. However, concurrent stimulation of CD4 and the iNKT TCR can result in enhanced cytokine secretion and calcium flux, whereas blocking CD4 inhibits cytokine secretion and iNKT proliferation but not cytotoxicity in response to $CD1d^+$ target cells, suggesting that the CD4 co-receptor is functional and responsible for mediating certain iNKT cell functions (Chen et al., 2004). Work done in murine cell lines showed that CD1d is constitutively expressed and restricted to the plasma membrane lipid rafts of

antigen-presenting cells, which is key to efficient signal transduction to iNKT cells without the need for co-receptors and high ligand densities (Park et al., 2005).

iNKT cells are frequently described as immunoregulatory cells that bridge innate and adaptive immunity due to their ability to rapidly activate cells of the innate system such as dendritic cells and cells of the adaptive immune system such as T helper cells (Cerundolo et al., 2009; Taniguchi, Seino, and Nakayama, 2003). This has led to them being implicated in many diseases and functions, such as autoimmunity (Miyake and Yamamura, 2007), allergy (Stock and Akbari, 2008), cancer (Konishi et al., 2004) and protective immunity against infectious agents (Sada-Ovalle et al., 2008; Sköld and Behar, 2003; van Dommelen and Degli-Esposti, 2004). Their ability to interact with a large repertoire of other cells and unique capability to activate T helper 1 (Th1), Th2, and Th17-type responses, as well as the paradoxical ability to simultaneously secrete both Th1 and Th2-type cytokines, have brought into focus their potential for boosting B and T cell responses in vaccination or as immunomodulators in disease therapy (Cerundolo and Salio, 2007).

In this thesis I have focused on CD1d-restricted iNKT cells, which are hereafter referred to as iNKT cells.

2.4.2 CD1d trafficking and antigen presentation.

CD1d belongs to the CD1 family of membrane glycoproteins and is a non-polymorphic MHC I-like molecule that specialises in presenting glycolipids to lipid-reactive T cells such as iNKT cells (Godfrey et al., 2000). CD1d is highly conserved among mammalian species (Brigl and Brenner, 2004) and is constitutively expressed on professional antigen-presenting cells in humans, such as dendritic cells (Gerlini et al., 2001), B cells in peripheral blood and lymph node mantle zones, monocytes and cortical thymocytes (Exley et al., 2000).

During synthesis in the endoplasmic reticulum of the cell, the CD1d molecule forms a heterodimeric complex with β2-microglobulin and is transported from the Golgi apparatus to the plasma membrane along the secretory pathway (Kang and Cresswell, 2002). For efficient presentation of lipid antigens to iNKT cells, both the lipid and CD1d have to be internalised by the antigen-presenting cell and subsequently traffick to the endosomal compartment where loading of the lipid onto CD1d occurs (Spada, Koezuka,

and Porcelli, 1998). This internalisation process is dependent on the cytoplasmic tail of CD1d (Chiu et al., 2002), which is responsible for governing trafficking to early and late endosomes (Jayawardena-Wolf et al., 2001).

2.4.3 Endogenous and exogenous iNKT cell ligands.

iNKT cells exhibit a degree of reactivity against self in a TCR-dependent manner that can be inhibited by blocking CD1d (Exley et al., 1997). This reactivity does not require the presence of exogenous antigen and it has been suggested that the iNKT cells recognise self-lipids that are presented on the CD1d molecule rather than direct recognition of CD1d (Gapin, 2010). The identity of the natural antigenic ligand for iNKT cells is still very much in debate. To date, the endogenous glycolipid recognised by iNKT cells has still not been identified, although conflicting reports point to isoglobotrihexosylceramide (iGb3) as a potential candidate (Christiansen et al., 2008; Speak et al., 2007; Zhou et al., 2004).

iNKT cells can also be activated by exogenous bacterial glycosphingolipids (Kinjo et al., 2005) and xenogeneic glycolipids such as α-galactosylceramide (α-GC) from the marine sponge *Agelas mauritianus*, which is known to be a potent activator of iNKT cells in mice (Kawano et al., 1997; Morita et al., 1995), and remains the ligand of choice for activating iNKT cells. These antigens share a similar structure for efficient CD1d presentation, consisting of a lipid tail bound to an α-linked sugar molecule (Borg et al., 2007; Zajonc et al., 2005). Several synthetic analogs of α-GC have thus been synthesized and tested for their ability to induce Th1 or Th2 responses in the hope of harnessing the therapeutic potential of iNKT cells (Bricard et al., 2010; Hogan et al., 2011; Miyamoto, Miyake, and Yamamura, 2001; Tashiro et al., 2010; Wu et al., 2005).

2.4.4 The role of iNKT cells in disease.

2.4.4.1 iNKT cells in infectious diseases.

iNKT cells are generally thought to have a protective role against viral and bacterial infections. Their ability to recognise a wide range of microbial lipids expressed on bacterial cell walls confers particular efficiency in dealing with bacterial infection. Studies with mice showed that α-GC-activated iNKT cells are able to inhibit replication of the

Mycobacterium tuberculosis bacteria and synergise with antibiotic treatment in the clearance of infection (Sada-Ovalle et al., 2010). Both murine and human iNKT cells are able to recognise glycosphingolipids from *Sphingomonas,* a gram-negative bacteria that does not contain lipopolysaccharide (LPS), indicating that iNKT cells may function in the clearance of bacteria that are not recognised by pattern recognition receptors such as the Toll-like receptors (Kinjo et al., 2005). iNKT cells are also reactive to bacterial glycolipids from the spirochete *Borrelia burgdoferi*, which causes Lyme disease. Presentation of these glycolipids to the iNKT TCR stimulates proliferation and cytokine production in both murine and human iNKT cells (Kinjo et al., 2006). $CD1d^{-/-}$ mice exhibit Lyme-disease associated arthritis, increased spirochete DNA in tissues and enhanced IgG2a antibody production normally associated with susceptibility, suggesting that a deficiency in lipid presentation to iNKT cells impairs host resistance and ability to deal with bacterial infection (Kumar et al., 2000).

iNKT cells play a role in anti-viral responses, both directly and indirectly by activating other immune cells such as cytotoxic T lymphocytes (or $CD8^+$ T cells). Infections of $CD1d^{-/-}$ murine models with respiratory syncytial virus (RSV) resulted in reduced $CD8^+$ T cell count and delayed viral clearance (Johnson et al., 2002), suggesting that the iNKT cells mediate viral clearance indirectly by contributing to efficient induction of $CD8^+$ T cell responses and amplification of anti-viral immunity through the secretion of IFN-γ early in the infection. In human T lymphotrophic virus 1 (HTLV-1) infection, iNKT cells are thought to play a direct role, as severe loss of iNKT cells correspond to defects in proliferative response to α-GC and are associated with disorders caused by HTLV-1 infection. In asymptomatic HTLV-1 carriers, iNKT cell expansion by in vitro α-GC stimulation decreased numbers of infected cells (Azakami et al., 2009).

2.4.4.2 iNKT cells in cancer.

iNKT cells can exert anti-tumour effects directly through cytotoxic activity by the induction of cell-death-associated molecules such as perforin, Fas/FasL and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), or indirectly by activating other cytotoxic immune cells such as natural killer cells and $CD8^+$ T cells (Carnaud et al., 1999; Kawano et al., 1998; Kawano et al., 1999; Teng et al., 2007). Their potent anti-tumour effects have led to a surge of studies examining how they can be harnessed in treating cancer. Adoptive transfer of wild-type iNKT cells into $J\alpha18^{-/-}$ mice (which are iNKT-deficient) successfully restored tumour surveillance and inhibition of methylcholanthrene-induced sarcoma growth in the liver (Crowe, Smyth, and Godfrey,

2002). Adoptive transfer of liver-derived iNKT cells into Jα18$^{-/-}$ mice that were inoculated with B16F10 lung melanoma and α-GC effectively inhibited metastases of B16F10 melanoma cells in the lungs. Interestingly, spleen-derived iNKT cells were less effective than liver-derived iNKT cells at reducing the number of metastases (Crowe et al., 2005). Adoptive transfer of α-GC-pulsed dendritic cells in mice has been shown to result in greater anti-tumour activity than direct administration of α-GC, with observations of prolonged and large expansion of IFN-γ-producing iNKT cells and greater resistance to B16 metastases (Fujii et al., 2002). In humans, reduced numbers of iNKT cells in cancer patients have been linked to poor clinical outcome (Molling et al., 2007). Increased iNKT cell infiltration into tumour sites has also been associated with decreased number of metastases and prolonged survival in colon cancer patients (Tachibana et al., 2005), suggesting that iNKT cells contribute to anti-tumour immunity in humans.

Following the success of the in vivo work, several studies have also tried to harness the anti-tumour properties of iNKT cells in humans but have so far been unsuccessful. Direct intravenous administration of α-GC in patients with solid tumours resulted in disappearance of the iNKT cells from peripheral blood after 24 hours, and multiple administrations at weekly intervals failed to achieve increases in iNKT cell numbers or positive clinical outcome (Giaccone et al., 2002). Injection of α-GC-pulsed monocyte-derived dendritic cells into patients with metastatic malignancies also resulted in decreases in circulating iNKT cells 1 – 2 days after administration, activation of NK cell cytotoxicity and induction of iNKT cell memory. However, apart from temporary exacerbation of inflammatory symptoms there was no positive clinical outcome (Nieda et al., 2003).

Current opinion is divided on whether iNKT cells can be harnessed in anti-cancer therapy. Decreased numbers and reduced function of iNKT cells have been observed in patients with various malignant diseases, and these have been observed to correlate with poor clinical outcome (Molling et al., 2007; Tahir et al., 2001). However, other studies have shown that circulating iNKT cells in cancer patients are still able to produce IFN-γ in reponse to anti-CD3 mAb stimulation despite being decreased in number (Motohashi et al., 2002).

2.4.4.3 iNKT cells in allergy and autoimmune disease.

iNKT cells have been implicated in promoting the development of allergen-induced airway hyperreactivity (AHR), a characteristic feature of asthma. iNKT-deficient mice fail to develop AHR when challenged with a combination of antigen and alum, but adoptive transfer of Vα14 iNKT cells producing IL-4 and IL-13 reversed the protection against AHR (Akbari et al., 2003). Similar work with adoptive transfer of wild-type iNKT cells into ovalbumin-immunised $J\alpha18^{-/-}$ mice showed that in response to ovalbumin challenge, iNKT cells amplify eosinophil recruitment to airways, Th2 cytokine and IgE production in a CD1d-restricted manner (Lisbonne et al., 2005). Subsequent research suggests that in the ovalbumin-induced asthma murine model Th2 cytokines synergise with IL-17A in inducing AHR (Wingender et al., 2011). In humans, iNKT cells were initially thought to be involved directly in the pathogenesis of asthma as iNKT cells were observed to constitute 60% of T cells in the bronchoalveolar fluid from the lungs of 14 asthma patients, suggesting that iNKT cells are increased in asthma patients (Akbari et al., 2006). However, several other studies have now shown that iNKT cells are not significantly increased in the bronchoalveolar fluid of asthma patients (Mutalithas et al., 2007, Vijayanand et al., 2007, Thomas et al., 2006), suggesting that iNKT cells probably have a modulatory role rather than a causative role in asthma (Thomas et al., 2010).

The role of iNKT cells in autoimmune disease is also unclear. The non-obese diabetic (NOD) mice is commonly used as a model in studies of type 1 diabetes and is characterised by autoimmune Th1 responses arising from deficiencies in $CD4^+$ Th2 cells (Delovitch and Singh, 1997). NOD mice exhibit reduced iNKT cell numbers and functional defects (Baxter et al., 1997; Gombert et al., 1996). In spite of their numerical and functional deficiencies, iNKT cells in NOD mice are protective as early treatment with α-GC (to activate iNKT cells) suppresses disease whereas a lack of CD1-restricted iNKT cells promotes development of diabetes (Wang, Geng, and Wang, 2001). Studies of iNKT cell frequencies in human type 1 diabetes patients are conflicting, with some reporting a decrease in iNKT cell frequencies (Kukreja et al., 2002, Wilson et al., 1998) while others reporting an increase (Oikawa et al., 2002) or no change (Lee et al., 2002) in iNKT cell frequencies. However, similar to studies in NOD mice, Kis et al observed a bias in Th1 (IFN-γ) cytokine production in the iNKT cell population, corresponding with a decrease in the $CD4^+$ subset (Kis et al., 2007). iNKT cells are also protective in collagen-induced arthritis (CIA) mice, a murine model for arthritis. Administration of OCH, a synthetic analog of α-GC, selectively induced Th2 cytokine production by iNKT

cells and promoted collagen-specific Th2 responses, which was correlated with protection against CIA (Chiba et al., 2004).

Similarly, the role of iNKT cells in systemic lupus erythematosus (SLE) is less clear in murine models, as they have been observed to be pathogenic in the NZB/W F_1 model, enhancing autoantibody titers and exacerbating disease when activated by α-GC (Zeng et al., 2003). However, in the MRL-lpr model, activation of iNKT cells by α-GC relieved inflammatory symptoms of dermatitis associated with lupus disease, portraying the iNKT cells in a protective role (Yang et al., 2003). Similar to the contradicting roles of iNKT cells reported in murine models of SLE, activation of iNKT cells by α-GC administration has been reported to be pathogenic in oxazolone-induced colitis in which colitis is induced by a T cell-mediated response against hapten-modified autologous proteins (Heller et al., 2002), but protective in colitis induced by dextran sodium sulphate which is directly toxic to colonic epithelial cells of the basal crypts (Saubermann et al., 2000, Wirtz et al., 2007). In humans, iNKT cells are decreased in the circulation and are also thought to be aberrant in function (van der Vliet et al., 2001, Kojo et al., 2001).

2.4.4.4 Potential of iNKT cells in immunotherapy for autoimmune diseases.

Studies listed in the previous sections (2.4.4.1 - 2.4.4.3) have shown that iNKT cells are implicated in many different diseases and as such they have been examined as attractive targets for immunotherapy, particularly so in autoimmune diseases where there is as yet no available cure. Resolution of Th1-mediated autoimmune diseases such as type 1 diabetes and collagen-induced arthritis in murine models has been relatively straightforward and easily mediated with α-GC or synthetic analogs that are able to selectively induce Th2 responses by iNKT cells, such as OCH (Chiba et al., 2004; Wang, Geng, and Wang, 2001). However, how we can harness iNKT cells is not as clear-cut in therapy for diseases such as SLE where the role of iNKT cells is less well-understood. An additional concern is whether the studies in mice can be translated effectively into clinical applications, as the effect of iNKT cell activation on limiting disease progression is influenced by a variety of parameters, such as the murine models utilised in the studies, the genetic background of the mice, route of α-GC administration and the stage of disease during administration of α-GC (Van Kaer, 2004). As such, further study of iNKT cell activation and pathology, as well as its interaction with other cells in the immune microenvironment in human autoimmune diseases are

necessary for insight into how iNKT cells can be harnessed for immunotherapy in these diseases.

2.5 B Lymphocytes

2.5.1 General Phenotype and Function

B lymphocytes are the principal cells that initiate antibody responses against extracellular pathogens and facilitate cell-mediated immunity by Fc receptor-bearing phagocytes, mast cells, eosinophils and natural killer cells (Belperron et al., 2007; Kenny and Herzberg, 1968; Pape et al., 2007). In human peripheral blood, they comprise 5 – 15% of the lymphocyte population. They are distinguished by their expression of CD19, which in combination with CD21, acts as a coreceptor to enhance B cell activation (Tedder, Zhou, and Engel, 1994) and promote B cell survival in a primary immune response (Barrington et al., 2005). They can also be identified by surface expression of CD20, a regulator of transmembrane Ca^{2+} conductance and cell cycle progression functioning in B cell activation, proliferation and differentiation (Tedder and Engel, 1994; Uchida et al., 2004). Interestingly, B cells in peripheral blood and lymph nodes also express CD1d (Exley et al., 2000), thus suggesting that they have the potential to present glycolipids to iNKT cells and modulate iNKT cell function.

B cells are potent antigen-presenting cells and are able to prime $CD4^+$ T cells without the participation of dendritic cells and macrophages (Rodriguez-Pinto, 2005). The B cell receptor (BCR) is antigen-specific and when it is cross-linked by an antigen with high affinity for the BCR, it endocytoses the antigen, processes it and concentrates it for efficient presentation on MHC class II to $CD4^+$ T cells (Rodriguez-Pinto, 2005). The B cell also upregulates CD86 expression, which is part of the B7-1/2 costimulatory complex needed for naïve T cell activation (Lenschow et al., 1994). This activates the $CD4^+$ T cells and causes them to upregulate CD154, which functions by interacting with CD40 on the B cell to sustain B cell activation and enhance their antigen-presenting capability (Faassen et al., 1995). The activated B cell proliferates and differentiates into short-lived antibody-secreting cells (also termed plasmablasts) (LeBien and Tedder, 2008). Memory B cells also develop in the process and they remain circulating in the body for a long time, primed to mount a rapid secondary immune response by differentiating into plasma cells upon subsequent antigen challenge (McHeyzer-Williams and McHeyzer-Williams, 2005).

Similar to dendritic cells, B cells have the ability to produce both T helper (Th) 1 and Th2 cytokines and can be polarised towards one or the other subsequent to interaction with $CD4^+$ Th1 or Th2 cells (Harris et al., 2000).

2.5.2 Regulatory B (Breg) cells

In recent years, the suppressive ability of a subset of B cells, loosely termed as "regulatory B (Breg) cells" was discovered. Janeway et al hinted at the existence of such a group of B cells when they showed that a genetic lack of B cells aggravated disease and impeded recovery in an experimental autoimmune encephalomyelitis (EAE) mouse model (Wolf et al., 1996). Subsequent work by other groups in several autoimmune disease mouse models (Fillatreau et al., 2002; Mauri et al., 2003) and virus-challenged mice (Madan et al., 2009) revealed that IL-10 production from these B cells was responsible for downregulating excessive immune responses.

Despite the recent explosion of studies, there is still a need to achieve a consensus on the definitive surface phenotype of Breg cells and elucidate the transcription factors controlling their development and/or function (Gray and Gray, 2010).

2.5.2.1 Breg cells in mice

The most well-characterised subset of Breg cells in mice are the $CD1d^{hi}CD5^+CD19^+$ subset, also termed as "B10 Breg cells". In response to T-cell mediated inflammation these cells increase IL-10 expression and migrate from the spleen to the blood circulation (Yanaba et al., 2008).

Another B cell subset to which Breg function has been attributed to is the splenic transitional 2-marginal zone precursor (T2-MZP) B cells, which exhibits the $CD21^{hi}CD23^{hi}CD24^{hi}IgM^{hi}CD1d^{hi}CD19^+$ phenotype. IL-10 production from these T2-MZP B cells were observed to suppress arthritis in an experimental mouse model by inhibiting antigen-specific T cell activation and downregulating the Th1 response (Evans et al., 2007). The mechanism by which IL-10-producing T2-MZP B cells inhibit inflammation has been speculated to be via the upregulation of $Foxp3^+$ regulatory T (Treg) cell frequencies, which in turn downregulate Th1 and Th17 cell frequencies (Carter et al., 2011).

2.5.2.2 Breg cells in humans

To date there is still a paucity of studies illuminating the function and phenotype of Breg cells in humans. The only definitive paper on human Breg cells identified the $CD24^{hi}CD38^{hi}CD19^{+}$ transitional B cell subset as having regulatory properties. In this paper the authors observed that in co-cultures of $CD4^{+}$ T cells with $CD24^{hi}CD38^{hi}CD19^{+}$ B cells, upregulation of CD154 on the activated $CD4^{+}$ T cells induced the $CD24^{hi}CD38^{hi}CD19^{+}$ B cells to produce IL-10, which reciprocally inhibited pro-inflammatory cytokine (IFN-γ and TNF-α) production from the $CD4^{+}$ T cells in a CD80- and CD86-dependent manner (Blair et al., 2010). Investigation of the $CD24^{hi}CD38^{hi}CD19^{+}$ B cells in SLE patients revealed that they were deficient in suppressive function and produced less IL-10 in response to stimulation. Interestingly the authors also observed that the $CD1d^{hi}CD5^{+}CD19^{+}$ B cells which have only been reported to be regulatory in mice, were also encompassed in this $CD24^{hi}CD38^{hi}CD19^{+}$ B cell subset.

Breg cells are also suspected to exist in the memory B cell subset. Recently, Iwata et al (Iwata et al., 2011) identified a subset of $CD24^{hi}CD27^{+}$ B cells that was able to inhibit cytokine production from monocytes via the release of IL-10. These B cells were found to be expanded in patients with autoimmune disease (SLE, primary Sjögren syndrome, multiple sclerosis and autoimmune vesiculobullous skin disease), mirroring that observed in autoimmune mouse models.

2.5.2.3 How can we activate Breg cells for therapy?

Due to their therapeutic potential, several studies have been conducted to find out how Breg cells can be activated to harness their immunoregulatory function in disease. Blair et al (Blair et al., 2009) demonstrated that the T2-MZP Breg cells could be selectively activated by agonistic anti-CD40 mAb stimulation of total splenic B cells to increase IL-10 production. Adoptive transfer of these activated T2-MZP Breg cells into MRL/lpr mice (a mouse model for SLE) decreased mortality and kidney damage in the mice. Interestingly there was also a shift in the isotype of anti-dsDNA antibody production from a Th1 pathogenic profile (IgG2a) to a Th2 profile (IgG1). Th2 cytokines such as IL-4 are known to induce transcription of IgG1 (Snapper et al., 1988), whereas Th1 cytokines such as IFN-γ induce transcription of IgG2a (Finkelman et al., 1988). In vivo

administration of anti-CD40 mAb into MRL/lpr mice also ameliorated disease activity and expanded T2-MZP Bregs in the mice (Blair et al., 2009).

A separate study by Mauri et al (Mauri et al., 2003) showed that activation of splenocytes from a chronic collagen-induced arthritic (CIA) mouse model with antigen and agonistic anti-CD40 mAb increased IL-10 and decreased IFN-γ production from these cells. Transfer of the B cells from the antigen/anti-CD40 stimulated splenocytes into the arthritic mice conferred protection from the arthritis. In contrast, a lack of CD40 expression on B cells led to deficiency in IL-10 production and aggravated disease in an EAE mouse model (Fillatreau et al., 2002). Taken together, these studies suggest that the CD40-CD154 pathway is crucial to the activation and function of Breg cells.

Breg cells can also be activated through TLR stimulation via LPS (Tian et al., 2001) or CpG (Brummel and Lenert, 2005), inducing TGF-β and IL-10 secretion respectively. A pioneering study by Rafei et al (Rafei et al., 2009) showed that murine Breg cells with suppressive, immunoregulatory properties could be generated by stimulation with a granulocyte-macrophage colony-stimulating factor and interleukin-15 fusokine (two cytokines fused together). Adoptive transfer of Breg cells generated through these methods has all been successful in suppressing autoimmune disease in mouse models. B cell activating factor (BAFF), a member of the TNF cytokine family and key regulator of B cell maturation and function, is also able to induce IL-10-producing B cells with a $CD1d^{hi}CD5^{+}$ phenotype (Yang et al., 2010). These BAFF-induced Breg cells differentiate from the marginal zone B cell subset and were demonstrated to be able to suppress T cell proliferation and Th1 cytokine production in vitro, as well as inhibit arthritis development in CIA mice in vivo (Yang et al., 2010).

Despite the expanse of knowledge about how we can activate Breg cells in mice there still is a dearth of studies about how Breg cells can be activated in humans, which is further compounded by the lack of defined Breg cell phenotypes in humans.

2.5.2.4 What is the mechanism of Breg cell suppression of immune responses?

IL-10 is known to be crucial to the regulatory activity of Breg cells but the mechanisms by which they suppress inflammation are largely unknown. In a mouse model of airway allergic inflammation, Breg cells were shown to induce recruitment of natural Treg cells

(Foxp3$^+$CD4$^+$CD25$^+$) to the lungs in an IL-10-dependent and TGF-independent manner (Amu et al., 2010). As such, the authors suggest that the suppression of inflammation was mediated by the Treg cells, relegating the role of the Breg cells to an indirect one. In a contrasting study, depletion of Treg cells in CIA mice prior to adoptive transfer of Breg cells did not impede the ability of Breg cells to ameliorate disease, suggesting that the Treg cells did not play a role in mediating suppression of autoimmune inflammation (Evans et al., 2007). In EAE, B10 cells downregulated the antigen-presenting function of dendritic cells and indirectly inhibited T cell proliferation. However, they were thought to control disease initiation whereas Treg cells were thought to be involved in controlling late-phase disease (Matsushita et al., 2010).

Apart from downregulating Th1 responses, Breg cells have also been shown to be able to induce maturation of effector T cells into "IL-10-producing CD4$^+$ T regulatory cells (Tr1)" (Gray et al., 2007). Antigen recognition by the BCR (Evans et al., 2007; Yanaba et al., 2008) and co-stimulatory signalling through CD86 and CD80 (Mann et al., 2007) were shown to be necessary for IL-10 production. It has not yet been proven that Breg cells inhibit Th17 effector functions, and due to their high expression of CD1d it has been proposed that they are likely to interact with iNKT cells (Mauri, 2010).

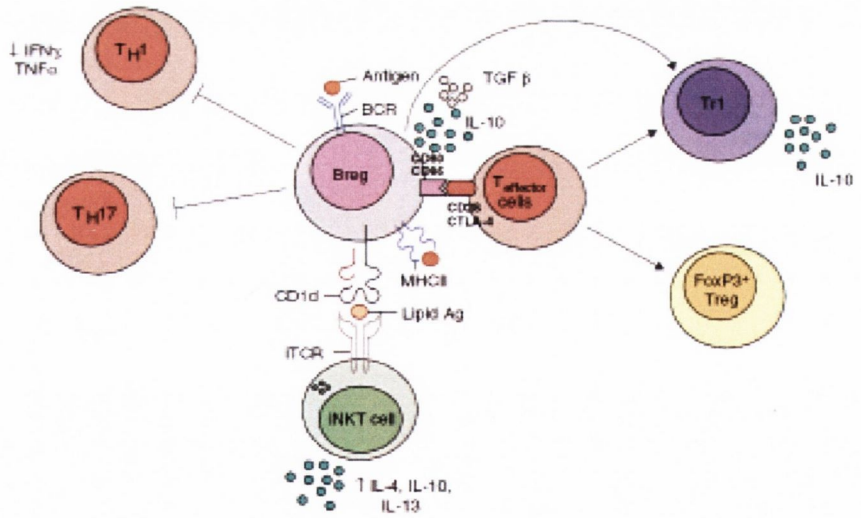

Figure 2.5.1. Possible effector mechanisms of Breg cells. Adapted from (Mauri, 2010). Breg cells are activated through crosslinking of BCR by antigens and CD80/CD86 co-stimulatory signalling, resulting in secretion of IL-10 and/or TGF-β. This results in induction of regulatory T cells (FoxP3$^+$ Treg cells, FoxP3$^-$ Tr1 cells), and suppression of Th1 and Th17 responses. Breg cells may also present lipids on CD1d to iNKT cells to enhance their regulatory functions.

2.5.3 Memory B cells

Memory B cells are phenotypically and functionally heterogeneous and many different subsets exist. Classical dogma divides memory B cells into two categories: (i) plasma cells, which are responsible for antibody production and also termed "effector memory", and (ii) long-lived memory B cells that remain in circulation, generating and replenishing plasma cells in the event of an immune response (Bachmann et al., 1996; Sanz et al., 2008; Schittek and Rajewsky, 1990). When the naïve B cell recognises its specific antigen and interacts with other immune cells such as $CD4^+$ T cells, macrophages and dendritic cells, it differentiates into either a short-lived plasma cell or migrates to the B cell follicle, seeding a germinal centre (Tangye and Tarlinton, 2009). In the germinal center B cells undergo a series of processes such as somatic hypermutation and class switch recombination of the immunoglobulin variable and constant region genes respectively, creating a repertoire of cells that differentiate into plasma cells and long-lived memory B cells for sustained serological memory (Ahmed and Gray, 1996; McHeyzer-Williams and McHeyzer-Williams, 2005).

2.5.3.1 Classification of memory B cells.

Human memory B cells are most commonly classified using surface expression of IgD and CD27 (Figure 2.5.2). CD27, a member of the TNF receptor superfamily, interacts with CD70 on the surface of activated $CD4^+$ T helper cells and facilitates memory B cell differentiation into plasma cells (Agematsu et al., 2000). According to the CD27/IgD classification, switched memory B cells are $CD27^+IgD^-$, unswitched memory B cells are $CD27^+IgD^+$, DN memory B cells are $CD27^-IgD^-$ and naïve B cells are $CD27^-IgD^+$.

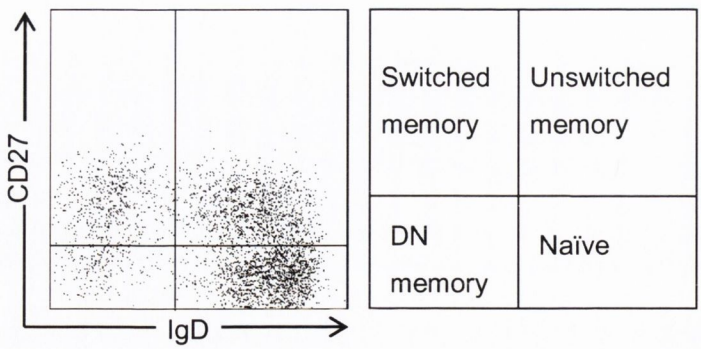

Figure 2.5.2. CD27/IgD classification scheme for memory B cells. Switched memory B cells are $CD27^+IgD^-$, unswitched memory B cells are $CD27^+IgD^+$, DN memory B cells are $CD27^-IgD^-$ and naïve B cells are $CD27^-IgD^+$.

In addition to a lack of IgD expression, class-switched memory B cells are known to express IgG, IgA or IgE (Bonilla, 2007; Warnatz et al., 2002), although a minor subset of $IgM^+CD27^+IgD^-$ B cells have also been observed (Weller et al., 2004). Conversely, IgM expression is known to exist on unswitched memory B cells and naïve B cells (Chaganti et al., 2008), although there is speculation that $CD27^+IgM^+IgD^+$ B cells may represent a recirculating fraction of marginal zone B cells instead of being true "memory B cells" (Weller et al., 2004). DN memory B cells, a relatively undefined subset of memory B cells, have been documented to express either IgM, IgG or IgA (Wei et al., 2007).

2.5.3.2 Isotype switching

Isotype switching is a classical feature of the humoral response whereby naïve B cells switch from IgM to other antibody isotypes, such as IgA and IgG, upon their first exposure to antigen (Janeway et al., 2005). This requires stimulation of the BCR and CD40-CD154 co-stimulatory signalling, and is regulated by the number of cell divisions and cytokines such as TGF-β, IL-4, IL-10 and IL-13 (Purkerson and Isakson, 1992; Tangye et al., 2002). During isotype switching, the constant region of the antibody heavy chain is changed while the variable region, which recognises the antigen, remains unchanged (Janeway et al., 2005). The purpose of isotype switching is to increase functional diversity for an efficient humoral response during immune challenge (Mizoguchi et al., 1999).

2.6 iNKT interactions with dendritic cells

Interactions between iNKT cells and dendritic cells (DCs) have been extensively studied, particularly in the context of how iNKT cells can regulate Th1/Th2 responses. Murine iNKT cells can be activated by α-GC presented on immature DCs and CD40/CD154 co-stimulation by direct contact between the iNKT cells and the DCs. This induces the iNKT cells to produce IFN-γ, which reciprocally induces IL-12 production by the DCs (Kitamura et al., 1999). Murine iNKT cells activated by α-GC also result in maturation of DCs in vivo, causing them to upregulate co-stimulatory molecules and differentiate into potent antigen-presenting cells that activate the adaptive immune response (Fujii et al., 2007). This polarisation of DCs towards a Th1 response has resulted in many studies being undertaken to examine how iNKT cells can be harnessed in anti-cancer therapy (Crowe et al., 2005, Giaccone et al., 2002, Nieda et al., 2003, Motohashi et al., 2009).

Despite the overall polarising Th1 response seen when polyclonal iNKT cells interact with DCs, iNKT cells have been shown to interact differently with DCs when separated into their subsets. $CD4^+$ iNKT cells, when stimulated by α-GC-loaded DCs, produced massive amounts of IL-4, IL-13 and IFN-γ, which synergised with CD40/CD154 co-stimulatory signalling to enhance IL-12 production by the DCs. DN iNKT cells, however, exhibited cytotoxicity against the DCs and downregulated IL-12 production by them, suggesting that the $CD4^+$ iNKT cells preferentially induce Th1 responses by DCs whereas the DN iNKT cells preferentially induce Th2 responses by DCs (Liu et al., 2008).

2.7 iNKT interactions with B cells

There is a paucity of studies with regards to how iNKT cells interact with and influence B cell function in humans, with a single study done by Galli et al, who observed that human iNKT cells can directly prime autologous B cells to proliferate and produce antibodies in a CD1d-dependent manner (Galli et al., 2003). Many studies have since emerged looking at iNKT-B cell interactions in mice, and due to the immunoregulatory properties of iNKT cells these have been focused upon the potential of iNKT cells to regulate, enhance and sustain humoral immune responses. These studies, along with other *in vivo* studies that explore the adjuvant effect of α-GC on antigen-specific antibody production, are further described in section 4.1.

2.8 Autoimmune disease

Under normal circumstances the existence of self-tolerance mechanisms, effected mainly during lymphocyte development, prevents the immune system from reacting against self. However, disruption of self-tolerance mechanisms results in development of autoimmune disease when the immune system becomes activated by autoantigens. Autoimmune diseases are broadly divided into organ-specific (Bach, 1995) and systemic. An example of organ-specific autoimmune disease is type 1 diabetes mellitus, which is caused by autoimmune destruction of the insulin-producing pancreatic β-cells and affects only the pancreas (Wherrett and Daneman, 2009) because the autoantigens are limited to the organ. Systemic autoimmune diseases, such as systemic lupus erythematosus, affect multiple organs and have a tendency to become chronic because of the ubiquitous nature of the autoantigen and continual perpetuation of self-reactive lymphocytes (Shlomchik, Craft, and Mamula, 2001).

2.8.1 iNKT cells in autoimmune disease

iNKT cells have been implicated in various autoimmune and allergic responses in mouse models that are mediated by B cells or autoantibodies (Dufour, Silveira, and Baxter, 2008; Lang, 2009). Sensitisation of mice by subcutaneous injection of ovalbumin, followed by challenge with ovalbumin in the airways, has been documented to cause allergic IgE responses through the CD1d- and IL-4-dependent priming of B cells by iNKT cells (Lisbonne et al., 2005). Numbers of autoantibody-producing B cells were increased while iNKT cell numbers were decreased simultaneously when autoimmune hepatitis in mice was induced by concanavalin A (Fujii et al., 2010).

In lupus-prone NZB/W mice, the expansion and hyperactivation of iNKT cells with increasing age has been deemed responsible for enhanced production of IgM and IgG autoantibodies by B1 and marginal zone B cells (Takahashi and Strober, 2008). However, in MRL-*lpr/lpr* mice, iNKT cell expansion with α-GC was associated with amelioration of inflammatory dermatitis (Yang et al., 2003). A recent paper demonstrated that iNKT cells (with the help of α-GC) in vitro have the ability to inhibit autoreactive (IgG anti-dsDNA) antibody and rheumatoid factor production from B cells in a murine model that spontaneously produces autoreactive antibodies (Yang et al., 2011). The iNKT cells also reduced IL-10 secretion from B cells in a contact-dependent manner but increased total IgG production and CD86 and CD69 expression on the B cells independent of contact (via soluble factors).

2.8.1.1 iNKT-B cell interactions in systemic lupus erythematosus (SLE).

SLE is a chronic, multi-organ autoimmune disease with extremely variable and diverse clinical manifestations (Kotzin, 1996). The development of disease is characterized by pathogenic IgG autoantibodies against nuclear antigens (e.g. ribonucleoprotein (RNP)) and double-stranded DNA (dsDNA) resulting in the formation of immune complexes (Kotzin, 1996), appearance of autoreactive $CD4^+$ T cells, and deficiencies in regulatory T and B cell subsets (Bonelli et al., 2008; Mok and Lau, 2003). However, the cause of SLE is largely unknown, although several factors such as exposure to sun (Jönsen et al., 2007), elevated sex hormones (Grimaldi, 2006), viral infection (Verdolini et al., 2002) and HLA genes (Martens et al., 2009) have been linked to susceptibility to SLE. The 1997 update (Hochberg, 1997) of the 1982 revised criteria (Tan et al., 1982) for classification of SLE from the American College of Rheumatology is frequently used to aid physicians in diagnosis of SLE (Table 2.8.1). An individual presenting 4 or more of

the criteria serially or simultaneously at any interval of observation is diagnosed to have SLE.

iNKT cells are known to be involved in development of lupus-like disease in SLE mouse models, but it is unclear if they contribute to disease pathogenesis or have a protective effect due to conflicting results from studies conducted in different genetic strains of SLE-like mice. In humans, the frequency of circulating iNKT cells is decreased in SLE patients (van der Vliet et al., 2001) and thought to be aberrant in function (Kojo et al., 2001). These studies are further described in section 6.1.1.2.

2.8.1.2 Memory B cells in SLE.

DN memory B cells constitute a small proportion of memory B cells in healthy individuals but are reportedly expanded and represent a large fraction of memory B cells in some SLE patients (Huang et al., 2002; Wei et al., 2007). Higher frequency of nephritis and increased titer of anti-dsDNA and anti-RNP/Sm antibodies are associated with expanded numbers of DN memory B cells, suggesting that they might contribute to disease pathogenesis (Wei et al., 2007). Low-affinity self-reactive antibodies that are frequently found in the serum of healthy individuals have been shown to originate from IgG^+CD27^+ memory B cells, presumably created during the transition from mature naïve B cells to IgG^+ memory B cells (Tiller et al., 2007). Indeed in a follow-up study, autoreactive IgG^+ memory B cells were characterized in SLE patients, and they were observed to produce antibodies that are highly specific for the extractable nuclear antigens (ENA) Ro and La (Mietzner et al., 2008). In SLE patients with active disease, frequencies of $CD27^+$ B cells (comprising mostly $CD27^{hi}$ plasma cells) were enhanced while naïve and memory B cells were reduced (Odendahl et al., 2000). IgM^+ unswitched memory B cells have also been reported to be decreased in frequency and activated phenotype in SLE patients (Rodríguez-Bayona et al., 2010).

Table 2.8.1. The 1997 update to the 1982 American College of Rheumatology revised criteria for diagnosis of SLE. Taken from (Ginzler and Tayar, 2008).

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions.
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation.
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician.
5. Nonerosive arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion.
6. Pleuritis or pericarditis	1. Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion. OR 2. Pericarditis--documented by electrocardigram or rub or evidence of pericardial effusion.
7. Renal disorder	1. Persistent proteinuria > 0.5 grams per day or > than 3+ if quantitation not performed. OR 2. Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed.
8. Neurologic disorder	1. Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance. OR 2. Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance.
9. Hematologic disorder	1. Hemolytic anemia--with reticulocytosis. OR 2. Leukopenia--< 4,000/mm^3 on $\geqslant$ 2 occasions. OR 3. Lymphopenia--< 1,500/ mm^3 on $\geqslant$ 2 occasions. OR 4. Thrombocytopenia--<100,000/ mm^3 in the absence of offending drugs.

Criterion	Definition
10. Immunologic disorder	1. Anti-DNA: antibody to native DNA in abnormal titer. OR 2. Anti-Sm: presence of antibody to Sm nuclear antigen. OR 3. Positive finding of antiphospholipid antibodies on: (i) an abnormal serum level of IgG or IgM anticardiolipin antibodies, (ii) a positive test result for lupus anticoagulant using a standard method, or (iii) a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test.
11. Positive antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs.

2.9 Phosphoinositide-3-kinases (PI3Ks) in lymphocyte function.

Phosphoinositide-3-kinases (PI3Ks) are lipid signalling kinases that are responsible for regulating many cellular processes such as proliferation, survival, adhesion and migration (Cantley, 2002). PI3Ks have been divided into 3 classes based on their structure and lipid substrate specificities. Class I PI3Ks generate phosphoinositide-3,4,5-trisphosphate (PIP_3) from phosphoinositide-4,5-bisphosphates, whereas classes II and III PI3Ks preferentially produce phosphoinositide 3-phosphates from phosphoinositide (Kok, Geering, and Vanhaesebroeck, 2009). Class I PI3Ks are heterodimeric in nature, consisting of a catalytic and regulatory subunit, and can be subdivided into class IA (p110α, p110β and p110δ) and class IB (p110γ) (Vanhaesebroeck et al., 2010). Little is known about the role of class II and III PI3Ks in immune function, although there is indirect evidence that inhibition of class III PI3K can induce IL-1β secretion through regulation of autophagy (Harris et al., 2011).

The class I PI3K isoform, p110δ (class IA) (or PI3Kδ) is documented to be involved in immune responses (Koyasu, 2003), being expressed exclusively in leukocytes (Vanhaesebroeck et al., 2001). p110δ is typically activated by receptor tyrosine kinases such as cytokine receptors, and costimulatory receptors such as CD28 on T cells (Wu et al., 2005b) and CD19 on B cells (Otero, Omori, and Rickert, 2001). PI3Kδ has been described as essential regulators of B and T cell proliferation, survival and function (Rommel, Camps, and Ji, 2007). PI3Kδ can mediate activation of mitogen-activated protein kinases (Okkenhaug et al., 2002) and inactivation of PI3Kδ leads to signalling defects in multiple cell types such as B cells and T cells.

B cells from PI3Kδ knockout mice exhibit impaired B cell receptor (BCR) signalling, resulting in inhibition of phosphorylation of downstream mediators such as Akt, Erk, FOXO3a and p70 S6K and failure to enter the cell cycle (Bilancio et al., 2006). Similar downregulation of intracellular kinase phosphorylation was observed by these authors when PI3Kδ in B cells was inhibited by its specific inhibitor, IC87114. Inhibition of PI3Kδ in B cells also impaired their proliferative response to anti-IgM, anti-CD40 stimulation and IL-4 signalling, enhancing their susceptibility to apoptosis instead (Bilancio et al., 2006; Clayton et al., 2002). Unlike in B cells, inhibition of PI3Kδ activity in murine T cells appears to only partially impede their proliferative response to anti-CD3, which could be rescued by co-stimulation with anti-CD28 (Okkenhaug et al., 2002). However, PI3Kδ appears to be important for $CD4^+$ T cell differentiation, as differentiation into Th1 and Th2 subsets was impaired in $p110\delta^{D910A/D910A}$ $CD4^+$ T cells (in which p110δ was inactivated by a point mutation) and could not be rescued by exogenous cytokines in vitro (Okkenhaug et al., 2006).

PI3Kδ has been shown to be vital for B and T cell activation and inactivation of PI3Kδ results in defective T-dependent and T-independent humoral responses. Levels of circulating antigen-specific antibodies in response to immunization with protein antigen, as well as the numbers of germinal centers are decreased (Okkenhaug et al., 2002). Levels of serum IgG and IgM were also reduced in $p110\delta^{D910A/D910A}$ mice, which suggest that PI3Kδ is involved in T-independent generation of natural antibody responses by B cells (Durand et al., 2009). As such, PI3Kδ has been investigated as a potential therapeutic target in autoimmune inflammatory diseases such as rheumatoid arthritis (Ghigo et al., 2010). Rheumatoid arthritis is an autoimmune disease involving both B cells and T cells, and is characterised by chronic inflammation in the joints. Using the K/BxN serum transfer model of arthritis, which results in joint inflammation as a result of transfer of arthritogenic serum, Randis et al showed that administration of athritogenic

serum to p110δ$^{-/-}$ mice resulted in significant reduction of paw edema and decreased eosinophil recruitment to inflamed tissues. (Randis et al., 2008). However, the role of PI3Kδ has not been explored in SLE, which is an autoimmune disease that also involves T cells and B cells.

3 Materials and Methods

3.1 List of reagents, materials and instruments used

Table 3.1.1. General reagents used.

Reagent	Company	Location
30% bovine serum albumin	Sigma-Aldrich	Poole, UK
30% hydrogen peroxide	Sigma-Aldrich	Poole, UK
37% formaldehyde (Molecular Biology Grade)	Sigma-Aldrich	Poole, UK
96-well round bottom plates Corning HTS Transwell-96 well permeable support plates with insert	Corning Life Sciences	MA, USA
α-galactosylceramide	Funakoshi co. ltd.	Tokyo, Japan
Anti-CD19, anti-iNKT, anti-PE, anti-APC magnetic beads	Miltenyi Biotech	Bergisch-Gladbach, Germany
Bicinchoninic acid (BCA) protein assay reagent	Thermo-Scientific Pierce	Rockford, IL, USA
Cell-based Human/Mouse/Rat Akt phosphorylation (S473) ELISA kit	R&D Systems	Abingdon, UK
Celltrace Violet Proliferation Kit	Invitrogen	Paisley, UK
CL-Xposure Xray film (5 x 7 in.)	Thermo-Scientific	Rockford, IL, USA
DuoSet ELISA	R&D Systems	Abingdon, UK
ECL western blotting substrate	Thermo-Scientific Pierce	Rockford, IL, USA
Human Phospho-MAPK Array kit	R&D Systems	Abingdon, UK
Hyclone fetal bovine serum	Thermo-Scientific	Logan, UT, USA
IC87114	Cayman Chemicals	Ann Arbor, MI, USA
Ionomycin	Sigma-Aldrich	Poole, UK
LIVE/DEAD Fixable Dead Cell Stain	Invitrogen	Paisley, UK
Lymphoprep	Nycomed Pharma AS	Oslo, Norway
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich	Poole, UK
Phytohemagglutinin (PHA)	Sigma-Aldrich	Poole, UK

Reagent	Company	Location
Poly-L-Lysine	R&D Systems	Abingdon, UK
RPMI 1640 + L-glutamax 200 mM (100X) L-glutamine 50 mM β-mercaptoethanol 1M Hepes 100 mM sodium pyruvate 50X MEM amino acids 100X MEM NEAA Penicillin-streptomycin Amphotericin B Fungizone	Gibco Invitrogen	Paisley, UK
Sodium azide	Sigma-Aldrich	Poole, UK
Staphylococcal enterotoxin B (SEB)	Sigma-Aldrich	Poole, UK
3,3',5,5' - tetramethylbenzidine (TMB) substrate solution	Thermo-Scientific Pierce	Rockford, IL, USA
Tuberculin PPD Batch RT50 lot 221	Statens Serum Institut	Copenhagen, Denmark

Table 3.1.2. Equipment and software used.

Equipment/Software	Company	Location
Agfa CP-1000 developer	Agfa Healthcare NV	Mortsel, Belgium
CyAN ADP flow cytometer	Beckman Coulter	High Wycombe, UK
Eclipse E200 fluorescence microscope	Nikon	NY, USA
FACSArray Bioanalyser	BD	Oxford, UK
FACSCalibur	BD	Oxford, UK
FlowJo v7.6 software	Treestar Inc	New Jersey, USA
Fluoroskan Ascent FI2.6	Thermo-Scientific	Logan, UT, USA
GraphPad Prism v5.0	GraphPad Software Inc	La Jolla, CA, USA
MoFlo XDP cell sorter	Beckman Coulter	High Wycombe, UK
Summit v4.3 software	Dako	Colorado, USA
TECAN Sunrise plate reader	TECAN Group Ltd.	Männedorf, Switzerland

Table 3.1.3. Antibodies directed against iNKT cells.

Specificity (Mouse anti-human)	Conjugate	Company	Location
Vα24	FITC	Immunotech	Marseille, France
Vβ11	PE	Immunotech	Marseille, France
6B11	PE	BD Biosciences	Oxford, UK
CD3	FITC	Immunotools	Friesoythe, Germany
CD3	PerCP, PE-Cy7	eBioscience	Hatfield, UK
CD4	FITC	Immunotools	Friesoythe, Germany
CD4	PE-Cy7	eBioscience	Hatfield, UK
CD4	PE	BD Biosciences	Oxford, UK
CD8	APC	BD Biosciences	Oxford, UK
CD154 (CD40L)	APC-eFluor780	eBioscience	Hatfield, UK

Table 3.1.4. Antibodies directed against regulatory B (Breg) cells.

Specificity (Mouse anti-human)	Conjugate	Company	Location
CD19	APC-eFluor780	eBioscience	Hatfield, UK
CD5	PerCP-eFluor710, PerCP-Cy5.5	eBioscience	Hatfield, UK
CD1d	PE	BD Biosciences	Oxford, UK
CD24	FITC	Immunotools	Friesoythe, Germany
CD38	PE-Cy7	eBioscience	Hatfield, UK

Table 3.1.5. Antibodies directed against memory B cells, naïve B cells and plasma cells.

Specificity (Mouse anti-human)	Conjugate	Company	Location
CD19	APC-eFluor780	eBioscience	Hatfield, UK
CD19	APC	Immunotools	Friesoythe, Germany
CD27	FITC	Immunotools	Friesoythe, Germany
IgD	PE	BD Biosciences	Oxford, UK
IgM	PE-Cy5	BD Biosciences	Oxford, UK
IgG	PE-Cy5	BD Biosciences	Oxford, UK
CD20	FITC	Immunotools	Friesoythe, Germany
CD5	FITC	Immunotools	Friesoythe, Germany
CD10	FITC	Immunotools	Friesoythe, Germany
CD38	FITC	Immunotools	Friesoythe, Germany
CD22	FITC	Immunotools	Friesoythe, Germany

Table 3.1.6. Antibodies against activation and co-stimulatory markers on B cells.

Specificity (Mouse anti-human)	Conjugate	Company	Location
CD58	FITC	Immunotools	Friesoythe, Germany
CD80	FITC, APC	Immunotools	Friesoythe, Germany
CD86	FITC, APC	BD Biosciences	Oxford, UK
CD40	PE, APC	Immunotools	Friesoythe, Germany
CD69	PE-Cy5	BD Biosciences	Oxford, UK
CD95	PE	eBioscience	Hatfield, UK
CD83	PE	BD Biosciences	Oxford, UK

Table 3.1.7. Purified antibodies for functional studies.

Specificity (Mouse anti-human)	Company	Location
CD3 (HIT3A)	BD Pharmingen	San Jose, CA, USA
CD1d	eBioscience	Hatfield, UK
CD40	eBioscience	Hatfield, UK
CD154	eBioscience	Hatfield, UK
IL-4	eBioscience	Hatfield, UK
IL-13	eBioscience	Hatfield, UK

Table 3.1.8. Antibodies against cytokines, used in intracellular flow cytometry.

Specificity (Mouse anti-human)	Conjugate	Company	Location
IL-10	eFluor450	eBioscience	Hatfield, UK
IFN-γ	APC	eBioscience	Hatfield, UK
IFN-γ	PE	BD Biosciences	Oxford, UK
IL-13	FITC	eBioscience	Hatfield, UK
IL-13	PE	BD Biosciences	Oxford, UK

3.2 Isolation of cells from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors or buffy coat packs by Ficoll-Hypaque density gradient centrifugation with Lymphoprep. Thirty-five millilitres of peripheral blood was layered carefully onto 15 ml of Lymphoprep in a 50 ml conical tube and centrifuged at 400xg for 25 minutes with the brakes off. After centrifugation the buffy coat layer containing the PBMCs was removed using a sterile pastette and transferred to a fresh 50 ml tube. The PBMCs were washed twice by topping up the tube to 50 ml with sterile 1X PBS, centrifuging at 400xg for 7 minutes and decanting the supernatant. PBMCs were subsequently resuspended in 10 ml of T cell medium (TCM) and cell counts were obtained (section 3.4). TCM consists of complete RPMI (cRPMI) medium supplemented with 0.05 mM L-glutamine, 1X MEM non-essential amino acids (NEAA) and 0.05 mM β-mercaptoethanol. cRPMI comprises RPMI 1640 + L-glutamax supplemented with 10% v/v HyClone fetal bovine serum, 0.02 M HEPES buffer, 100 U/ml penicillin and 100 µg/ml streptomycin and 2.5 µg/ml Amphotericin B Fungizone.

3.3 B cell and iNKT cell enrichment by magnetic bead separation

To isolate B cells, PBMCs were stained with CD19 microbeads which are anti-CD19 mAb coated magnetic beads that bind to the CD19 molecule on B cells. The bead-cell mixture was incubated at 4°C for 15 minutes. Excess unbound beads were washed away by adding 10 ml of PBS + 2% fetal calf serum (FCS), centrifuging at 400xg for 7 minutes and discarding the supernatant. Positive selection of B cells by magnetic bead sorting was performed by running the PBMC-bead mix through the provided column inserted into a magnet, followed by five washes with PBS + 2% FCS to remove non-B cells from the column. The purified B cells were eluted from the column by running through 5 ml of PBS + 2% FCS after removing the column from the magnet. Purity of B cells was determined to be >99% when assessed by flow cytometric analysis of CD20 expression.

iNKT cells were sorted from PBMC by magnetic bead separation (same method as B cell separation) using either (i) anti-iNKT-PE and anti-PE magnetic beads or (ii) iNKT microbeads (anti-iNKT mAb-coated magnetic beads that recognise the iNKT cell receptor). Purity of iNKT cells was determined to be >50% when assessed by flow cytometric analysis of 6B11 and CD3 expression. The enriched iNKT cells were subsequently expanded in culture on 96-well round bottom plates using two different protocols (see section 3.6, first and second protocols).

3.4 Cell viability

To count cells, the ethidium bromide/acridine orange (EB/AO) reagent was used as described in (Curry et al., 2000) at a concentration of 16 µg/ml ethidium bromide and 10 µg/ml acridine orange in PBS. Cells resuspended in TCM were diluted in EB/AO at 1:20 or 1:40 and mounted on an improved Neubauer Haemocytometer for visualisation and counting using a fluorescence microscope. Ethidium bromide stains DNA of dead cells orange and is excluded by live cells whereas acridine orange stains DNA of live cells green. Hence only green cells were considered live and counted.

3.5 Antibodies and flow cytometry

Antibodies used are listed in tables 3.1.2 – 3.1.5 (section 3.1). These were titrated to determine appropriate concentration for use. Isotype control antibodies were purchased from the respective companies whose antibodies were used in the staining. FMO (fluorochrome minus one) controls and isotype controls were used in the flow cytometry experiments.

3.5.1 Principle of flow cytometry

In flow cytometry, cells are hydrodynamically-focused into a stream of single-cell suspension in a sheath fluid (usually PBS). A combination of laser beams hits the cells as they flow through and based on the pattern of light scatter and emission detected by the multiple detectors, multi-parametric data about the size, granularity and fluorescence is collected (Figure 3.5.1) (Fulwyler, 1980).

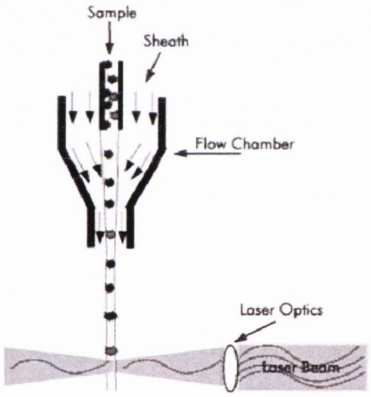

Figure 3.5.1. Cells to be analysed are hydrodynamically-focused into a single-cell suspension. Light scattering and emission as a result of laser beams hitting the cell provides quantifiable data about cell morphology and fluorescence. Taken from (Nolla).

To identify molecules expressed on a cell's surface, antibodies recognising the particular molecule, conjugated to a fluorophore, can be used to stain the cell. Fluorophores are fluorescent molecules that absorb and re-emit energy at specific wavelengths. Detection of these emission wavelengths provides information on whether the cell expresses the particular molecule of interest on its surface and the intensity of fluorescence translates to quantity of molecule expressed on the cell surface (Loken,

1990). Therefore detection of the expression of multiple cell-surface molecules can be used to identify specific subsets of cells within the sample population.

3.5.2 Cell staining and data acquisition

1 x 10^5 cells were labelled with mAbs (tables 3 to 6 in section 3.1), incubated in the dark at room temperature (RT) for 10 minutes, then washed with 2 ml PBA buffer, which consists of 1X phosphate buffered saline (PBS), 1.5% bovine serum albumin (BSA) and 0.02% sodium azide. Cells were resuspended in PBA buffer after centrifugation to remove excess antibodies and then analysed by flow cytometry with a CyAN ADP flow cytometer. Acquired data was analysed with the Summit v4.3 software and FlowJo v7.6.

3.6 Isolation and culture of iNKT cells

iNKT cells obtained from human peripheral blood needed to be expanded in culture prior to use as they constitute only 0.01 – 1% of lymphocytes (Porcelli and Modlin, 1999). This was done by first enriching iNKT cells from PBMCs using positive selection. Subsequently a variety of methods can be used to expand the enriched iNKT cells and these are broadly categorised into 2 groups: (i) non-specific stimulation with T cell polyclonal mitogens (e.g. PHA or anti-CD3 mAb), (ii) specific stimulation with α-GC and irradiated feeder cells acting as antigen-presenting cells (Exley, Balk, and Wilson, 2003). However the following protocols used to expand iNKT cells are variations of the first method and the second method was not used in this thesis.

In the first protocol, magnetic-bead enriched iNKT cells were stimulated once with 1 μg/ml plate-bound anti-CD3 (HIT3A) mAb and cultured in TCM. To coat plates with anti-CD3 mAb, 100 μl of anti-CD3 mAb diluted in PBS was pipetted into each well and the plate incubated overnight at 4°C. PBS was removed and the plate warmed to 37°C prior to seeding cells. The iNKT cells were given 100 U/ml of IL-2 for the first two weeks of culture, then subsequently fed with 50 U/ml of IL-2 weekly. Purity of iNKT culture after 4 weeks was assessed to be >97% by flow cytometric analysis of 6B11 and CD3 expression.

In the second protocol, magnetic-bead enriched iNKT cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-Vα24 and phycoerythrin (PE)-conjugated Vβ11 and further enriched by sorting for double-positive events with the MoFlo XDP cell sorter. Purity of $V\alpha24^+V\beta11^+$ iNKT cells was determined to be ≥98%. The enriched iNKT cells

were then cultured with irradiated (25 Gy) allogeneic PBMC feeders at ratios of 1:100 – 1:1000 in TCM supplemented with 250 U/ml IL-2 and 1 μg/ml phytohemagglutinin (PHA). PHA in the cultures was diluted during the first two days of culture by replacing the medium with PHA-free, IL-2-supplemented TCM. Purity of iNKT cells after 3 weeks was assessed to be ≥99% by flow cytometric analysis of 6B11 and CD3 expression.

A third protocol was used to expand iNKT cells from the peripheral blood of SLE patients. iNKT cells were sorted from PBMC by magnetic bead separation using anti-iNKT magnetic beads, and then expanded using the first protocol in section 3.6. Irradiated allogeneic feeder cells were also used as starting cell numbers were significantly smaller than with healthy donors. After 4 weeks of culture, flow cytometric analysis revealed low purity of iNKT cells (approximately 5%). Hence the expanded cells were enriched again by magnetic bead separation and cultured for 4 more weeks with anti-CD3 mAb and IL-2 stimulation (using the same protocol in section 3.6). The average purity of the iNKT lines obtained from the second round of expansion was between 7 – 55% pure.

3.6.1 Principle of flow sorting of cells

Sorting of cells by flow cytometry, also termed fluorescence-activated cell sorting (FACS), separates cells according to the amount and combination of fluorescent dyes bound to each cell. Similar to flow cytometry, the cells in the sample are hydrodynamically focused into a single-cell stream, and are rapidly passed through a small area intensely illuminated by a laser beam. Thereafter the cells are isolated into droplets, with each droplet containing a single cell. The droplets are electrostatically charged and deflected into different collecting reservoirs based on their charge (Herzenberg, Sweet, and Herzenberg, 1976). (Figure 3.6.1)

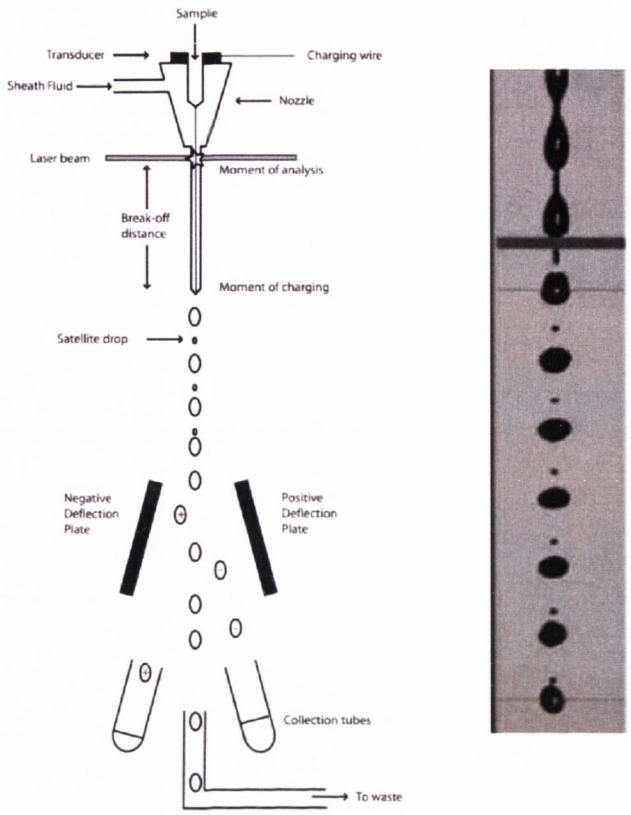

Figure 3.6.1. Schematic of a general cell sorter. Stroboscopic illumination of the stream shows the breakoff point into charged droplets. Taken from (Davies, 2001).

3.7 Co-culture of expanded iNKT cells with Hela-CD1d cells.

To test the functionality of expanded iNKT cells, they were co-cultured in equal numbers with Hela-CD1d cells (Chang et al., 2007) at densities of 10^6 cells/ml for 3 days in the absence or presence of 100 ng/ml of α-galactosylceramide (α-GC). Hela-CD1d cells are Hela cells that have been transfected with a vector expressing CD1d. As negative controls, the expanded iNKT cells were also co-cultured with Hela-mock cells (mock-transfected with empty vector) in the absence or presence of α-GC. All cells were plated on 96-well round-bottom plates. Supernatants were harvested and analysed for IFN-γ, IL-4, IL-10 and IL-13 production by enzyme-linked immunosorbent assay (ELISA).

3.7.1 Maintenance of Hela cell lines

Hela-CD1d and Hela-mock cells were cultured at 37°C in flasks with DMEM supplemented with 10% FCS. Both were kindly provided by Prof Steven Porcelli, Dept

of Microbiology and Immunology, Albert Einstein College of Medicine, New York, USA. The Hela cells were trypsinized with 0.25% trypsin and incubated at 37 °C for 2 minutes to remove their adherence to the tissue culture flasks. Resuspended Hela cells were transferred to a fresh 50 ml tube, topped up with 10 ml of cRPMI and centrifuged at 400xg for 7 minutes to inactivate and remove the trypsin prior to use in experiments.

3.7.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a colorimetric assay used to quantify the amount of antigen or antibody present in a sample and the most commonly used technique is the "sandwich ELISA" (Engvall and Perlmann, 1971). ELISA was performed using the DuoSet ELISA kit for the respective cytokines, concentrations for antibodies and other reagents were obtained from the manufacturer's protocol (which differ depending on the cytokine being assayed).

Flat-bottomed 96-well plates were coated at 4°C overnight with 40 µl/well of capture antibodies (diluted in PBS) which recognise a specific epitope on the analyte of interest. Plates were blocked with 100 µl/well of PBS + 1% bovine serum albumin (BSA) for 1 hour at RT. Supernatant samples and standards (40 µl/well) were applied to the plate for 2 hours at RT, followed by 100 µl/well of detection antibodies (which recognise a separate epitope on the analyte of interest) for 2 hours at RT. Plates were washed with PBS + 0.5% Tween in between each step listed above. The plates were then incubated with 100 µl/well of a 1:200 dilution of streptavidin-horse radish peroxidase (HRP) for 20 minutes and TMB substrate solution for 20 – 40 minutes at RT in the dark. 20 µl/well of sulphuric acid ("Stop" solution) was added to stop colorimetric development. Absorbances from each well were read at 450 nm with the TECAN Sunrise microplate reader. Standard curves were drawn using GraphPad Prism v5.0 to determine concentrations of analytes in the samples.

3.8 Co-culture of B cells with iNKT or non-iNKT cells

iNKT cells were co-cultured with allogeneic or autologous B cells in order to study the reciprocal interaction between these cells. Most of the experiments in this thesis were done in an allogeneic system due to the difficulty in obtaining autologous iNKT and B cells. Some experiments were done using autologous iNKT-B cell co-cultures in order to determine if the response observed in the allogeneic system were due to alloreactivity.

B cells were co-cultured with allogeneic or autologous iNKT or non-iNKT cells for 3 or 10 days at densities of 10^6 cells/ml at ratios of 1:1. All cells were plated on 96-well round-bottom plates. Stimuli or glycolipids added were in the following concentrations: 100 ng/ml of α-galactosylceramide (α-GC), 10 ng/ml of phorbol-12-myristate-13-acetate (PMA) and 1 μg/ml of ionomycin. Supernatants were harvested and frozen at -20°C for further analysis of cytokine and immunoglobulin production. Cells were resuspended in PBA for analysis by flow cytometry.

3.9 Cell-cell contact dependence by transwell assay

To investigate if iNKT-B cell interactions require cell-cell contact, B cells and iNKT cells were co-cultured in the absence or presence of 100 ng/ml α-GC for 3 days or 10 days according to the following setup in a 96-well plate with the Corning HTS Transwell-96 well permeable support insert (Figure 3.9.1). The insert contains a membrane with a pore size of 0.3 μm, which allows soluble factors but not cells to cross the membrane.

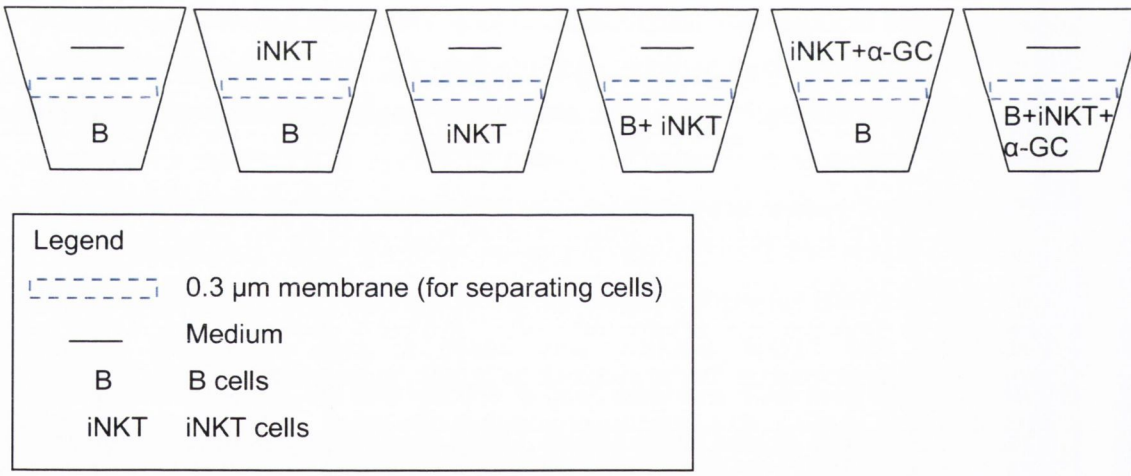

Figure 3.9.1. Schematic of experimental setup to assay for cell-cell contact dependence using a 96-well plate with the Corning HTS Transwell-96 permeable support insert.

After 3 days, cells were resuspended in PBA for analysis by flow cytometry. After 10 days, supernatants were harvested and frozen at -20°C until they were analysed for immunoglobulin production.

3.10 Multiplex detection of cytokines and immunoglobulins

Multiplex cytometric bead array (CBA) kits were used to analyse the supernatants from the co-cultures for cytokines and immunoglobulins. In chapter 4 of this thesis, cytokines assayed for were IL-2, IL-5, IL-6, IL-12p70, IL-10, IL-4, IFN-γ and IL-13. Immunoglobulins assayed for were IgA, IgM, total IgG, IgG_1, IgG_2 and IgE. In chapters 5 and 6 of this thesis, cytokines were assayed for using the Th1/Th2 kit and comprised IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ and IL-13. Immunoglobulins assayed for were IgA, IgM, total IgG, and IgG_2.

The assays were carried out according to the manufacturer's instructions. Flow cytometric data from the arrays were acquired with the FACSCalibur or CyAN ADP. Data was analysed with Summit v4.3, and GraphPad Prism v5.0 was used to draw standard curves and obtain sample concentration values.

3.10.1 Principle of the CBA assay

The CBA assay is similar to a multiplex ELISA. Capture beads containing antibodies that bind the analyte of interest were incubated with samples or standards for 1 hour at RT. Detector antibodies, which are conjugated to PE and specific for the analyte of interest were then added to the mixture and incubated for 2 hours at RT. Unbound beads and detector antibodies were washed off by adding excess wash buffer, centrifuging at 200xg for 5 minutes and decanting the supernatant. The resulting bead mixture was resuspended in wash buffer for flow cytometric analysis. Capture beads corresponding to a particular analyte have a unique position when visualised in the FL3-FL4 plot (taking the example of the FACSCalibur), allowing for many different analytes to be assayed for simultaneously. The intensity of fluorescence in the FL2 channel for each bead position corresponds with the quantity of analyte present in the sample.

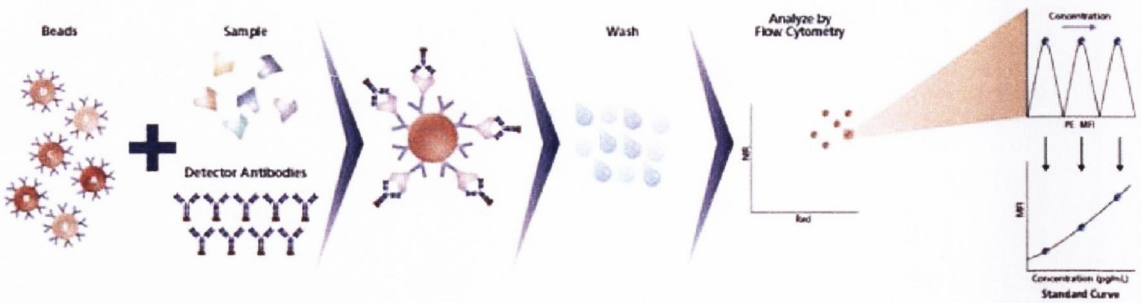

Figure 3.10.1. Overview of the CBA assay. Taken from the BD CBA brochure (Biosciences, 2009). Capture beads and detection antibodies are incubated with samples or standards, binding to the analyte of interest. Excess capture beads and detection antibodies are washed off with wash buffer and the resulting bead mixture is analysed by flow cytometry. The FL2 fluorescence in each bead position corresponds to the quantity of analyte present in the sample.

3.10.2 Flow cytometric acquisition and analysis

A high threshold was set on the forward scatter (FSC) and side scatter (SSC) to exclude debris caused by beads clumping. Based on FSC and SSC parameters, the beads of interest are clustered in the population in R1 (Figure 3.10.2). As each analyte occupies a unique position when viewed on the FL3-FL4 plot, separate gates can be created for each analyte to obtain their corresponding fluorescence intensities in the FL2 channel. For creation of a standard curve, the beads were incubated with varying concentrations of the standards (provided in the array). The FL2 intensities for each standard concentration were obtained and fitted into a sigmodal dose-response (variable slope) curve in GraphPad Prism. Unknown concentrations for the samples were then extrapolated from the standard curve. (Figure 3.10.2)

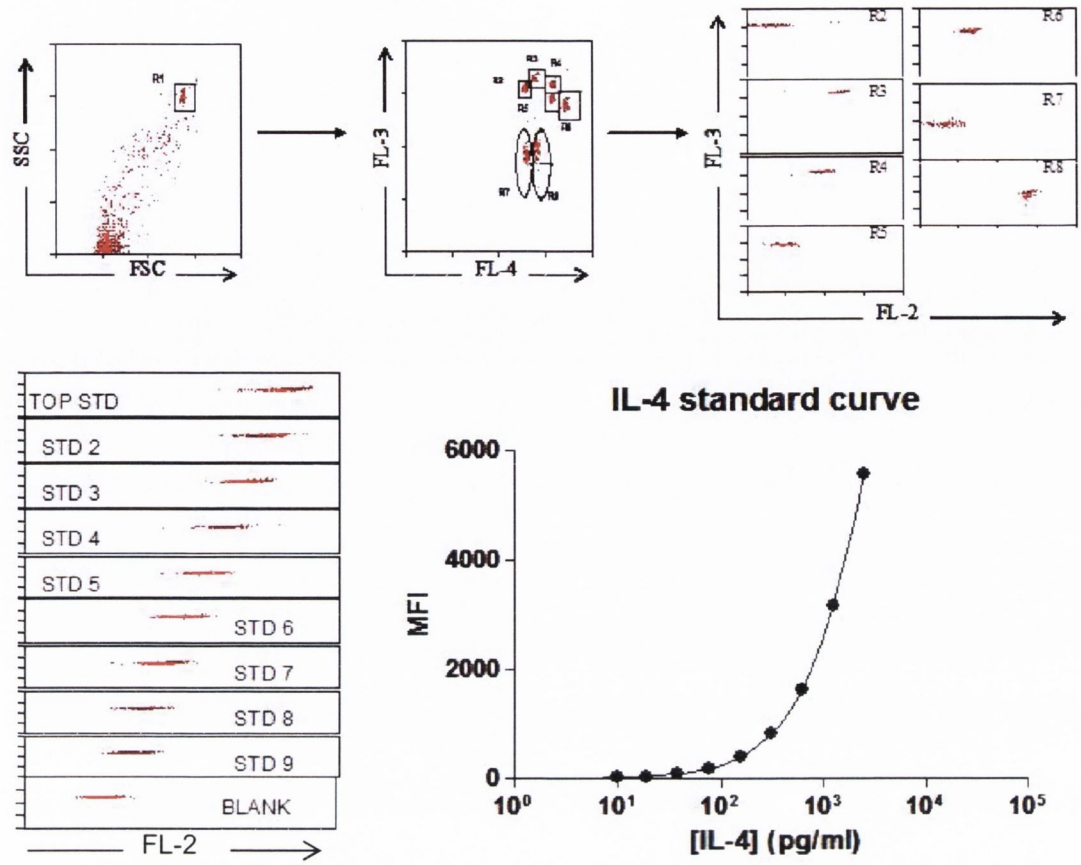

Figure 3.10.2 Overview of data acquisition on the flow cytometer and plotting of the standard curve to extrapolate concentrations of samples. The bead mixture is clustered in the R1 population on the FSC/SSC dot plot, which can be resolved into individual populations in the FL3/FL4 dot plot, each population corresponding to a particular analyte. Each bead population is then gated on and the FL2 intensity is obtained, which corresponds to the quantity of the analyte in the sample being assayed. This is performed for both a pre-determined set of standards and the supernatant samples. A standard curve is created using GraphPad Prism from which unknown concentrations of the samples can be extrapolated.

3.11 Antigen presentation studies

To test if iNKT cells were able to induce maturation of B cells into antigen-presenting cells, antigen presentation studies were carried out. B cells were co-cultured with autologous iNKT cells at a density of 10^6 cells/ml and in equal numbers in the presence or absence of 100 ng/ml of α-GC for 3 days. B cells and iNKT cells were also cultured separately in medium alone as negative controls. The co-cultured cells were harvested and subsequently co-cultured with Celltrace Violet-labelled (see section 3.11.1) autologous or allogeneic T cells in the ratio of 1:3 with the following stimuli/antigens: medium only (negative control), 10 µg/ml tuberculin PPD, 1 µg/ml SEB and 5 µg/ml PHA

(positive control). Proliferation of the T cells was assayed by flow cytometry after 6 – 8 days in culture. Acquired data was analysed using FlowJo v7.6.

3.11.1 Labelling T cells with Celltrace Violet dye

A 5 mM Celltrace Violet stock solution was prepared immediately prior to use by dissolving the contents in one vial of lyophilized Celltrace Violet in 20 µl of DMSO. The stock solution was diluted to 1 mM in pre-warmed PBS. Medium was removed from the T cells, which were then resuspended in pre-warmed PBS and incubated for 20 minutes at 37 °C. TCM was added to the T cells at five times the original staining volume to quench unbound dye, after which cells were centrifuged and resuspended in the appropriate volume of TCM for the experiment. As the cells proliferate and divide, each daughter cell receives half the amount of Celltrace dye that the parent had, resulting in a series of peaks decreasing exponentially in fluorescence intensity.

3.12 Co-culture of B cells with $CD4^+$, $CD8^+$ and DN iNKT subsets

iNKT cells used in this experiment were enriched from peripheral blood of healthy donors by density-gradient centrifugation as in section 3.2, enriched by magnetic bead separation as in section 3.3 and expanded using the second protocol in section 3.6.

B cells were co-cultured with sorted $CD4^+$, $CD8^+$ and DN iNKT cells to investigate if the three iNKT subsets have different effects on B cells.

To separate the $CD4^+$, double negative (DN) and $CD8^+$ iNKT subsets, expanded polyclonal iNKT lines were stained with phycoerythrin (PE)-conjugated anti-CD4 and allophycocyanin (APC)-conjugated CD8, incubated in the dark on ice for 30 minutes and washed with PBS + 2% FBS. Labelled cells were sorted into their subsets using the MoFlo XDP cell sorter. If the percentage of $CD4^+$ or $CD8^+$ iNKT subsets were low (<10%), the corresponding subset was enriched by labelling with anti-PE or anti-APC magnetic beads, followed by magnetic bead separation with the autoMACS separator prior to cell sorting. The autoMACS separator is a benchtop automated magnetic cell sorter containing its own columns and carries out magnetic bead separation (as outlined in section 3.3) automatically.

B cells were co-cultured with $CD4^+$, DN and $CD8^+$ iNKT cells for 3 or 10 days at densities of 10^6 cells/ml in the ratio of 1:1. All cells were plated on 96-well round-bottom plates in a total volume of 200 µl/well. The B cells were co-cultured in the absence or presence of 100 ng/ml of α-galactosylceramide (α-GC). Supernatants were harvested and frozen at -20°C until they were analysed for immunoglobulin production. Cells were resuspended in PBA for analysis by flow cytometry.

3.12.1 Analysis of intracellular cytokine production.

To determine which cytokines are produced by iNKT cells and B cells, individually and in co-culture, cells were stained with mAb that detect cell surface phenotypes and intracellular cytokines and analysed by flow cytometry.

$CD4^+$ iNKT cells were co-cultured with equal numbers of B cells at equal numbers for 3 days. B cells and $CD4^+$ iNKT cells were individually cultured in medium alone as negative controls. On the third day, 0.05 mM of monensin was added to the co-cultured cells for 4 hours to prevent the cells from releasing the cytokines produced into the supernatant.

After 4 hours, cells were washed once with PBS and incubated with LIVE/Dead Fixable Dead Cell Stain for 30 minutes at room temperature to exclude dead cells during flow cytometry analysis. Cells were subsequently stained with fluorochrome-conjugated antibodies (see tables 3, 5 and 8 in section 3.1) for surface markers delineating iNKT cells and Breg cells, followed by fixation with 4% paraformaldehyde for 10 minutes at room temperature. Cells were permeabilised with 0.2% saponin for 10 minutes at room temperature and stained with FITC- or PE-conjugated anti-human IL-13, eFluor450-conjugated anti-human IL-10 and APC-conjugated anti-human IFN-γ for 20 minutes at room temperature. Cells were washed once with PBA and then resuspended in 200 µl PBA for analysis on the CyAN ADP.

3.13 Co-culture of B cells with iNKT cells and blocking antibodies

To investigate the mediating factors facilitating iNKT-B cell interactions, antibodies were directed against CD1d, CD40, CD154, IL-4 and IL-13 in an attempt to block them during co-cultures.

B cells were co-cultured with iNKT cells for 3 or 10 days at a density of 10^6 cells/ml in the ratio of 1:1, in the absence or presence of 100 ng/ml α-GC. Blocking antibodies were added in the following concentrations: 10 µg/ml anti-CD1d, 10 µg/ml anti-IL-4, 10 µg/ml anti-IL-13, 10 µg/ml anti-CD40 and 10 µg/ml anti-CD154. Supernatants were harvested and frozen at -20°C until they were analysed for cytokine and immunoglobulin production. Cells were resuspended in PBA for analysis by flow cytometry.

3.14 Investigating the role of the δ isoform of phosphoinositide-3-kinase (PI3K) in mediating iNKT-B cell interactions

As PI3Kδ (or p110δ) is known to play a vital role in B cell and T cell function, we investigated their role in mediating iNKT-B cell interactions in the presence of α-GC.

3.14.1 Determining IC50 of IC87114

IC50, also known as the half maximal inhibitory concentration, is a measure of efficacy of a compound in inhibiting a biological process or an enzyme. It is determined by generating a dose-response curve with a range of concentrations of the inhibitor and obtaining the value at which the response is halved (Neubig et al., 2003).

To determine the IC50 of the PI3Kδ-specific inhibitor, IC87114, varying concentrations of IC87114 was added to purified human B cells and the corresponding inhibition of Akt phosphorylation was measured using the cell-based Human Akt phosphorylation ELISA kit. The Human Akt phosphorylation ELISA kit quantifies intracellular phosphorylated Akt and total Akt in the cells simultaneously by using specific detection antibodies that permeabilise the immobilised cells on the ELISA plate. To immobilise the cells on the microplate, it was first incubated with 10 µg/ml of Poly-L-Lysine for 30 minutes at 37°C. The Poly-L-Lysine was then decanted, the plate washed twice with PBS and then seeded with B cells at 10^6/ml, 200µl/well. The B cells were stimulated with 10 ng/ml of PMA and 1 µg/ml of ionomycin, then treated with titrating concentrations of IC87114 from 0 – 300 µM. After 3 days, the rest of the steps were carried out according to the manufacturer's protocol. Fluorometric emission from the plate was read using the Fluoroskan Ascent Fl2.6. Phosphorylated Akt was read at 578/604 (ex/em) nm and normalised with corresponding readings of total Akt at 355/460 (ex/em) nm. The normalised readings were used to plot an inhibition curve in GraphPad Prism v5.0 and the IC50 value calculated by the software was 0.1567 µM. It is recommended that the

inhibitor be used at a concentration 5 – 10 times higher than the IC50 value for maximal inhibition (2009).

3.14.2 Co-culture of B cells with iNKT cells and IC87114

To observe the effect of IC87114 on surface expression of B cells and iNKT cells, B cells were co-cultured in a 1:1 ratio with iNKT cells at a density of 10^6 cells/ml, in the absence or presence of 100 ng/ml α-GC. IC87114 was added at a concentration of 1 μM and cells were analysed by flow cytometry after 3 days of co-culture.

A similar setup was used to observe the effect of IC87114 on protein expression in the co-cultured B and iNKT cells. 5 x 10^6 B cells were co-cultured with 5 x 10^6 iNKT cells in the absence or presence of 100 ng/ml α-GC. 10 x 10^6 B cells or iNKT cells were cultured in medium alone as negative controls. 1 μM IC87114 was also added to the co-cultures. After 3 days, co-cultured B and iNKT cells were separated by staining with anti-iNKT beads and magnetic bead separation. Purity of separated fractions was determined to be ≥ 70% based on flow cytometric analysis of CD19 and CD3 expression. The separated fractions and negative controls were washed once with PBS, then lysed at a density of 10^7/ml with Lysis buffer 6 (provided in the Human PhosphoMAPK array kit).

3.14.3 Protein quantitation with bicinchoninic acid (BCA) protein assay

Working BCA reagent was reconstituted by combining BCA reagent A with BCA reagent B in the ratio of 50:1. Cell lysates and BSA standards were added to working BCA reagent at a ratio of 1:20 and incubated for 2 hours at 37°C. Colorimetric absorbance readings at both 492 and 620 nm were obtained using the TECAN Sunrise plate reader. GraphPad Prism v5.0 was used to draw the standard curve and obtain protein concentration values for the cell lysates.

3.14.4 Multiplex western blots with the Human Phospho-mitogen-activated protein kinase (MAPK) array kit

The Human PhosphoMAPK array kit allows for simultaneous determination of 26 different MAPKs on a single blot. Antibodies specific for each MAPK are spotted in duplicate on a nitrocellulose membrane, which capture the corresponding MAPK. B cell lysates and iNKT cell lysates from the co-cultures were incubated overnight at 4°C on a rocking platform with the provided antibody mix containing antibodies specific to the phosphorylated form of the 24 MAPKs. Streptavidin-HRP and chemiluminescent reagent (ECL western blotting substrate) were then added according to manufacturer's instructions. Blots were washed three times with 1X Wash Buffer (diluted from 25X Wash Buffer provided with kit, composition not specified in manufacturer's product insert) in between additions of streptavidin-HRP and ECL. Membranes were covered with plastic wrap, exposed to CL-Xposure Xray film for 1 – 30 minutes and developed with the Agfa CP-1000 developer. Developed film was scanned using the Canon MP460 scanner and pixel density (mean gray value) of each spot was analysed using Photoshop CS4. Pixel density for each pair of duplicate spots representing each kinase was averaged and an averaged background signal (obtained from negative control spots in the blot) was subtracted from that to obtain the final values. Values were graphed using GraphPad Prism v5.0 to compare relative change in protein phosphorylation between the samples.

3.15 Sample collection from SLE patients

Patients attending the SLE clinic at St James' Hospital were recruited for the study by a dedicated physician. A total of 32 samples were received but only 15 patients with a firm diagnosis of SLE were included in the study. The SLE patients (mean age 39 years; age range 25 – 70 years) were classified based on the American College of Rheumatology revised classification criteria for lupus (Tan et al., 1982) and patients with both active and inactive disease were included. Table 3.20.1 describes the characteristics of the SLE patients and Table 3.20.2 shows the medications that the patients were on. The ethics committee of St James' Hospital in Dublin, Ireland, approved this study, patients and healthy volunteers were recruited after obtaining informed consent.

3.16 PBMC isolation from SLE patients and healthy controls

Peripheral blood mononuclear cells (PBMC) were isolated from 18 ml of peripheral blood from SLE patients and healthy controls as outlined in section 3.2.

3.17 Phenotyping PBMC from SLE patients and healthy controls

PBMC from SLE patients and healthy donors were analysed by flow cytometry for the following cell types: iNKT cells, Breg cells ($CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$), memory B cells (unswitched, switched and double negative) and naïve B cells. CD40 and CD80 expression was phenotyped on the B cell subsets, whereas CD154 expression was phenotyped on the iNKT cell subsets. (Table 3.17.1)

Table 3.17.1 Panel of antibodies used to phenotype iNKT cells and B cell subsets.

	FITC	PE	PerCP/ PE-Cy5	PerCP-Cy5.5/ PerCP-eFluor 710	PE-Cy7	APC	APC-eFluor 780
Breg cells	CD24	CD1d	-	CD5	CD38	CD40/CD80	CD19
Memory B cells	CD27	IgD	IgM/IgG	-	-	CD40/CD80	CD19
iNKT cells	CD4	6B11	CD3	-	-	CD8	CD154

3.18 Isolation and culture of iNKT lines from SLE patients

Similar to healthy donors, iNKT cells from SLE patients were expanded for use in functional studies. The third protocol in section 3.6 was used to expand the iNKT cells.

3.19 Co-culture of healthy B cells with iNKT lines from SLE patients

To test if iNKT cells from SLE patients interact with B cells similarly to iNKT cells from healthy donors, B cells were purified from PBMC of healthy blood donors using CD19 microbeads, then co-cultured with iNKT lines grown from SLE patients in the

presence or absence of α-GC. After 3 days, the co-cultured cells were resuspended in PBA and analysed by flow cytometry. Regulatory B cells and memory B cell subsets were analysed for changes in CD40 and CD80 expression, whereas iNKT cells were analysed for changes in CD154 expression. Supernatants were harvested from 3-day and 10-day co-cultures and frozen at -20°C until they were analysed for cytokine and immunoglobulin production by CBA respectively.

3.20 Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.0. For comparison between 2 groups, the Mann-Whitney U test was used to compare unpaired data and the Wilcoxon matched-pairs test was used to compare paired data. For comparison between 3 or more groups, the Kruskal-Wallis test was used to compare unpaired data and the Friedman's test was used to compare paired data. Dunn's multiple comparison tests were performed post-hoc to compare individual groups within an experiment. Two-way ANOVA with post-hoc Bonferroni's test was used to compare the effect of treatments (e.g. B only against B + iNKT). *, ** and *** represent $p<0.05$, $p<0.01$ and $p\leq0.0001$ respectively.

Table 3.20.1. Table of characteristics of SLE patients enrolled in study (n=15).

Identifier	Age	Diagnosis	ESR	CRP	C3	C4	Lymphocyte count	mAb against dsDNA	PBMC isolated (10^6)/ml of blood
SLE 13	52	SLE	28	3.81	1.46	0.33	1.4 (L)	NA	1.167
SLE 14	41	SLE	54*	5.46	1.47	0.15 (L)	0.8 (L)	+	0.789
SLE 15	25	SLE + Sjogren's	24	4.82	NA	NA	0.9 (L)	+	0.700
SLE 16	38	SLE(N)+AP	19	<1.0	0.84	0.07 (L)	2.5	+	2.022
SLE 18	41	SLE + Sjogren's	25	0.59	1.06	0.23	1.6	+	1.583
SLE 19	29	SLE + AP	NA	NA	NA	NA	NA	NA	2.097
SLE 20	30	SLE (N)	32*	1.74	0.78 (L)	0.02 (L)	0.4 (L)	+	0.728
SLE 21	38	SLE + Sjogren's	7	2.73	NA	NA	1.6	NA	2.042
SLE 23	35	SLE	NA	<1.0	NA	NA	1.1 (L)	+	2.028
SLE 24	36	SLE	NA	NA	NA	NA	1.2 (L)	+	1.611
SLE 25	41	SLE	24	1.24	NA	NA	1.1 (L)	+	0.628
SLE 28	70	SLE + Sjogren's	NA	NA	NA	NA	NA	+	1.194
SLE 30	29	SLE	9	1.92	0.88	0.14 (L)	1.0 (L)	+	1.483
SLE 31	30	SLE	4	2.71	NA	NA	1.8	NA	3.578
SLE 32	39	SLE(N)+AP	54*	2.9	1.16	0.09 (L)	NA	+	2.717

Values with asterisks (*) indicate elevated levels, (L) indicates lowered levels, '+' indicates positive, '-' indicates negative and 'NA' indicates data not available. 'AP' = anti-phospholipid syndrome, 'N' = nephritis, 'ESR' = erythrocyte sedimentation rate, 'CRP' = C reactive protein, 'C3' and 'C4' = complement components.

Table 3.20.2. Medications taken by the SLE patients.

Identifier	Steroids	Anti-malarial	Immunosuppressants
SLE 13	None	Hydroxychloroquine	None
SLE 14	Prednisolone	None	Azathioprine
SLE 15	None	None	None
SLE 16	None	Hydroxychloroquine	None
SLE 18	None	Hydroxychloroquine	None
SLE 19	Prednisolone	Hydroxychloroquine	Mycophenolate motefil
SLE 20	Prednisolone	None	Mycophenolate
SLE 21	Corticosteroid inhaler	Hydroxychloroquine	None
SLE 23	None	Hydroxychloroquine	None
SLE 24	None	None	None
SLE 25	None	Hydroxychloroquine	Mycophenolate
SLE 28	None	None	None
SLE 30	NA	NA	NA
SLE 31	NA	NA	NA
SLE 32	Prednisolone	None	Mycophenolic acid

'NA' indicates data not available. 'None' indicates type of medication was not taken by the patient.

4 iNKT cells activate and induce B cell antibody production but may inhibit antigen presentation.

4.1 Introduction

4.1.1 Current literature on iNKT cell interactions with B cells

4.1.1.1 In vitro studies

The first and only definitive study demonstrating that human iNKT cells can directly prime autologous B cells to proliferate and secrete antibodies in a CD1d-restricted manner was done by Galli et al in 2003 (Galli et al., 2003). In that study the authors suggest that $CD4^+$ and DN iNKT cells were similar in their ability to induce proliferation of naïve and $CD27^+$ memory B cells, but $CD4^+$ iNKT cells were more efficient than DN iNKT cells at inducing IgM and IgG_1 production by the B cells. The effect mediated by iNKT cells was dependent on CD1d but not α-GC, prompting the authors to suggest that the iNKT cells may recognise endogenous ligands presented by CD1d on the B cells. Subsequently, Rossignol et al showed that $CD4^+$ T cells, but not DN T cells, enhanced the production of IgG and IgE in response to α-GC-loaded B cells and also increased the percentage of $CD27^+$ B cells (presumably memory B cells) (Rossignol et al., 2007). However, there are several drawbacks in their study, such as the confounding presence of conventional T cells in the experiments and the use of the Vα24 marker solely to demarcate iNKT cells, which is not optimal as there are also non-iNKT cells (non-CD1d-restricted) that can also express Vα24 (Metelitsa, 2004).

4.1.1.2 In vivo studies

There has been a recent surge of studies looking at iNKT-B cell interactions in mice, with a particular interest in the potential of iNKT cells to regulate, enhance and sustain humoral immune responses. Following their in vitro work with human iNKT cells, Galli et al demonstrated that simultaneous activation of murine iNKT cells by α-GC with injection of exogenous protein antigens elicited higher antibody titers in response to the particular antigen, but did not polarise the immune response and instead induced production of both Th1-type (IgG_2) and Th2-type (IgG_1) antibody production from the B cells (Galli et al., 2007). These mice which were immunised with antigen and α-GC were subsequently challenged with the pathogen they were immunised against and they

exhibited higher survival rates than mice that were immunised with antigen alone, demonstrating the adjuvant effect of activating iNKT cells in vaccination. In an experimental asthma murine model, production of allergen-specific IgE was found to be abrogated in iNKT-deficient mice and when CD1d was blocked with anti-CD1d blocking antibodies (Lisbonne et al., 2005), providing evidence that iNKT cells are responsible for eliciting IgE production by B cells in the murine model. CD1d expression on murine B cells was found to be absolutely essential for iNKT-enhanced antibody production against T-dependent antigens as B-cell-deficient μMT mice that were reconstituted with $CD1d^{-/-}$ B cells produced significantly lower antibody titers than wild-type mice when administered with exogenous antigen and α-GC (Lang, Devera, and Lang, 2008). The importance of CD1d in the adjuvant effect of iNKT cells on antibody production suggests that direct contact between iNKT cells and B cells is necessary for iNKT-enhanced antibody production.

Several other studies have also found iNKT cells to be vital for a protective humoral response against parasites, bacteria and viruses. In mice inoculated with circumsporozoite proteins from the malaria parasite, the iNKT-B cell interaction was vital for mounting reactive IgG responses (Schofield et al., 1999). $CD1d^{-/-}$ mice infected with the *Borrelia* spirochete suffered from impaired production of specific antibodies against the bacterial pathogens and exhibited high bacterial load in comparison to wild-type controls expressing CD1d, due to the inability to clear the pathogen effectively (Belperron, Dailey, and Bockenstedt, 2005; Kumar et al., 2000). Intranasal co-administration of the iNKT cell activating ligand, α-GC together with influenza hemagglutinin antigen (HA) was shown to have an adjuvant effect on the HA-specific antibody response and hence confer protection from influenza viral infection, suggesting that iNKT cell activation may be instrumental to the success of mucosal vaccines (Ko et al., 2005).

The adjuvant effect of α-GC on antigen-specific antibody production was further confirmed by Lang et al (Lang, Exley, and Lang, 2006), who found that in addition to IgM and IgG_3, there was also selective induction of antigen-specific IgG_1, implying that the iNKT-B cell interaction can be harnessed to produce a desired subclass of antibodies (Lang, 2009). They also reported a dichotomy in antibody isotype production depending on the nature of the antigen co-administered with α-GC, with IgG_{2b} being preferentially induced by T-dependent antigens and IgG_1 being preferentially induced by T-independent antigens. In comparison to TLR ligands and conventional adjuvants like alum, the adjuvant effect of activating iNKT cells with α-GC was found to be equally

effective at enhancing primary and memory antibody responses through the induction of long-lived antibody-secreting plasma cells (Devera et al., 2008). This effect was again shown to be CD1d-dependent as $CD1d^{-/-}$ mice displayed reduced induction of plasma cells in recall responses to immunized antigen. Apart from the requirement for CD1d, iNKT help to B cells requires costimulation via CD40L (or CD154) and CD80/86 (Lang, Devera, and Lang, 2008; Leadbetter et al., 2008). However, current reports are conflicting as to whether IL-4 is necessary for the interaction and reciprocal activation of iNKT and B cells (Kitamura et al., 2000; Leadbetter et al., 2008).

4.2 Aims and hypothesis

The overall aim of this chapter was to characterise the outcomes and requirements of human iNKT-B cell interactions *in vitro*. Based on current literature, we hypothesized that human iNKT cells would activate B cells in co-culture and result in B cell maturation and antibody production. The activation of B cells by iNKT cells could also result in an enhancement of their antigen-presenting function as activation is a requirement for antigen-presentation (Kakiuchi et al., 1983). In addition, the role of the iNKT ligand α-GC in iNKT help to B cells was also investigated as results from the Galli study are in discord with the murine studies with regards to the requirement for α-GC.

To test the hypothesis, iNKT cells from the peripheral blood mononuclear cells (PBMCs) of healthy donors were expanded and co-cultured with allogeneic or autologous B cells. Changes in expression of co-stimulatory and activation markers on the B cells were examined by flow cytometry and cytokine and immunoglobulin production were analysed by multiplex cytometric bead arrays. Allogeneic and autologous co-cultures were compared to determine if alloreactivity contributes to the outcome of iNKT-B cell interactions.

To investigate if the observed iNKT cell-induced B cell activation also enhanced antigen-presentation by B cells, co-cultures of iNKT cells and B cells were incubated with autologous and allogeneic naïve T cells in the presence of various antigens.

As several reports have found that the iNKT-B cell interaction is CD1d-dependent, we examined whether cell-cell contact is required for iNKT-induced B cell activation. iNKT cells were co-cultured with B cells in a 96-well plate with a transwell insert containing a membrane to separate the cells from each other.

4.3 Results

4.3.1 Generation of expanded iNKT cell lines

4.3.1.1 Phenotype of iNKT cell lines expanded by anti-CD3 mAb and IL-2 stimulation.

Due to the low frequency of iNKT cells in human peripheral blood (0.01 – 1%) (Eger et al., 2006), iNKT cells enriched from PBMC by magnetic bead separation were expanded *in vitro* by stimulation with anti-CD3 mAb and cultured with IL-2. Flow cytometric analysis was performed on the PBMC before separation, enriched iNKT cells after separation and expanded iNKT lines after 4 weeks of culture to assess the frequency of iNKT cells. iNKT cell frequency was enhanced approximately 750 – 1000 fold after magnetic bead separation, and purity of iNKT lines ($6B11^+CD3^+$) was 87.2 ± 10.4% (mean ± SD, n=6) (**Figure 4.3.1**). Yields of iNKT cells were highly variable, culminating in 50 – 500 fold expansion in comparison to starting cell numbers. Cells remained viable for up to 7 weeks when maintained in close contact with each other in 96-well plates. As non-CD1d-restricted $V\alpha24^+$ T cells have previously been found to be picked up by anti-CD3/6B11 staining (unpublished data), we compared the use of 6B11/CD3 with the canonical Vα24/Vβ11 staining (Godfrey et al., 2000) and 6B11/Vβ11 staining (Hou et al., 2003). It was observed that all three methods were comparable for the enumeration of iNKT purity (**Figure 4.3.1**B). iNKT lines were also phenotyped for expression of CD4 and CD8 to elucidate proportions of $CD4^+$, double negative (DN) and $CD8^+$ iNKT cells. The percentages of iNKT cell lines that expressed $CD4^+$, DN and $CD8^+$ phenotypes were 20.1 ± 21.2%, 67.7 ± 25.6% and 10.5 ± 8.2% (**Figure 4.3.1**). Hence it appears that the DN subset is the most predominant population in the iNKT lines, followed by the $CD4^+$ subset and the $CD8^+$ subset (**Table 4.3.1**).

Table 4.3.1. Purity and phenotype of iNKT lines from 6 healthy donors, expanded using anti-CD3 mAb/IL-2 stimulation.

iNKT lines	$6B11^+CD3^+$	$CD4^+$	DN	$CD8^+$
G161209	97.38	8.64	88.17	2.71
G060110.2	98.18	18.62	67.34	11.2
G210110.1	81.01	13.09	76.82	7.04
G210110.2	78.09	5.69	83.96	10.06
G270110.1	71.43	29.31	40.53	28.11
G270110.2	90.91	1.8	92.05	5.96
G030210.2	93.52	63.51	24.76	8.66

Data show percentages of $6B11^+CD3^+$ T lymphocytes (2nd column) and percentages of $6B11^+CD3^+$ cells expressing $CD4^+$, $CD8^+$ and DN phenotypes.

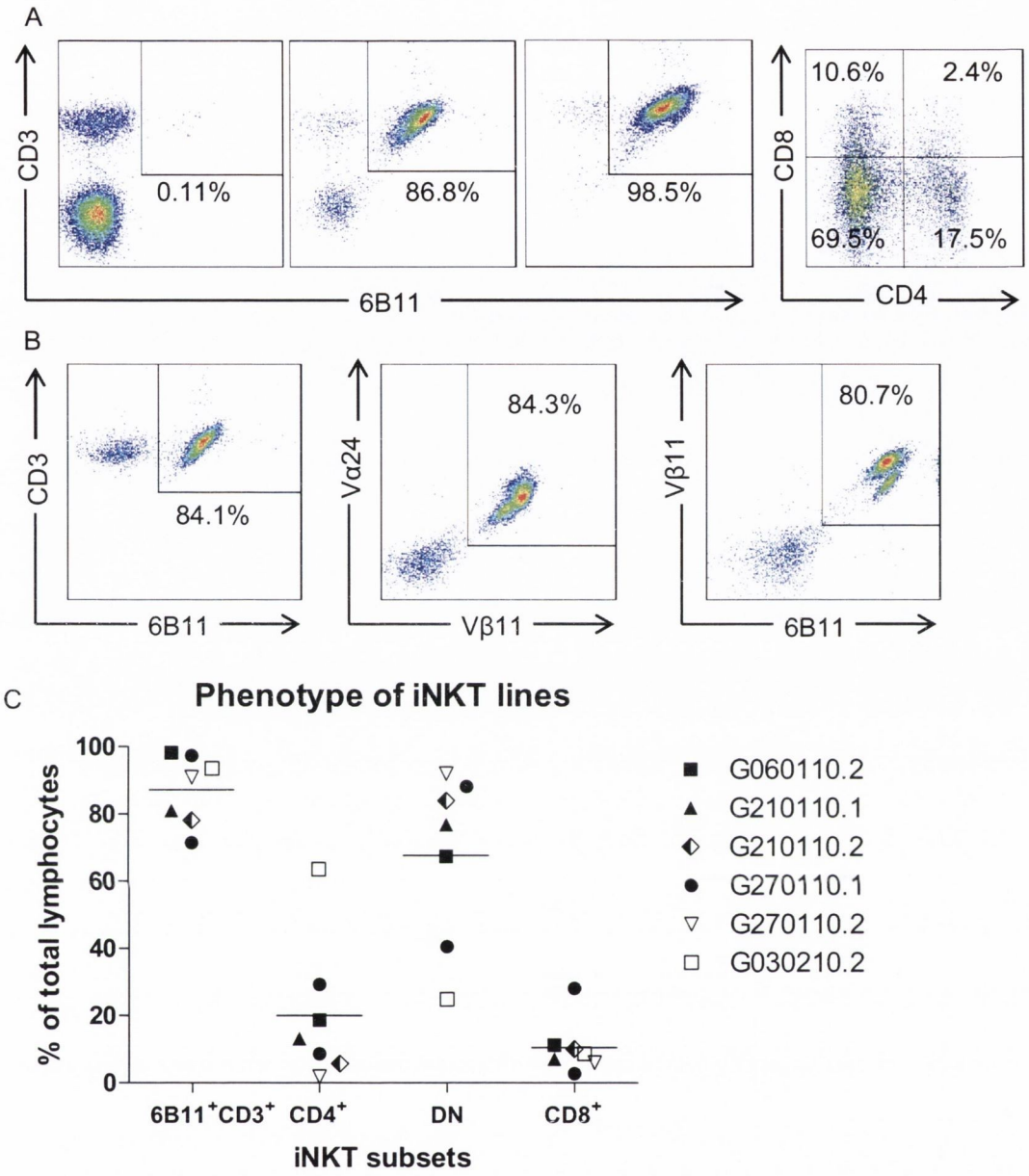

Figure 4.3.1. Flow cytometric assessment of iNKT cell frequencies and phenotype in iNKT lines cultured by anti-CD3 mAb and IL-2 stimulation.

(A) PBMC before magnetic bead separation (left), after separation (second from left), and after four weeks in culture with anti-CD3 and IL-2 stimulation (second from right). Cells were stained with antibodies against the iNKT receptor CDR3 loop (6B11) and CD3. Expanded iNKT cells are polyclonal, expressing $CD4^+$, $CD8^+$ or DN phenotypes (right). Flow cytometry plots shown are representative of 6 different iNKT lines set up from healthy donors.

(B) 6B11/CD3 staining is comparable with Vα24/Vβ11 and Vβ11/6B11 staining for enumeration of iNKT purity. iNKT lines were stained with antibodies against 6B11, CD3, Vα24 and Vβ11. Flow cytometry plots shown are representative of 6 different iNKT lines set up from healthy donors.

(C) Percentage of total ($6B11^+CD3^+$), $CD4^+$, DN and $CD8^+$ iNKT cell subsets in 6 iNKT lines. iNKT lines were generated as described above; expression of CD4 and CD8 by $6B11^+CD3^+$ cells was analysed by flow cytometry. Purity of iNKT is 87.2 ± 10.4% (mean ± sd). Percentages of $CD4^+$, DN and $CD8^+$ subsets are 20.1 ± 21.2%, 67.7 ± 25.6% and 10.5 ± 8.2% respectively.

4.3.1.2 Phenotype of iNKT cell lines expanded by PHA and irradiated feeder cells.

iNKT cells were enriched from PBMC by magnetic bead separation, then purified further by cell sorting to obtain purities of >99% $V\alpha24^+V\beta11^+$ iNKT cells. The purified iNKT cells were then cultured *in vitro* with irradiated allogeneic feeder cells, PHA and IL-2 stimulation. Flow cytometric analysis was performed on the PBMC before separation, purified fraction after sorting and iNKT lines after 3 weeks of culture to assess the frequency of iNKT cells. (**Figure 4.3.2**A and B)

iNKT frequencies were enhanced approximately 4000-fold by sorting, and the purity of iNKT lines ($6B11^+CD3^+$) was 99.8 ± 0.22% (mean ± SD, n=7). Yields of iNKT cells were typically higher when expanded with PHA, feeders and IL-2 than with anti-CD3 mAb/IL-2 stimulation, culminating in 1000 – 2000 fold expansion in comparison to starting cell numbers. Cells remained viable for up to 12 weeks when maintained in close contact with each other in 96-well plates. iNKT lines were also phenotyped for expression of CD4 and CD8 to elucidate proportions of $CD4^+$, DN and $CD8^+$ iNKT cells. The mean percentage of cells expressing $CD4^+$, DN and $CD8^+$ phenotypes are 33 ± 26.6%, 51.3 ± 21.1% and 14 ± 8.71% (**Figure 4.3.1**C). Hence it appears that similar to the anti-CD3 and IL-2 stimulation method, the DN subset is the most predominant population in the iNKT lines, followed by the $CD4^+$ subset and the $CD8^+$ subset.

Table 4.3.2. Purity and phenotype of iNKT lines from 7 healthy donors, expanded using irradiated allogeneic feeders, PHA and IL-2 stimulation.

iNKT subsets	$6B11^+CD3^+$	$CD4^+$	DN	$CD8^+$
G1	99.6	3.18	83.5	11.8
G2	99.4	24.2	60	15.7
SS	99.7	4.89	63.5	30.5
VR	99.9	73.2	21.7	4.72
G3	100	26.3	58.2	11.5
G4	100	37.9	41	18.2
G5	99.8	61.4	31.5	5.93

Data show percentages of $6B11^+CD3^+$ T lymphocytes (2nd column) and percentages of $6B11^+CD3^+$ cells expressing $CD4^+$, $CD8^+$ and DN phenotypes.

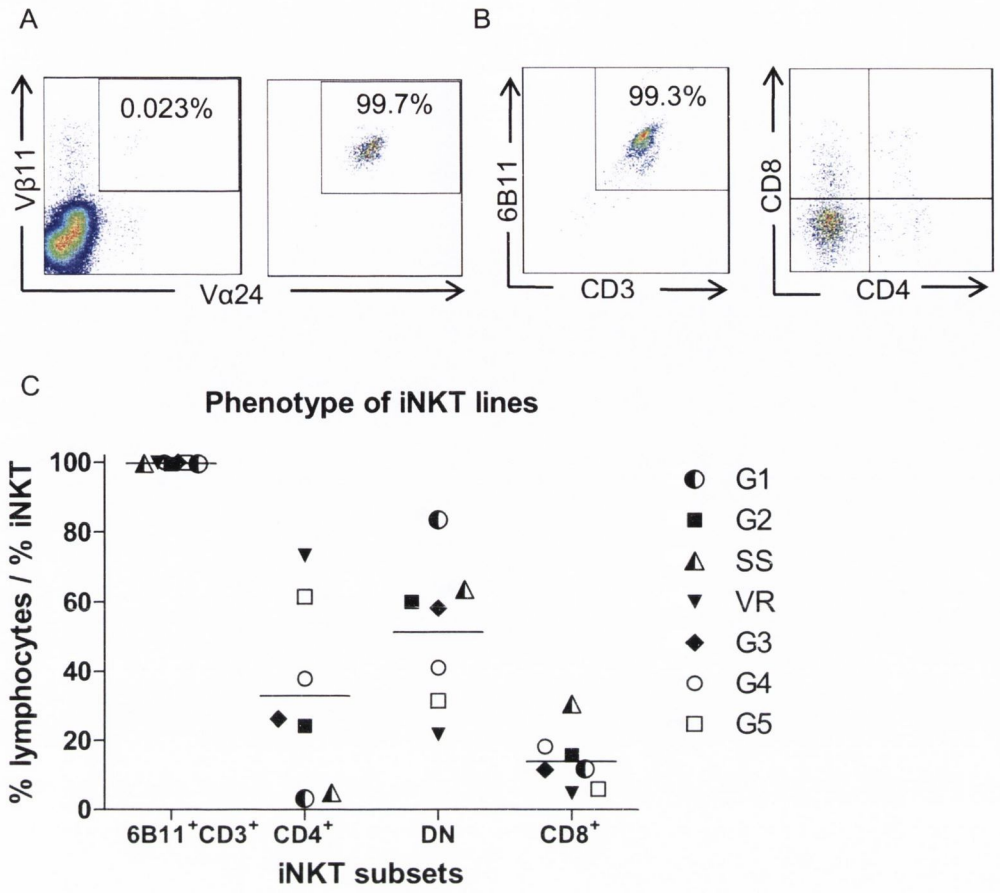

Figure 4.3.2. Flow cytometric assessment of iNKT cell frequencies and phenotypes in iNKT lines cultured by stimulation with feeders, PHA and IL-2.

(A) PBMC before magnetic bead separation (left), purified iNKT cells after separation and cell sorting (right). Cells were stained with antibodies against the iNKT receptor Vα24 and Vβ11.

(B) iNKT lines after 3 weeks of culture with irradiated allogeneic feeders, PHA and IL-2 (left). Expanded iNKT lines were heterogenous in CD4 and CD8 expression (right). Cells were stained with antibodies against the iNKT receptor CDR3 loop (6B11) and CD3. Expanded iNKT cells are polyclonal, expressing $CD4^+$, $CD8^+$ or DN phenotypes. Flow cytometry plots shown in (A) and (B) are representative of 7 different iNKT lines set up from healthy donors.

(C) Percentage of $CD4^+$, DN and $CD8^+$ iNKT cell subsets in 7 iNKT lines. iNKT lines were generated as described above; expression of CD4 and CD8 by $6B11^+CD3^+$ cells was analysed by flow cytometry. Purity of iNKT ($6B11^+CD3^+$) is 99.8 ± 0.22% (mean ± sd). Percentages of $CD4^+$, DN and $CD8^+$ iNKT subsets are 33 ± 26.6%, 51.3 ± 21.1% and 14 ± 8.71% respectively.

4.3.1.3 Expanded iNKT lines release Th1 and Th2 cytokines in response to CD1d and α-GC.

iNKT cells in both mice and human have been reported to be able to produce both Th1 and Th2 cytokines (Bendelac et al., 1997; Spada, Koezuka, and Porcelli, 1998). To test if the expanded iNKT lines function similarly, the iNKT cells were co-cultured with equal numbers of CD1d-transfected Hela cells in the absence or presence of α-GC for 3 days. The CD1d-transfected Hela cells express CD1d exogenously on the cell surface and are able to present α-GC to the iNKT cells (Chang et al., 2007). As negative controls, the iNKT cells were also co-cultured with equal numbers of mock-transfected Hela cells (transfected with empty vector) in the absence or presence of α-GC for the same duration. Supernatants were analysed by ELISA for IFN-γ, IL-4, IL-10 and IL-13 production.

There was significant production of IFN-γ, IL-4 and IL-13 both in the absence ($p<0.01$ for IFN-γ and IL-4, $p<0.05$ for IL-13) and presence of α-GC ($p<0.01$ for all). IL-10 production was not significantly enhanced in the co-cultures. Interestingly, IL-4 and IL-13 production, but not IFN-γ production, were significantly enhanced in the presence of α-GC in comparison to co-cultures in the absence of α-GC ($p<0.01$ for IL-4 and IL-13). (**Figure 4.3.3**)

These results suggest that the expanded iNKT cells were fully functional and able to induce production of both Th1 and Th2 cytokines. The iNKT-induced IL-4 and IL-13 production was enhanced by α-GC, indicating that the expanded iNKT cells are responsive to α-GC.

4.3.2 iNKT cells induce B cell activation and immunoglobulin production.

4.3.2.1 CD1d is uniformly expressed across all B cell subsets.

To determine if all B cell subsets expressed CD1d uniformly and hence have similar potential to present glycolipid antigens to iNKT cells, PBMC were obtained from seven healthy donors and analysed for CD1d expression on different B cell subsets by flow cytometry. Samples were stained with APC-conjugated anti-CD19 mAb and PE-conjugated anti-CD1d mAb, and one of the following: FITC-conjugated anti-CD5 mAb,

anti-CD22 mAb, anti-CD38 mAb or anti-CD27 mAb, which corresponds with B1, mature, plasma and memory B cells respectively. CD1d expression was compared between the samples for each B cell subset and the corresponding FMO controls, which were not stained with PE-conjugated anti-CD1d mAb. (**Figure 4.3.4**A)

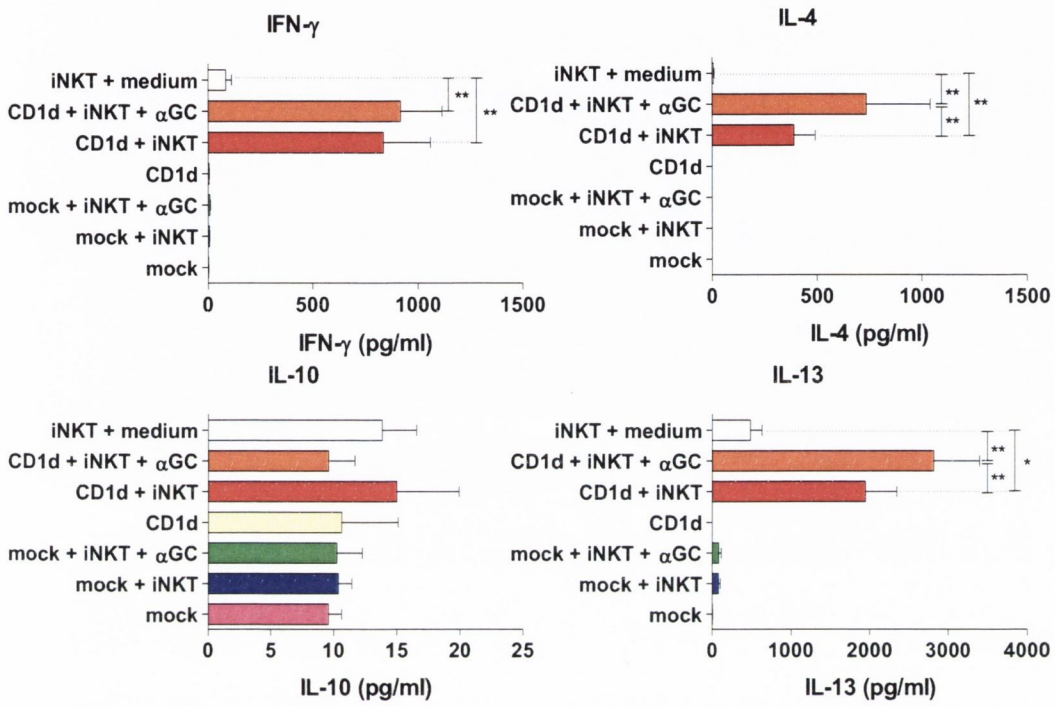

Figure 4.3.3. Expanded iNKT lines are able to induce both Th1 and Th2 cytokines and respond to α-GC. The expanded iNKT cells were co-cultured with CD1d-transfected Hela cells ("CD1d") in the presence or absence of α-GC for 3 days. As negative controls, the iNKT cells were also co-cultured with mock-transfected Hela cells ("mock") in the presence or absence of α-GC for 3 days. Supernatants from the co-cultures were analysed by ELISA for IFN-γ, IL-4, IL-10 and IL-13 production. Bars represent mean ± SEM from 4 independent experiments. Statistical analysis was performed using the Wilcoxon matched pairs test, *$p<0.05$, and **$p<0.01$.

On average, between 75% - 85% of each B cell subset expresses CD1d on the surface, and the CD1d expression on each B cell subset was relatively uniform between subsets. Plasma cells ($CD38^+CD19^+$) appeared to have the highest proportion of CD1d expression. (**Figure 4.3.4**C) In contrast, the mean fluorescence intensity of CD1d expression on mature ($CD22^+CD19^+$), plasma and memory B cells was slightly higher than that on B1 ($CD5^+CD19^+$) B cells. (**Figure 4.3.4**D) However, there was no significant difference between the mean proportions of CD1d expression, and the mean fluorescence intensity of CD1d expression between all B cell subsets.

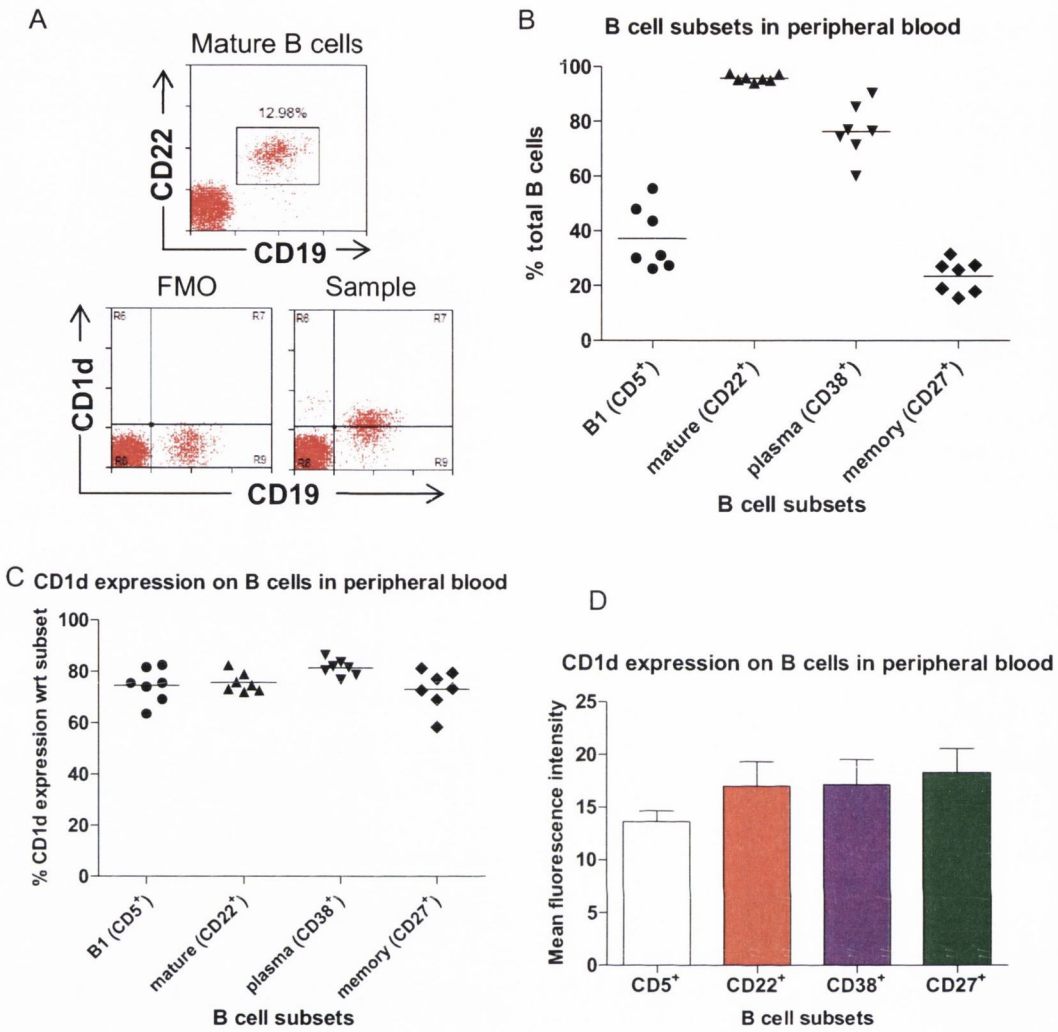

Figure 4.3.4. CD1d is uniformly expressed across all human B cell subsets.
(A) Representative plot generated from flow cytometric analysis of CD1d expression in B cell subsets from peripheral blood. Top: Subset of $CD22^+$ B cells; Bottom left: FMO control lacking anti-CD1d PE mAb; bottom right: Sample containing anti-CD1d PE mAb.
(B) Proportions of B cell subsets in peripheral blood.
(C) Cell surface CD1d expression by different B cell subsets in human peripheral blood. Horizontal lines represent the arithmetic mean from seven donors.
(D) Mean fluorescence intensity of CD1d expression on B cell subsets. Error bars represent mean ± SEM. Statistical analysis was performed on (C) and (D) using Friedman's test with post-hoc Dunn's multiple comparison test and there was no observed significant difference in percentage and MFI of CD1d expression between each subset.

4.3.3 Interactions between B cells and allogeneic iNKT cells *in vitro*.

4.3.3.1 iNKT interaction with B cells causes upregulation of activation and co-stimulatory markers on the B cells.

B cells and iNKT cells were sorted from PBMC from healthy donors by magnetic bead separation (**Figure 4.3.5**). iNKT cells were further expanded by anti-CD3 mAb and IL-2 stimulation to obtain sufficient numbers for experiments. To assess whether iNKT cells interact with B cells and cause quantifiable morphological changes on their cell surfaces, iNKT and B cells were co-cultured in equal numbers for 3 to 10 days. B cells were also cultured in medium only as a negative control and stimulated with PMA and ionomycin (PMA/ionomycin) as a positive control for the same duration. The B cells were also co-cultured with non-iNKT cells (total PBMC lacking iNKT cells, expanded by anti-CD3 mAb and IL-2 stimulation) to account for changes in surface marker expression due to possible alloreactivity. Changes in the surface markers CD40, CD58, CD86, CD69, CD83, CD95 and CD80 expression by B cells were analysed by flow cytometry.

We observed considerably increased expression of CD40 ($p<0.05$), CD69, CD83, CD95 ($p<0.01$) and CD86 ($p<0.05$) on B cells relative to negative control (medium only) within 3 days of co-incubation with iNKT cells (**Figure 4.3.6**). After 10 days of co-incubation with iNKT cells, expression of CD40 and CD83 were still increased. The expression of CD58 and CD80 on B cells were not significantly increased at both 3 days and 10 days relative to negative control.

With non-iNKT cells, we observed increased expression of CD83, CD86 and CD95 on the B cells after 3 days of co-culture (**Figure 4.3.6**). Upregulation of B cell activation and co-stimulatory markers appear to be more pronounced when co-cultured with iNKT cells than with non-iNKT cells ($p<0.05$ for CD83 at 10 days).

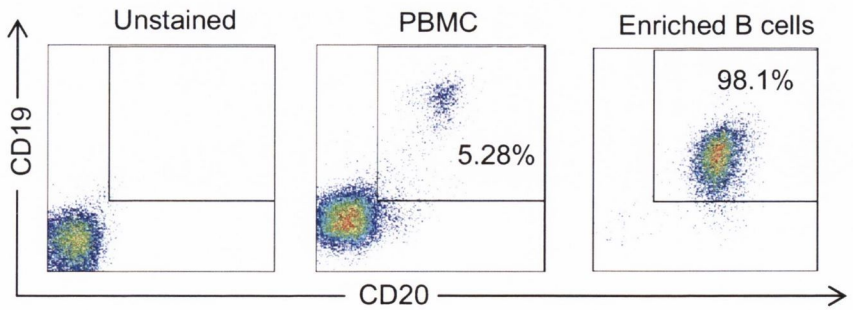

Figure 4.3.5. Magnetic bead separation of B cells from PBMCs. Flow cytometric analysis of B cell ($CD19^+CD20^+$) frequencies before separation (middle) and after separation (right). Unstained PBMCs were used as a negative control for setting the $CD19^+CD20^+$ gate.

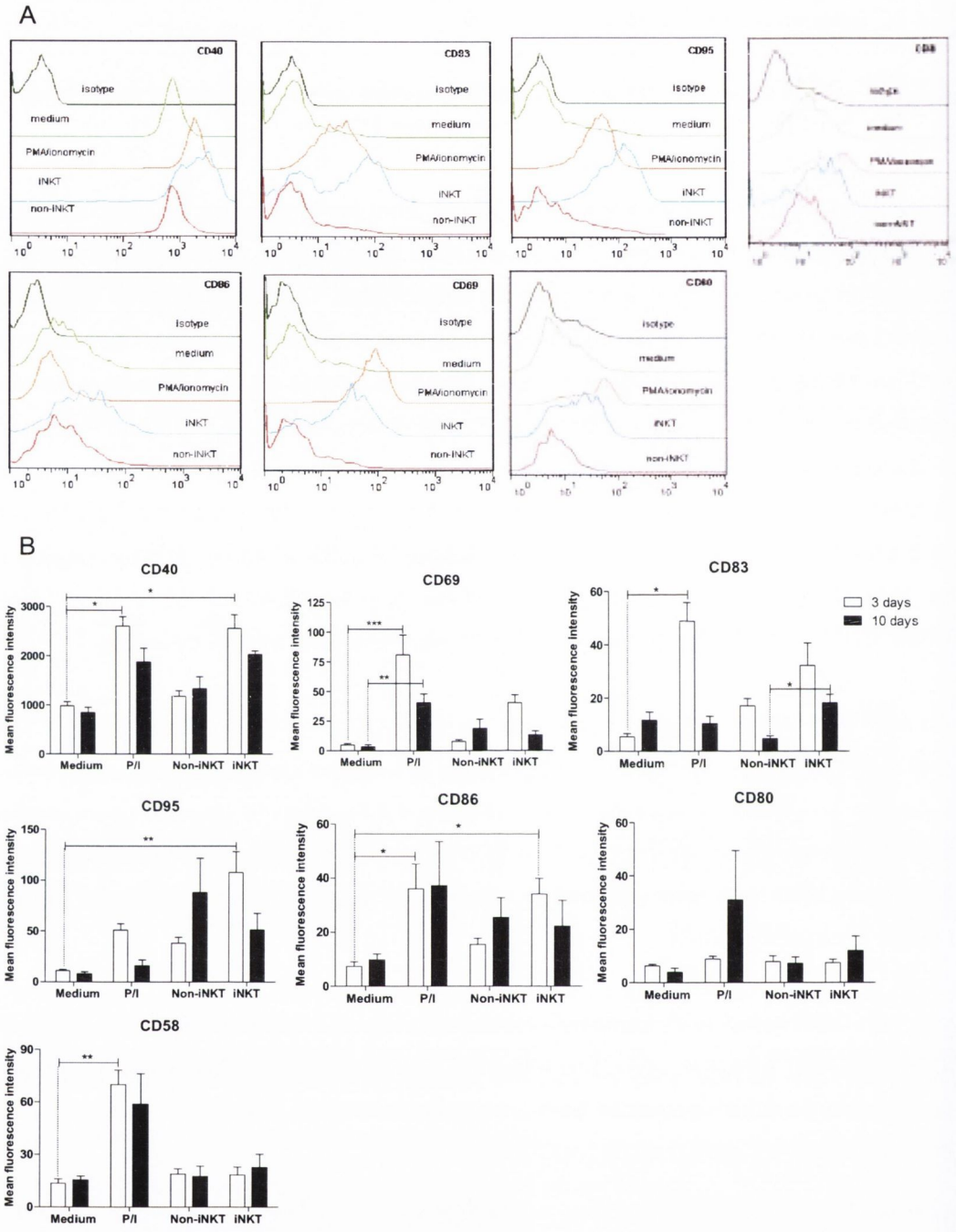

Figure 4.3.6. iNKT interaction with B cells causes upregulation of activation and co-stimulatory markers on the B cells. Changes observed in mean fluorescence intensity (MFI) of surface expression markers on B cells after 3 or 10 days of co-incubation with iNKT or non-iNKT cells in the absence of α-GC, as measured by flow cytometry. Upregulation of CD40, CD69, CD83, CD86 and CD95 was observed after 3 days, upregulation of CD58 and CD80 were observed after 10 days. (A) Offset histogram overlays of marker expression for isotype, medium, PMA/ionomycin, co-culture with iNKT cells and co-culture with non-iNKT cells for 3 days. (B) Changes in MFI for B cells that were incubated with medium only (negative control), P/I: PMA and ionomycin (positive control), non-iNKT cells and iNKT cells. N=4 for 3 days, N=3 for 10 days. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test. *, ** and *** represent $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

4.3.3.2 iNKT cell interactions with B cells induce the production of IgG, IgA, and IgM, but not IgE.

Supernatants from the co-culture of B cells with allogeneic iNKT or non-iNKT cells were assayed for immunoglobulin production by multiplex cytometric bead arrays. Relative to negative controls, there was increased production of total IgG, IgM and IgA after 10 days of B cell co-culture with iNKT cells (**Figure 4.3.7**). The amount of antibody produced in iNKT-B cell co-cultures appears to be greater than that produced in B cell-non-iNKT co-cultures. No IgE was detected in any of the stimulations or co-cultures (data not shown). No visible difference in antibody production between negative controls and iNKT-B cell or non-iNKT-B cell co-culture (except for total IgG production) was observed when cells were co-cultured for 3 days, suggesting that 10 days was optimal for observing differences in antibody production.

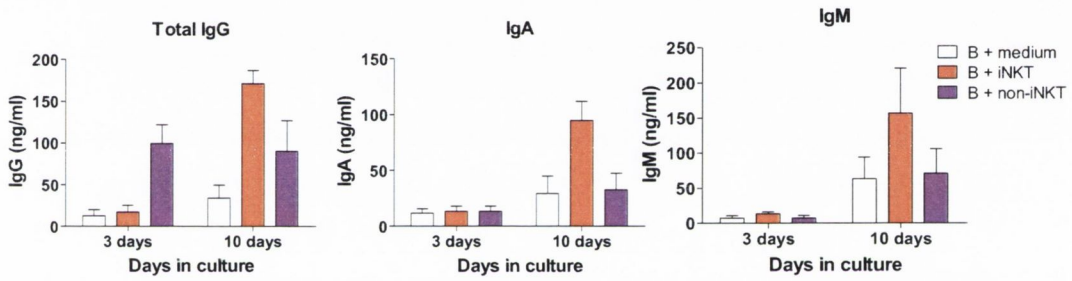

Figure 4.3.7. Production of IgM, IgA and IgG was observed in co-cultures of iNKT with allogeneic B cells after 10 days. Supernatants from 10-day co-cultures of B cells with allogeneic iNKT cells or allogeneic non-iNKT cells were analysed by multiplex cytometric bead arrays for total IgG, IgA and IgM. Bars represent mean ± SEM from 3 independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test. Results were not statistically significant.

Supernatants from 3 day co-cultures of B cells with allogeneic iNKT or non-iNKT cells were also assayed for cytokine production (IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-12p70 and IL-13) by multiplex cytometric bead arrays. The expanded iNKT cell lines produced copious amounts of IL-5 and IL-13 prior to co-culture. However, co-culture of iNKT cells with allogeneic B cells resulted only in increased production of IL-6 ($p<0.05$) and minimal changes in production of other cytokines (Appendix 9.1.1).

4.3.4 Interactions between B cells and autologous iNKT cells in vitro.

4.3.4.1 Autologous iNKT cells induce upregulation of CD40 and CD86 expression on total B cells.

B cells and iNKT cells were sorted from PBMC from healthy donors by magnetic bead separation. iNKT cells were further expanded by stimulation with irradiated allogeneic feeder cells, PHA and IL-2 to obtain sufficient numbers for experiments. To assess whether iNKT cells interact with B cells similarly in an autologous system, autologous iNKT and B cells were co-cultured in equal numbers for 3 to 10 days. B cells were also cultured in medium only as a negative control for the same duration. The B cells were also co-cultured with autologous non-iNKT cells (T cells expanded by PHA, IL-2 and irradiated feeder cells) to compare against the effect of iNKT help. Changes in the expression of surface markers CD40 and CD86 by B cells were analysed by flow cytometry.

Upregulation of CD40 was observed with iNKT help (at both 3 and 10 days), and was enhanced with the addition of α-GC. CD86 expression was unchanged with iNKT help at 3 days but upregulated after 10 days, and further upregulation was observed when α-GC was added. The effect of α-GC on causing upregulation was more pronounced at 10 days ($p<0.05$ for CD40) compared to 3 days. Non-iNKT help resulted in higher expression of CD40 and CD86 than iNKT help (in the absence of α-GC), and was comparable to iNKT help in the presence of α-GC. This trend was maintained at both 3 days and 10 days. (**Figure 4.3.8**)

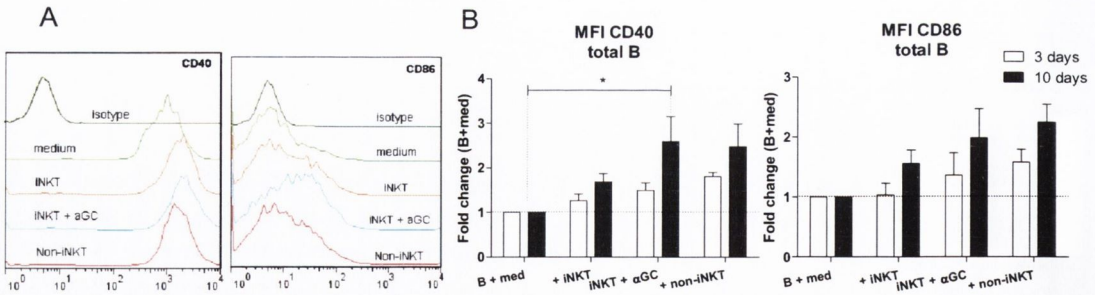

Figure 4.3.8. Autologous iNKT-B cell interaction induced upregulation of CD40 and CD86 on total B cells. iNKT cells were co-cultured with B cells in the absence and presence of α-GC for 3 days and 10 days. Changes in CD40 and CD86 expression on total B cells were analysed by flow cytometry. (A) Offset histogram overlays of marker expression on B cells for isotype, medium, co-culture with iNKT cells ± α-GC and co-culture with non-iNKT cells. (B) Changes in MFI for B cells that were incubated with medium only (negative control), iNKT cells, iNKT cells + α-GC and non-iNKT cells. N=4 for both 3 days (white bars) and 10 days (black bars). Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, *$p<0.05$.

The effect of iNKT cell help on various regulatory B cell (Breg) subsets, memory B cell subsets (unswitched, switched and DN) and naïve B cells were next examined.

4.3.4.2 Enumeration of B cell subsets

The flow cytometry plots below (**Figure 4.3.9**) show how the various B cell subsets were classified in the thesis. As human Breg cells do not yet have a defined phenotype, we looked at three possible Breg subsets: the $CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells. $CD24^{hi}CD38^{hi}$ B cells were previously reported to have regulatory properties (Blair et al., 2010), and $CD1d^{hi}CD5^{+}$ B cells, which are well-defined Breg cells in mice (Yanaba et al., 2008), were found within this subset (Blair et al., 2010). For convenience of naming, these three B cell subsets are denoted as "Breg subsets" in this thesis.

To elucidate memory B cell and naïve B cell subsets, the CD27/IgD classification was used. Based on the four different combinations, $CD27^{-}IgD^{+}$ B cells are classified as naïve B cells, $CD27^{+}IgD^{+}$ B cells are unswitched memory B cells, $CD27^{+}IgD^{-}$ B cells are switched memory B cells, and $CD27^{-}IgD^{-}$ are DN memory B cells. IgM and IgG expression on these B cell subsets were also defined. For the sake of differentiating between DN memory B cells and DN iNKT cells, DN memory B cells are referred to as $CD27^{-}IgD^{-}$ memory B cells in this thesis.

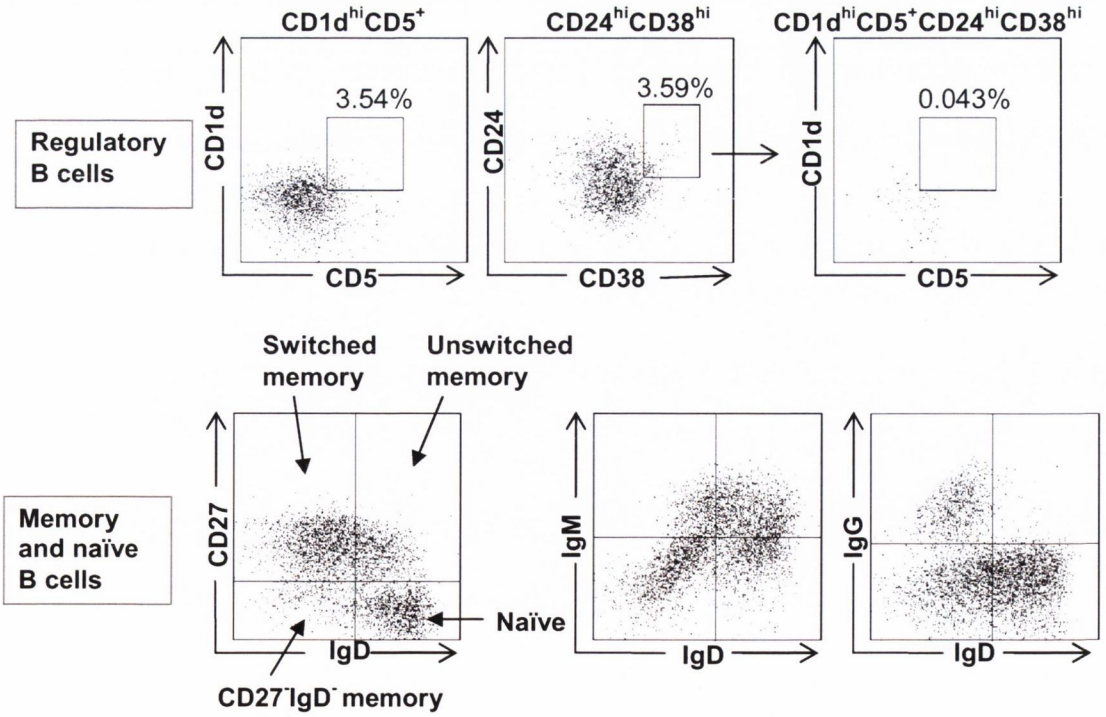

Figure 4.3.9. Enumeration of B cell subsets. Flow cytometry dot plots depicting regulatory B cell subsets (top row), naïve and memory B cell subsets (bottom row).

4.3.4.3 Autologous iNKT cells induce expansion and activation of Breg cells.

Slight increases in the proportions of $CD1d^{hi}CD5^{+}$ B cells, relative to B cells cultured with medium only, occurred after 3 days and 10 days of iNKT help. There was little change in proportions of $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells after 3 days, but a 3-fold increase was observed after 10 days. With the addition of α-GC, the proportions of $CD1d^{hi}CD5^{+}$ B cells remained similar whereas the proportions of $CD24^{hi}CD38^{hi}$ B cells were observed to have increased at 10 days relative to co-cultures where α-GC was absent. Non-iNKT cells were comparably efficient at inducing increases in all three Breg subsets. (**Figure 4.3.10**A)

After 3 days of iNKT help in the absence of α-GC, CD40 expression was slightly downregulated on $CD1d^{hi}CD5^{+}$ B cells but slightly upregulated on $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells. After 10 days, CD40 expression was slightly upregulated for $CD1d^{hi}CD5^{+}$ and $CD24^{hi}CD38^{hi}$ B cells but remained at the same level for $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells. The enhancing effect of α-GC on CD40 expression was most pronounced at 10 days ($p<0.05$ for $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells). Non-iNKT help was comparable to iNKT help for all three B cell subsets at both 3 days and 10 days but slightly inferior to iNKT help in the presence of α-GC at 10 days.

iNKT help had little effect on CD86 expression by the three B cell subsets, although upregulation was observed for $CD24^{hi}CD38^{hi}$ B cells at 10 days in the presence of α-GC. Conversely non-iNKT help caused upregulation of CD86 for $CD1d^{hi}CD5^{+}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells at 3 days, which then decreased back to the same level after 10 days as B cells that were cultured in medium only. Non-iNKT cells were also similarly proficient compared to iNKT cells in upregulating CD86 expression on $CD24^{hi}CD38^{hi}$ Breg cells at 10 days. (**Figure 4.3.10**B)

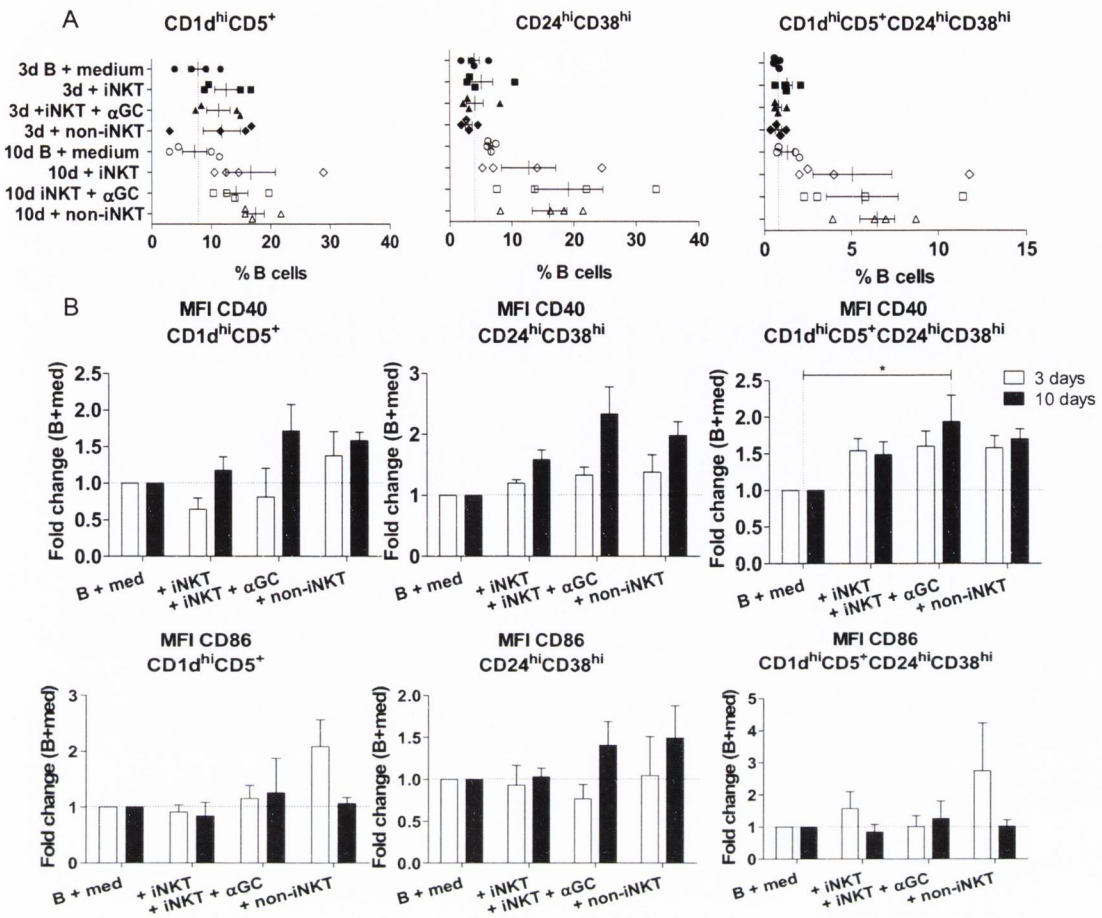

Figure 4.3.10. Breg cells are increased in frequency and activated by iNKT help. (A) Changes in proportion of $CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells when cultured for 3 and 10 days in medium only (negative control), with iNKT cells, with iNKT cells plus α-GC, and with non-iNKT cells. (B) Corresponding fold change in MFI of CD40 and CD86 for the Breg subsets. N=4 for both 3 and 10 days. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, *$p<0.05$.

4.3.4.4 iNKT cells induce expansion and upregulation of CD40 and IgM expression on autologous unswitched memory ($CD27^{+}IgD^{+}$) B cells.

Frequencies of total unswitched memory ($CD27^{+}IgD^{+}$) B cells were unchanged at 3 days but increased at 10 days with iNKT help. At both 3 and 10 days the proportion of IgM^{+} unswitched memory B cells were increased (relative to B cells cultured in medium alone), suggesting that iNKT cells induced the expression of IgM on the unswitched memory B cells. The presence of α-GC did not result in much change in frequency for either total, IgM^{+} or IgM^{-} unswitched memory B cells. Similar results were observed with non-iNKT cells. (**Figure 4.3.11**A)

iNKT cells caused significant upregulation of CD40 expression on total, IgM^+ and IgM^- unswitched memory B cells after 10 days of co-culture in the presence of α-GC ($p<0.05$ for all). The iNKT cells appeared to be more proficient than non-iNKT cells in upregulating CD40 expression on the unswitched memory B cells at 10 days. In contrast, iNKT cells were slightly less efficient than non-iNKT cells in upregulating CD86 expression on all three subsets at both 3 days and 10 days. (**Figure 4.3.11**B)

4.3.4.5 iNKT cells enhance CD40, CD86 and IgG expression on autologous switched memory ($CD27^+IgD^-$) B cells.

Frequencies of total switched memory B cells were unchanged with iNKT and non-iNKT help at both 3 days and 10 days. At 3 days, the proportions of IgM^+ and IgG^+ switched memory B cells were similar when cultured with iNKT cells (both with and without α-GC) relative to B cells cultured in medium alone. At 10 days, iNKT help had little effect on the proportion of IgM^+ switched memory B cells but resulted in an increase in IgG^+ switched memory B cells. Non-iNKT help also resulted in moderate increases in the frequencies of IgM^+ and IgG^+ switched memory B cells as compared to iNKT help (at 10 days only). (**Figure 4.3.12**A)

At 3 days, unremarkable changes in CD40 expression on total switched memory B cells were observed with iNKT help both in the presence or absence of α-GC, while CD86 expression was unchanged with iNKT help. CD40 and CD86 expression on total switched memory B cells were upregulated at 10 days by iNKT help, with the presence of α-GC causing a higher upregulation than iNKT help in the absence of α-GC. Non-iNKT help was as good as iNKT help at upregulating CD40 and CD86 expression on total switched memory B cells at both 3 days and 10 days. Significant upregulation of CD40 but not CD86 expression, on IgM^+ and IgG^+ switched memory B cells was induced by iNKT cells in the presence of α-GC at 10 days. (**Figure 4.3.12**B)

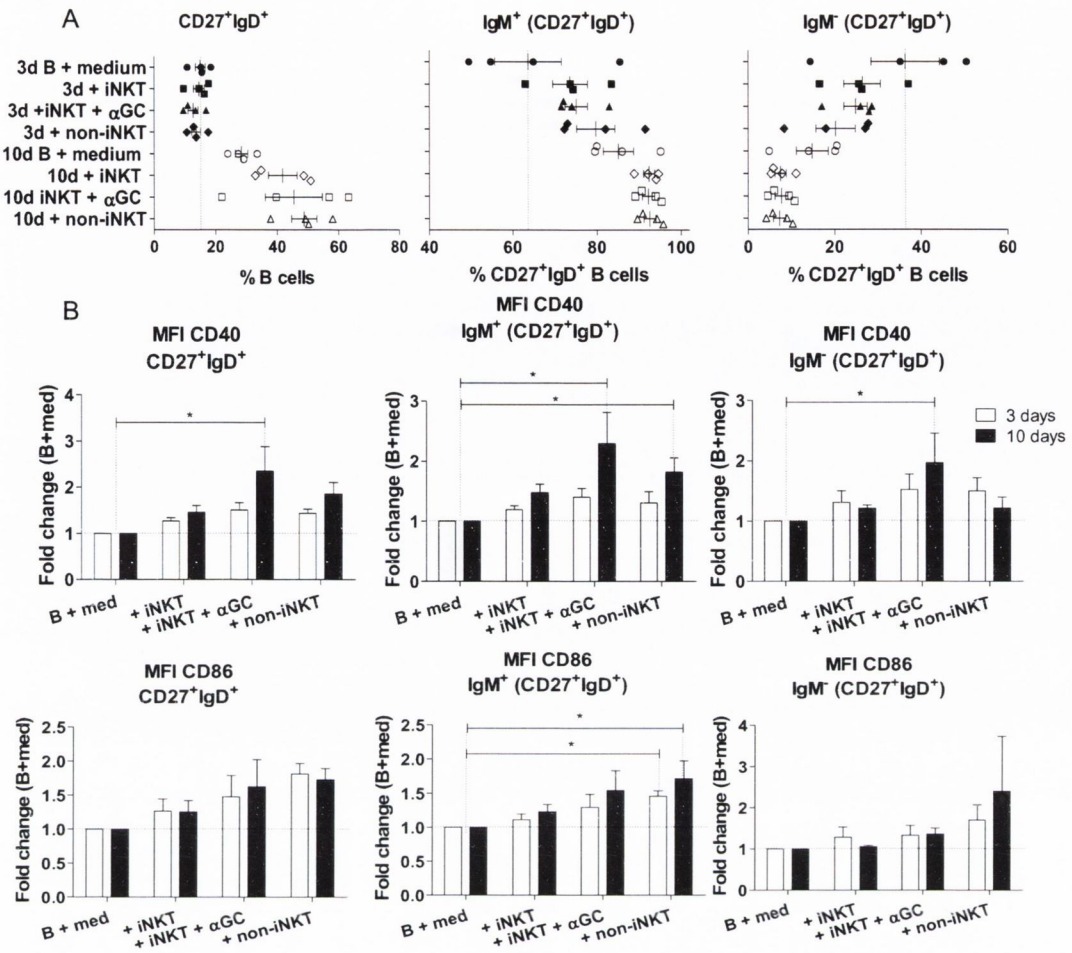

Figure 4.3.11. iNKT cells induce expansion and upregulation of CD40 and IgM expression on autologous unswitched memory B cells. (A) Changes in proportion of total ($CD27^+IgD^+$), IgM^+ and IgM^- unswitched memory B cells when cultured for 3 and 10 days in medium only (negative control), with iNKT cells, with iNKT cells plus α-GC, and with non-iNKT cells. (B) Corresponding fold change in MFI of CD40 and CD86 for the unswitched memory B cell subsets. N=4 for both 3 and 10 days. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, *$p<0.05$.

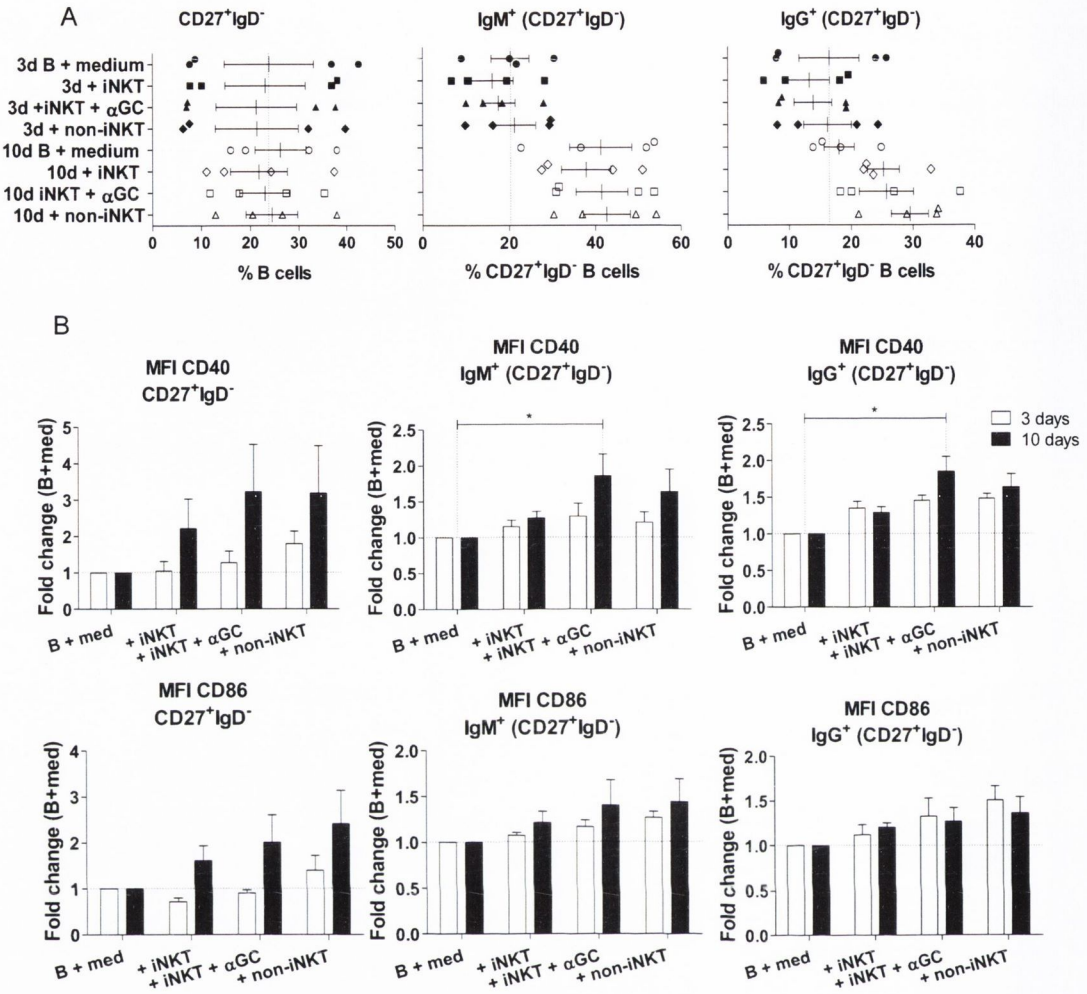

Figure 4.3.12. iNKT cells enhance CD40, CD86 and IgG expression on switched memory B cells. (A) Changes in proportion of total ($CD27^+IgD^-$), IgM^+ and IgG^+ switched memory B cells when cultured for 3 and 10 days in medium only (negative control), with iNKT cells, with iNKT cells plus α-GC, and with non-iNKT cells. (B) Corresponding fold change in MFI of CD40 and CD86 for the switched memory B cell subsets. N=4 for both 3 and 10 days. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, *$p<0.05$.

4.3.4.6 iNKT cells enhance CD40 and IgM expression on autologous naïve ($CD27^-IgD^+$) B cells.

Total naïve ($CD27^-IgD^+$) B cells were slightly increased in frequency at 3 days but decreased after 10 days with iNKT help. This observed decrease at 10 days was more pronounced in the presence of α-GC. IgM^+ naïve B cells progressively increased in proportion from 3 days to 10 days whereas IgM^- naïve B cells progressively decreased

in proportion, suggesting that iNKT help causes an increase in IgM expression on the naïve B cells. The same was observed with non-iNKT help. (**Figure 4.3.13**A)

Both iNKT cells and non-iNKT cells induced upregulation of CD40 and CD86 expression on total, IgM^+ and IgM^- naïve B cells. However this upregulation only reached significance ($p<0.05$) for CD40 expression on IgM^+ naïve B cells after culture for 10 days with iNKT cells and α-GC. Non-iNKT cells were comparably efficient at upregulating CD40 expression as iNKT cells in the presence of α-GC at 3 days but not as potent as the latter at 10 days. However, both iNKT and non-iNKT cells were equivalent in their ability to upregulate CD86 expression on the naïve B cells. (**Figure 4.3.13**B)

4.3.4.7 iNKT cells enhance CD40 expression on autologous $CD27^-$ IgD^- memory B cells.

Total $CD27^-IgD^-$ memory B cells were progressively decreased in frequency from 3 days to 10 days by iNKT help (both in the absence and presence of α-GC). This decrease was also observed with non-iNKT help. In the absence of iNKT or non-iNKT help, the proportion of IgM^+ $CD27^-IgD^-$ memory B cells spontaneously increased with time in culture whereas the proportion of IgG^+ $CD27^-IgD^-$ memory B cells decreased with time. iNKT help (plus α-GC) and non-iNKT help had little further effects on the proportions of total, IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells. (**Figure 4.3.14**A)

iNKT help in the absence of α-GC did not significantly affect CD40 and CD86 expression on total $CD27^-IgD^-$ memory B cells. Addition of α-GC resulted in moderate upregulation of both markers at 10 days. In contrast, non-iNKT help resulted in moderate but progressive upregulation of CD40 and CD86 at both 3 days and 10 days. iNKT cells were comparably proficient at upregulating CD40 expression ($p<0.05$ for IgM^+ at 3 days in the absence of α-GC) on both IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells. Upregulation of CD86 expression on IgM^+ $CD27^-IgD^-$ memory B cells was most efficient with iNKT help in the presence of α-GC, whereas upregulation of CD86 expression on IgG^+ $CD27^-IgD^-$ memory B cells was most efficient with iNKT help in the absence of α-GC. (**Figure 4.3.14**B) However, these observed effects of iNKT cells on CD86 expression by $CD27^-IgD^-$ memory B cells were not statistically significant.

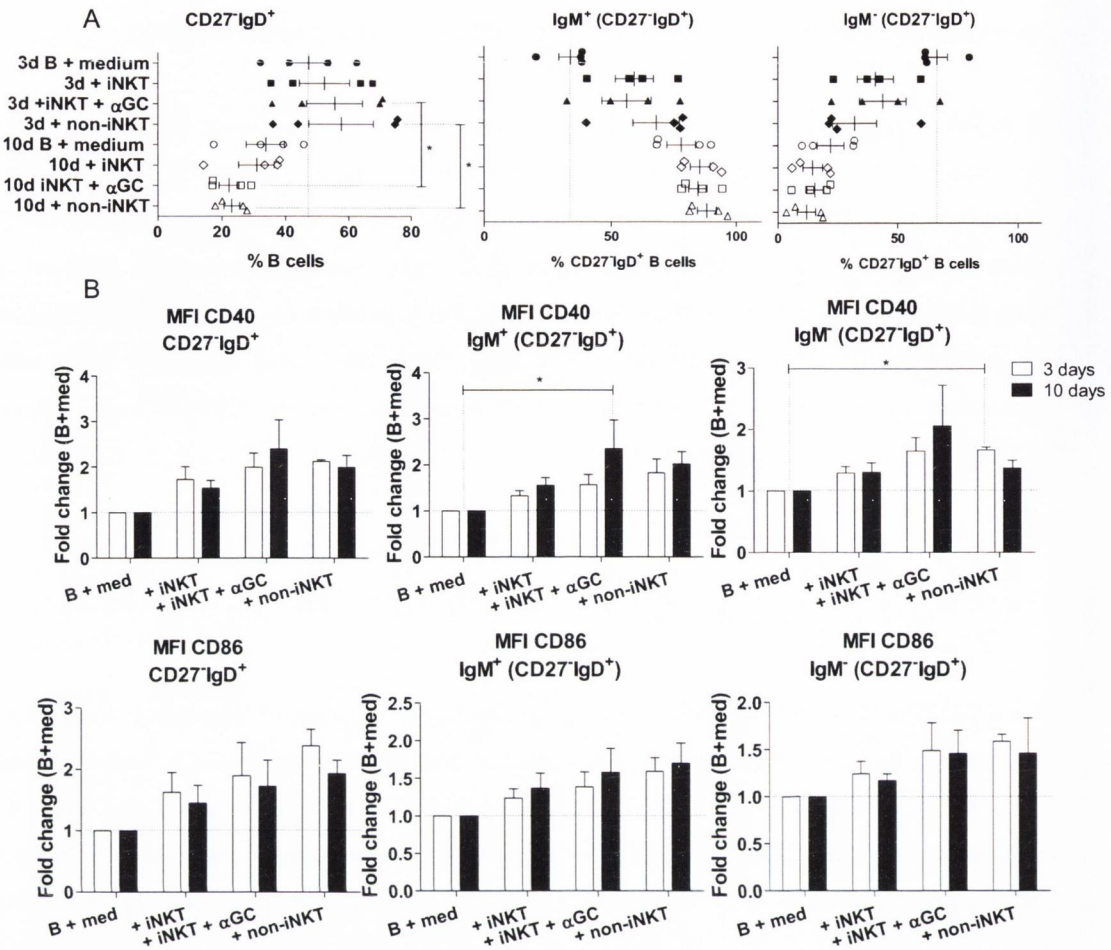

Figure 4.3.13. iNKT cells enhance CD40 and IgM expression on autologous naïve (CD27⁻IgD⁺) B cells. (A) Changes in proportion of total ($CD27^-IgD^+$), IgM^+ and IgM^- naïve B cells when cultured for 3 and 10 days in medium only (negative control), with iNKT cells, with iNKT cells plus α-GC, and with non-iNKT cells. (B) Corresponding fold change in MFI of CD40 and CD86 for the naïve memory B cell subsets. N=4 for both 3 and 10 days. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, *$p<0.05$.

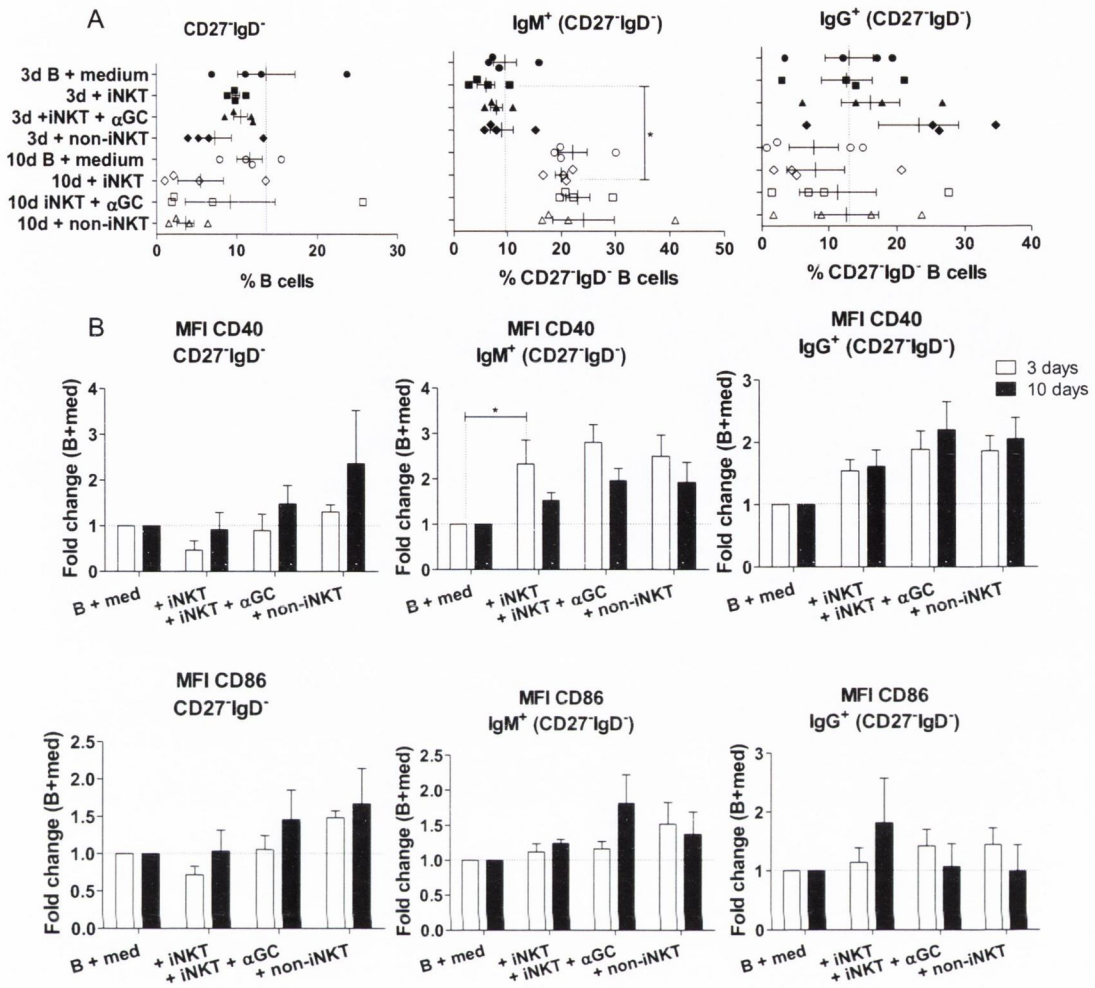

Figure 4.3.14. iNKT cells enhance CD40 expression on autologous CD27⁻IgD⁻ memory B cells. (A) Changes in proportion of total ($CD27^-IgD^-$), IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells when cultured for 3 and 10 days in medium only (negative control), with iNKT cells, with iNKT cells plus α-GC, and with non-iNKT cells. (B) Corresponding fold change in MFI of CD40 and CD86 for the $CD27^-IgD^-$ memory B cell subsets. N=4 for both 3 and 10 days. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, *p<0.05.

4.3.4.8 iNKT cells enhance immunoglobulin production by autologous B cells.

Supernatants from the co-culture of B cells with autologous iNKT or non-iNKT cells were assayed for immunoglobulin production by multiplex cytometric bead arrays (CBA). Relative to negative controls, release of total IgG, IgG_2, IgA and IgM was observed after 10 days of autologous iNKT co-culture with B cells. However when α-GC was present in the co-cultures, downregulation in immunoglobulin production was observed relative to

co-cultures in which α-GC was absent. Autologous non-iNKT cells were less proficient than iNKT cells at inducing total IgG and IgM production, but were more proficient at inducing IgG_2 production. iNKT and non-iNKT cells were comparably proficient at inducing IgA production. (**Figure 4.3.15**)

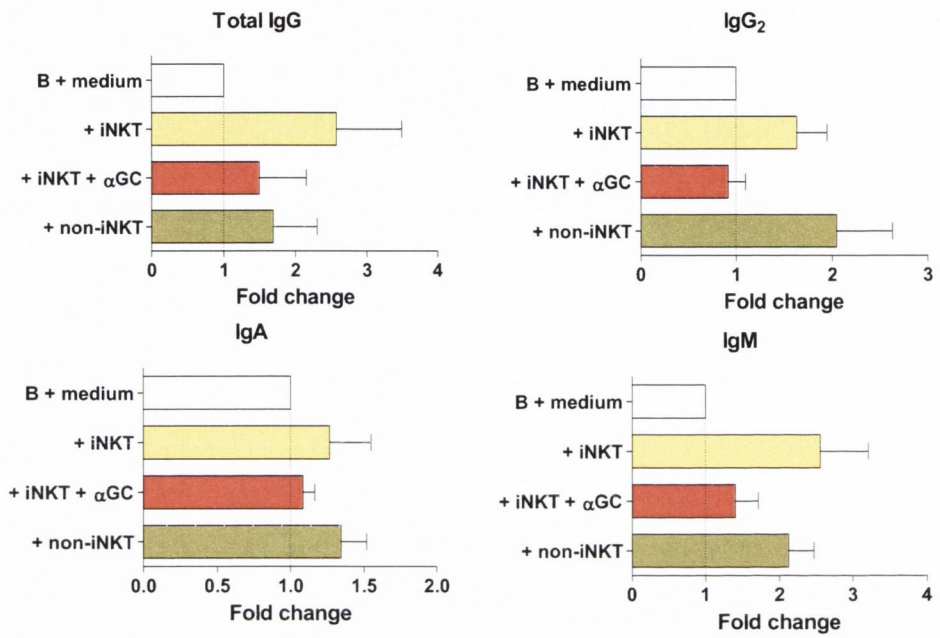

Figure 4.3.15. iNKT cells enhanced production of total IgG, IgG_2, IgA and IgM by autologous B cells. Supernatants from 10 day co-cultures (N=4) of autologous iNKT/non-iNKT and B cells in the presence or absence of α-GC were assayed by multiplex cytometric bead arrays for total IgG, IgG_2, IgA and IgM production. Bars represent mean ± SEM of fold changes in antibody production relative to B cells in medium alone. Fold changes were plotted because absolute values of antibody production from B cells in medium alone differed greatly between independent experiments.

Supernatants from 3 day co-cultures of B cells with autologous iNKT or non-iNKT cells were also assayed for cytokine production by multiplex cytometric bead arrays. The iNKT cell lines expanded by PHA did not produce significant amounts of IL-5 and IL-13 prior to co-culture, unlike the iNKT cells in the allogeneic co-cultures which were expanded by anti-CD3 mAb stimulation. Similar to the allogeneic co-cultures, there was minimal change in cytokine production, with slight increases in TNF-α, IL-4 and IL-13 in co-cultures where α-GC was present (Appendix 9.1.2).

4.3.5 The iNKT ligand, α-GC, enhances B cell activation and cytokine production but decreases immunoglobulin production.

Data from seven independent 3-day iNKT-B cell co-culture experiments was collated and the effect of α-GC on B cell surface marker expression (CD40 and CD86) and cytokine production was assessed. Supernatants from corresponding 10-day iNKT-B cell co-culture experiments were assessed for immunoglobulin production. The Wilcoxon matched pairs test was used to compare differences between co-cultures of iNKT and B cells in which α-GC was absent and those in which α-GC was present.

4.3.5.1 CD40 and CD86 expression on B cells are upregulated further when α-GC is present in iNKT-B cell co-cultures.

Our previous experiments showed that iNKT cells induce upregulation of CD40 and CD86 on B cells, both in the presence and absence of α-GC. To evaluate if the presence of α-GC in the iNKT-B cell interaction significantly enhances iNKT-induced upregulation of CD40 and CD86 expression on B cells, data from 7 such co-culture experiments was collated.

Addition of α-GC to the iNKT-B cell co-cultures significantly enhanced upregulation of CD40 and CD86 ($p<0.05$ for both) on total B cells in comparison to co-cultures where α-GC was absent, suggesting that α-GC induces more potent activation of B cells. (**Figure 4.3.16**)

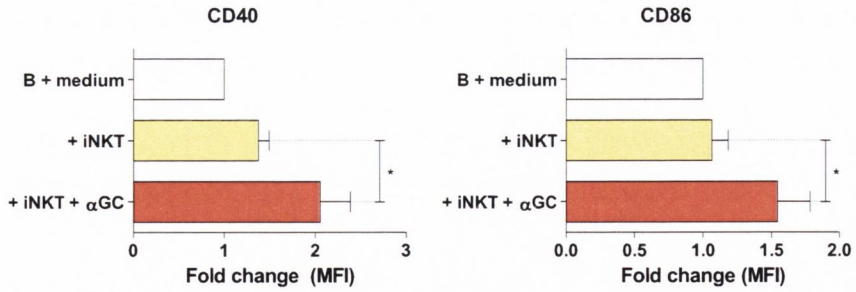

Figure 4.3.16. CD40 and CD86 expression on B cells are further upregulated when α-GC is present in iNKT-B cell co-cultures. MFI of CD40 and CD86 expression on B cells from seven independent 3-day iNKT-B cell co-culture experiments were collated. Bars represent mean ± SEM. The Wilcoxon matched pairs test was used to compare differences between co-cultures in which α-GC was absent and those in which α-GC was present. *$p<0.05$.

4.3.5.2 α-GC enhances production of TNF-α, IL-4 and IL-13 in the iNKT-B cell interaction.

In our previous experiments we showed that there were no significant changes in cytokine production, except for slight increases in TNF-α, IL-4 and IL-13 in co-cultures where α-GC was present. Multiplex cytometric bead array data from 7 independent iNKT-B cell co-culture experiments was collated to determine if α-GC influences cytokine production in the co-cultures. We observed that the presence of α-GC in iNKT-B cell co-cultures significantly enhanced production of TNF-α, IL-4 and IL-13 ($p<0.05$ for all) in comparison to co-cultures that did not have α-GC added. IL-2 production was unchanged, production of IFN-γ and IL-5 were increased whereas IL-10 was decreased (not significant). (**Figure 4.3.17**)

4.3.5.3 Immunoglobulin secretion is decreased when α-GC is present in the iNKT-B cell interaction.

In previous experiments we demonstrated that iNKT cells enhance antibody production by B cells. Multiplex cytometric bead array data from 7 independent iNKT-B cell co-culture experiments was collated to determine the effect of α-GC on antibody production by B cells.

It was observed that the presence of α-GC in iNKT-B cell co-cultures decreased production of total IgG, IgG_2, IgA and IgM ($p<0.05$), in comparison to co-cultures where α-GC was absent. (**Figure 4.3.18**)

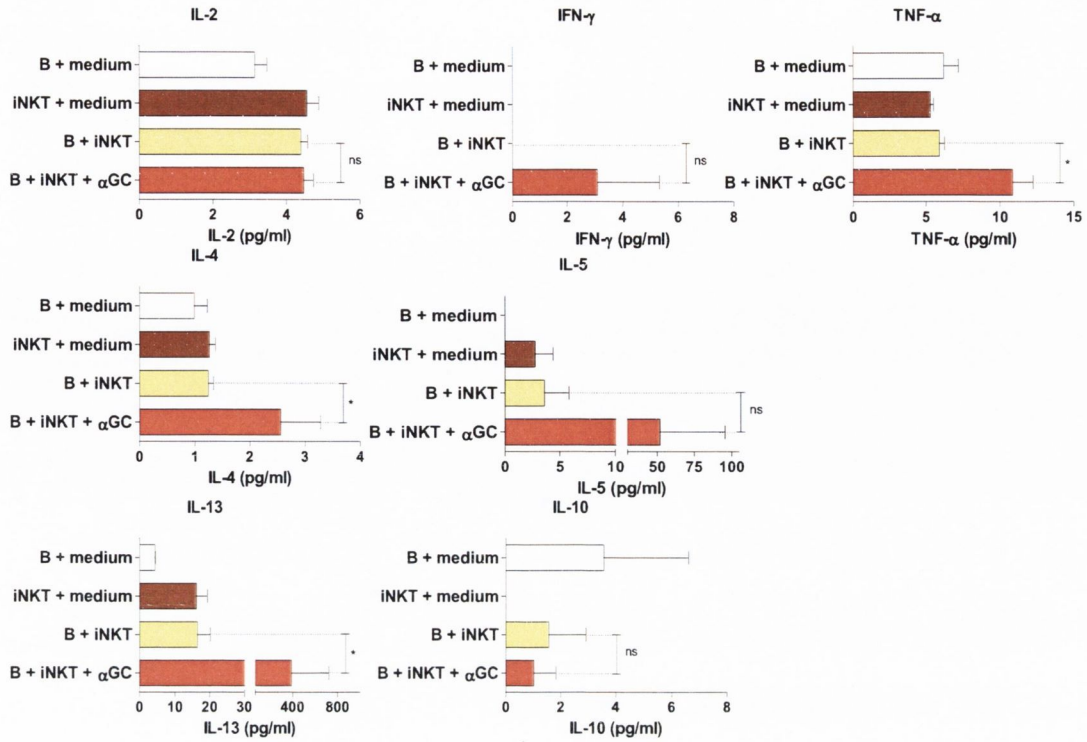

Figure 4.3.17. α-GC enhances production of TNF-α, IL-4 and IL-13 in 3-day iNKT-B cell co-cultures. CBA-measured cytokine production from seven independent 3-day iNKT-B cell co-culture experiments was collated. Bars represent mean ± SEM. The Wilcoxon matched pairs test was used to compare differences between co-cultures in which α-GC was absent and those in which α-GC was present. *p<0.05.

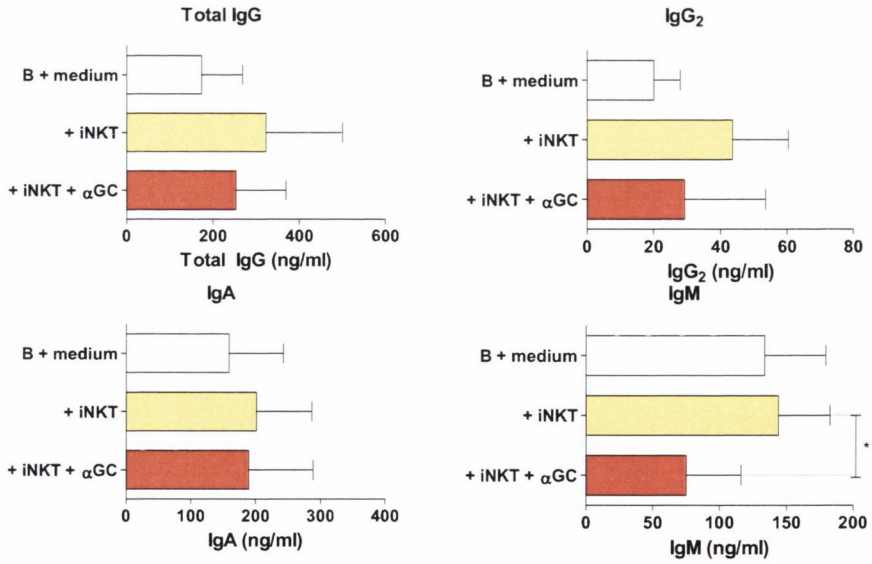

Figure 4.3.18. Immunoglobulin secretion is decreased when α-GC is present in iNKT-B cell co-cultures. CBA-measured immunoglobulin production from seven independent 10-day iNKT-B cell co-culture experiments was collated. Bars represent mean ± SEM. The Wilcoxon matched pairs test was used to compare differences between co-cultures in which α-GC was absent and those in which α-GC was present. *p<0.05.

4.3.6 iNKT cells may inhibit the antigen-presenting capability of B cells.

We observed previously that iNKT help to B cells induces the upregulation of CD86 and CD80 expression on the B cells, suggesting that iNKT help may cause the B cell to mature into antigen-presenting cells. In order to examine this hypothesis, we tested whether iNKT-activated B cells were able to present various antigens – tuberculin purified protein derivative (PPD) and the superantigen staphylococcal enterotoxin B (SEB) – and hence cause proliferation of pre-labelled T cells. PPD, derived from *Mycobacterium tuberculosis*, was used as an antigen because the lymphocytes used in this experiment were derived from BCG-vaccinated donors and were hence expected to have T cells that would mount a response against PPD. SEB elicits polyclonal T cell activation and was used as an alternate in the event that PPD-specific T cell proliferation could not be observed. PHA was added to co-cultures of B cells with autologous or allogeneic T cells as a positive control for T cell proliferation.

Similar to previous experimental setups, iNKT cells were co-cultured with autologous B cells in equal numbers for 3 days. B cells and iNKT cells were also cultured separately in medium only for the same period of time as negative controls. The co-cultured cells and negative controls were then cultured with Celltrace Violet dye (a dye that works similarly to CFSE)-labelled autologous or allogeneic T cells in a ratio of 1:3, along with the various antigens. As an additional negative control for proliferation, the autologous or allogeneic labelled T cells were left in medium alone without any co-cultured cells. After 6 days, division of Celltrace Violet-labelled T cells was assessed by flow cytometry.

4.3.6.1 iNKT cells may inhibit antigen presentation by B cells to autologous and allogeneic T cells.

B cells that were cultured in medium alone were able to induce proliferation of autologous and allogeneic T cells in the absence of antigen, and to a greater degree in the presence of PPD, SEB and PHA. However, T cell proliferation in response to B cells that were pre-activated by iNKT cells was greatly reduced in the absence of antigens and in the presence of PPD and SEB. Non-specific and PPD-specific T cell proliferation was eradicated, whereas SEB-specific T cell proliferation was not. When α-GC was present in iNKT-B cell co-cultures, SEB-specific T cell proliferation was slightly reduced. These observations were consistent for both autologous and allogeneic T cells. Interestingly, iNKT cells cultured in medium alone were able to cause SEB-specific

autologous T cell proliferation (but not allogeneic T cell proliferation) in one out of three experiments. (**Figure 4.3.19** and **Figure 4.3.20**)

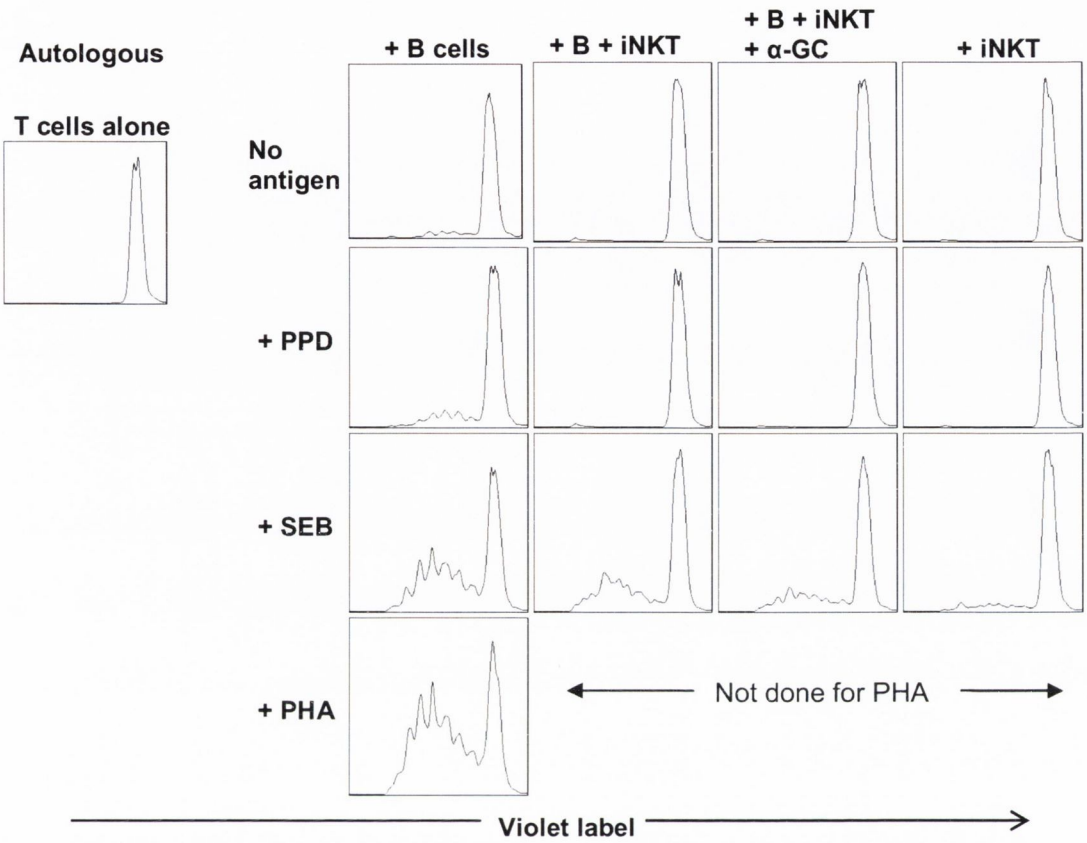

Figure 4.3.19. iNKT-activated B cells were reduced in their ability to cause autologous T cell proliferation in the presence of antigen. B cells were co-cultured with iNKT cells in the absence or presence of α-GC for 3 days. As negative controls, B cells and iNKT cells were separately cultured in medium alone for the same period of time. The co-cultured cells and negative controls were then co-cultured with pre-labelled autologous T cells with or without PPD, SEB or PHA. T cell division was visualized as successive reduction of Celltrace Violet dye by flow cytometry. Graphs are representative of three independent experiments.

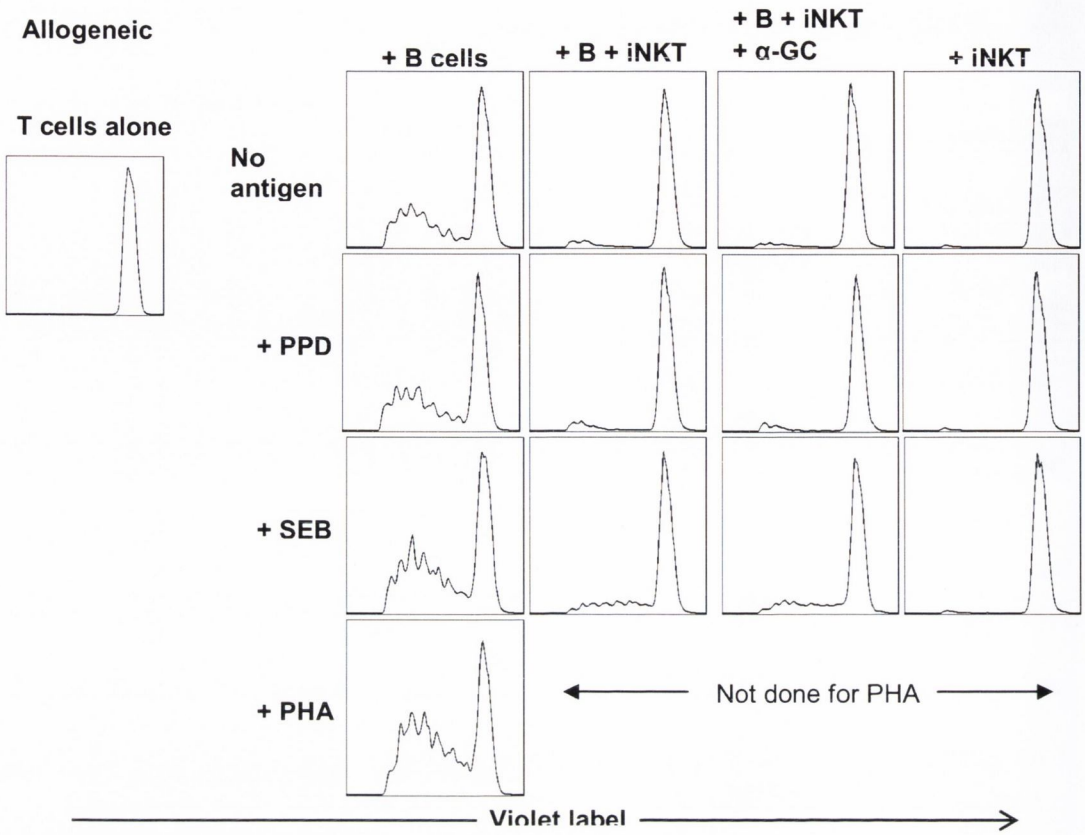

Figure 4.3.20. iNKT-activated B cells were reduced in their ability to cause allogeneic T cell proliferation in the presence of antigen. B cells were co-cultured with iNKT cells in the absence or presence of α-GC for 3 days. As negative controls, B cells and iNKT cells were separately cultured in medium alone for the same period of time. The co-cultured cells and negative controls were then co-cultured with pre-labelled allogeneic T cells with or without PPD, SEB or PHA. T cell division was visualized as successive reduction of Celltrace Violet dye by flow cytometry. Graphs are representative of three independent experiments.

4.3.6.2 $CD4^+$ T cells proliferated better than $CD8^+$ and DN T cells in response to antigen presentation by B cells.

Separate analysis of proliferation of $CD4^+$, $CD8^+$ and DN T cell subsets elucidated that B cells that were cultured in medium only (not pre-activated by iNKT cells) induced more proliferation of the $CD4^+$ T cells relative to $CD8^+$ and DN T cells. When looking at SEB-specific T cell proliferation, we observed that the B cells that were pre-activated by iNKT cells induced mostly proliferation of $CD4^+$ T cells whereas proliferation of $CD8^+$ and DN T cells were minimal. (**Figure 4.3.21**) This is expected since B cells are known to selectively activate $CD4^+$ T cells (Ciechomska et al., 2011; Reinhardt, Liang, and Locksley, 2009; Schmidt et al., 2011) and induce tolerization of $CD8^+$ T cells (Bennett et al., 1998). Also, because SEB binds directly to MHC II (Acharya et al., 1994), it is

expected that only MHC II-restricted $CD4^+$ T cells and not MHC I-restricted $CD8^+$ T cells would proliferate in response to SEB.

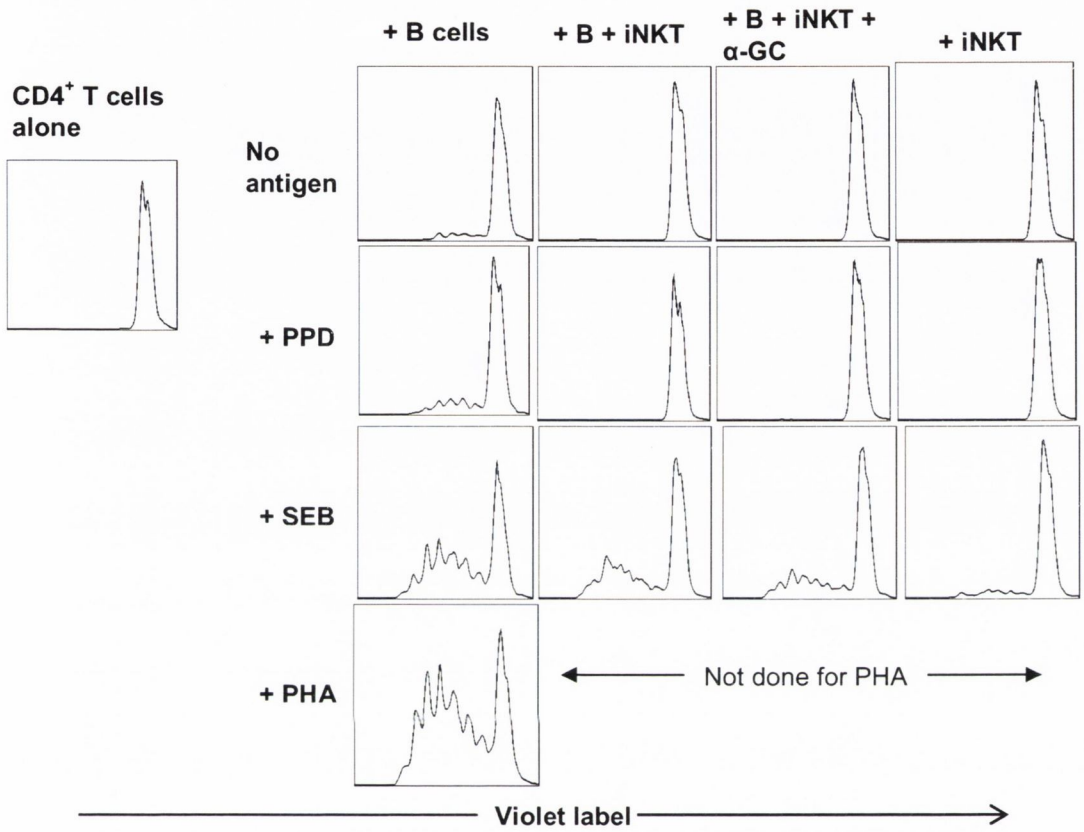

Figure 4.3.21. iNKT cells inhibit antigen presentation by B cells to $CD4^+$ T cells. Histogram overlays of Celltrace Violet-labelled $CD4^+$ T cells co-cultured with iNKT-activated B cells in the absence or presence of PPD, SEB or PHA. Graphs are representative of three experiments.

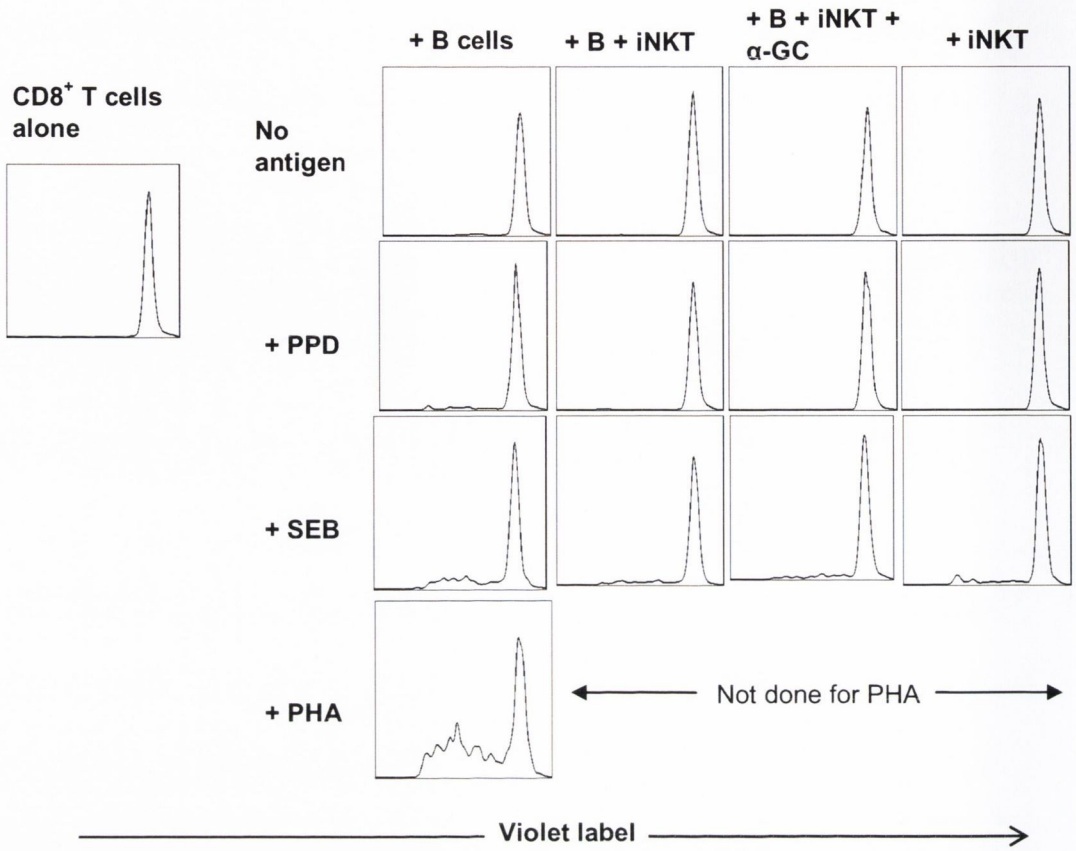

Figure 4.3.22. Little proliferation is observed when B cells present antigens to CD8$^+$ T cells. Histogram overlays of Celltrace Violet-labelled CD8$^+$ T cells co-cultured with iNKT-activated B cells in absence or presence of PPD, SEB or PHA. Graphs are representative of three experiments.

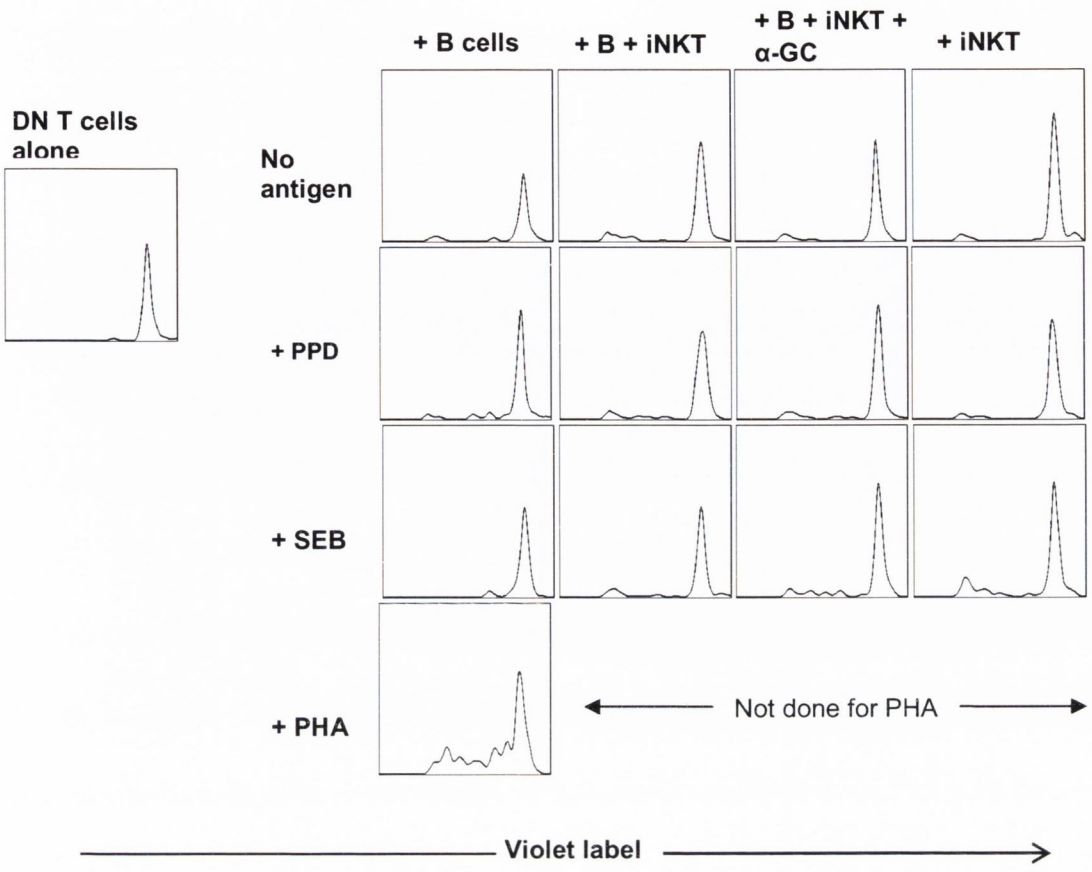

Figure 4.3.23. Little proliferation is seen when B cells present antigens to DN T cells. Histogram overlays of Celltrace Violet-labelled DN T cells co-cultured with iNKT-activated B cells in the absence or presence of PPD, SEB or PHA. Graphs are representative of three experiments.

4.3.7 Cell-cell contact is not required for reciprocal activation during iNKT-B cell interaction but contact enhances their activation state.

To determine if cell-cell contact is necessary for the iNKT-B cell interaction, iNKT cells and B cells were co-cultured for 3 and 10 days and were separated with a membrane by means of a transwell insert or cultured together. As negative controls, B cells and iNKT cells were separately cultured in medium only for the same duration. Flow cytometric analysis was performed on the cells after 3 days and supernatants from 10-day cultures were analysed by multiplex CBA for immunoglobulin production.

4.3.7.1 iNKT cells can induce slight upregulation of CD40 and CD86 expression on total B cells independent of cell-cell contact.

Flow cytometric analysis of total B cells revealed slight upregulation of CD40 and CD86 expression, relative to B cells in medium alone, when iNKT cells were co-cultured with the B cells but not in contact with them. CD40 and CD86 expression were further upregulated when iNKT cells were in contact with the B cells. These data suggest that cell-cell contact is not necessary for iNKT cells to activate B cells. (**Figure 4.3.24**)

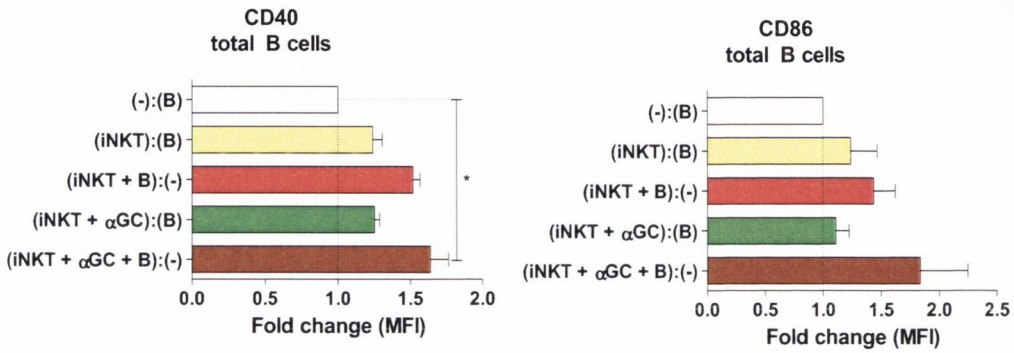

Figure 4.3.24. iNKT cells can induce slight upregulation of CD40 and CD86 expression on total B cells independent of cell-cell contact. iNKT cells were co-cultured with B cells in the absence or presence of α-GC for 3 days in a transwell system containing a membrane in the middle, preventing cell-cell contact. The cells were also co-cultured together in contact to compare between the two. Flow cytometric analysis of CD40 and CD86 expression on total B cells was performed and changes were plotted as fold changes relative to B cells in medium alone. In the figure axes, brackets represent each side of the membrane, with the colon symbol (:) representing the membrane. Bars represent mean ± SEM, data was obtained from three independent experiments. Friedman's test with post-hoc Dunn's multiple comparison test was used to compare differences in means between the different treatments, *$p<0.05$.

4.3.7.2 iNKT cell-induced expansion and downregulation of CD40 expression on $CD1d^{hi}CD5^{+}$ B cells is contact-dependent.

The increased frequencies of $CD1d^{hi}CD5^{+}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells observed when B cells were co-cultured in contact with iNKT cells were largely abrogated in the absence of cell-cell contact. This was observed in both the presence and absence of α-GC. Conversely, there was minimal change in frequencies of $CD24^{hi}CD38^{hi}$ B cells whether the cells were in contact or not. (**Figure 4.3.25**A)

When the iNKT cells and B cells were in contact, CD40 expression was downregulated whereas CD86 expression was slightly upregulated on $CD1d^{hi}CD5^{+}$ B cells. However when contact was prevented, CD40 expression was not downregulated in the absence or presence of α-GC. There was no significant change in levels of CD40 and CD86

expression on $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells in the absence or presence of contact with iNKT cells. (**Figure 4.3.25**B) These results suggest that cell-cell contact is more important for iNKT regulation of $CD1d^{hi}CD5^{+}$ B cells than of $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells.

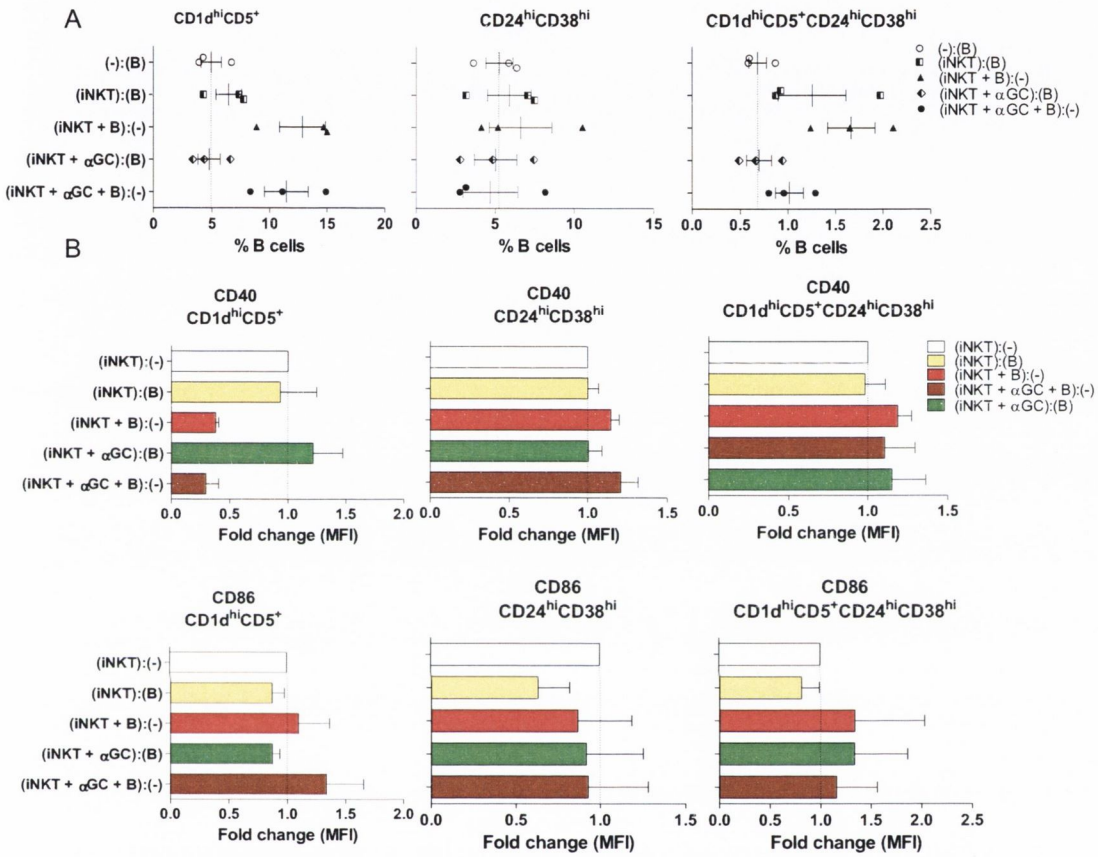

Figure 4.3.25. iNKT cell-induced expansion and downregulation of CD40 expression on $CD1d^{hi}CD5^{+}$ B cells is contact-dependent. iNKT cells were co-cultured with B cells in the absence or presence of α-GC for 3 days in a transwell system containing a membrane in the middle, preventing cell-cell contact. The cells were also co-cultured together in contact to compare between the two. In the figure legends, brackets represent each side of the membrane, with the colon symbol (:) representing the membrane. (A) Changes in proportions of $CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$, and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells were assayed by flow cytometry. (B) Corresponding fold changes in MFI of CD40 and CD86 expression for the respective B cell subsets. Bars represent mean ± SEM, data was obtained from three independent experiments . Friedman's test with post-hoc Dunn's multiple comparison test was used to compare differences in means between the different treatments, results were not statistically significant.

Changes in frequencies, CD40 and CD86 expression on memory and naïve B cell subsets were also analysed (results in Appendix 9.4 – 9.6). In comparison to when the cells were in contact, no significant changes were observed when cell-cell contact was prevented. In summary, the results show that none of the iNKT-induced changes on the

B cell subsets (except for $CD1d^{hi}CD5^{+}$ B cells) require cell-cell contact. iNKT cells themselves exhibited slightly higher CD154 expression when not in contact with B cells, suggesting that B cells may mediate regulation of iNKT cells in a contact-dependent manner (Appendix 9.3).

4.3.7.3 iNKT cell-induced IgM and total IgG production by B cells is contact-dependent.

Supernatants from 10 day co-cultures were analysed by multiplex CBA for total IgG, IgG_2, IgA and IgM. Similar to previous results, immunoglobulin production was upregulated when the B cells were co-cultured with iNKT cells ($p<0.05$ for IgM) and immunoglobulin production was slightly decreased when α-GC was present. In the absence of iNKT-B cell contact, total IgG and IgM were not upregulated whether α-GC was present or not. (**Figure 4.3.26**) IgG_2 and IgA were not detected in any of the experiments, and overall levels of immunoglobulin production appeared lower than usual, suggesting that the transwell insert had an inhibitory effect on immunoglobulin production. Our data suggest that cell-cell contact is necessary for iNKT cell-induced antibody production by B cells.

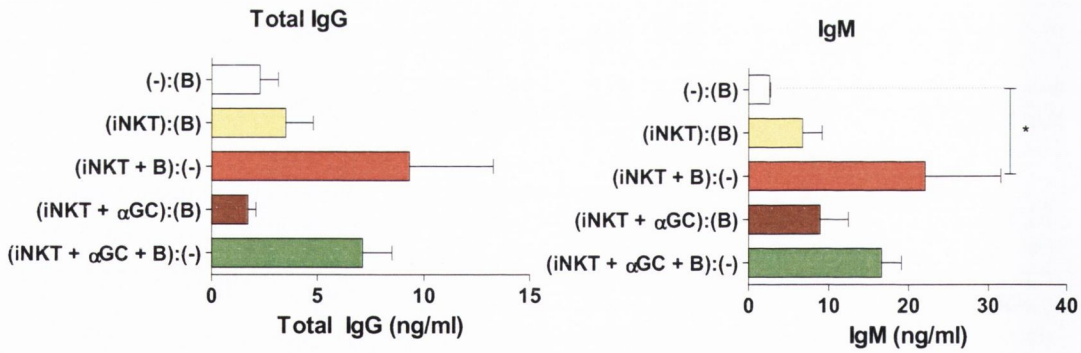

Figure 4.3.26. iNKT cell-induced IgM and total IgG production by B cells is contact-dependent. iNKT cells were co-cultured with B cells in the absence or presence of α-GC for 10 days in a transwell system containing a membrane in the middle, preventing cell-cell contact. The cells were also co-cultured together in contact to compare between the two. In the figure legends, brackets represent each side of the membrane, with the colon symbol (:) representing the membrane. Supernatants were analysed by multiplex CBA for total IgG, IgG_2, IgM and IgA. IgG_2 and IgA were not detected in the experiments. Bars represent mean ± SEM, data was obtained from three independent experiments. Friedman's test with post-hoc Dunn's multiple comparison test was used to compare differences in means between the different treatments, *$p<0.05$.

4.4 Discussion

iNKT cells have been described as potent immunoregulators (Wu et al., 2009) and are able to interact with a large variety of cells from both the innate and adaptive immune system, resulting in activation or suppression of these cells (Caielli, Sorini, and Falcone, 2011; Terabe and Berzofsky, 2007). They are well-characterised in their ability to induce Th1 responses by dendritic cells and cause their maturation into antigen-presenting cells in response to α-GC (Kitamura et al., 1999, Fujii et al., 2007, Liu et al., 2008). Their ability to induce B cell proliferation and antibody production in vitro has also been described (Galli et al., 2003) but little else is known about the effects of iNKT cells on B cells in humans. Hence in this chapter the effects of iNKT cells on B cell surface expression, antigen-presenting function, and antibody production was further characterised.

4.4.1 Generation of iNKT cell lines.

Working with human iNKT cells presents an innate difficulty as the circulating iNKT cell population usually constitutes only 0.01 – 1% of lymphocytes in peripheral blood (Porcelli and Modlin, 1999; Porcelli et al., 1993) and it is intrinsically more difficult to obtain samples from other richer sources of iNKT cells, such as the liver and omentum, than it would be in a murine model. Hence a myriad of protocols for expanding human iNKT cells have been developed based on tried-and-tested protocols for T cell expansion. These protocols can be broadly classified into 2 categories: (i) expansion by specific antigenic stimulation, and (ii) expansion by non-specific mitogenic stimulation.

The most potent iNKT ligand known is the marine sponge-derived glycolipid α-GC (Kawano et al., 1997). iNKT cells are specifically activated by TCR recognition of α-GC being presented on CD1d of antigen-presenting cells (Yu and Porcelli, 2005). When expanding iNKT cells by using α-GC there is a requirement for antigen-presenting cells to also be present, and thus irradiated autologous PBMCs are conventionally used for this purpose (Exley, Balk, and Wilson, 2003). An alternative is to use plate-bound CD1d-Fc fusion dimers to present α-GC to iNKT cells (Brigl et al., 2006; Watarai et al., 2008). Expansion of murine iNKT cells by repeated α-GC stimulation in vitro has been documented to result in robust proliferation and high yields of up to a 10,000-fold increase in iNKT cell numbers within 8 weeks (Chiba et al., 2009). Despite the merits of using specific antigenic stimulation to expand iNKT cells, this method was not utilised as

the iNKT cells from some human donors have been observed to respond poorly to α-GC (current literature and own observations), resulting in the creation of two categories of reproducible donor response patterns to expansion by α-GC – "strong responders" and "poor responders" (Croudace et al., 2008).

In this thesis, iNKT cells were expanded using two different methods with polyclonal T cell mitogens: (i) anti-CD3 mAb stimulation with IL-2, and (ii) PHA stimulation with irradiated feeder cells and IL-2. Both methods require prior enrichment of iNKT cells from PBMCs due to the non-iNKT-specific nature of stimulation.

Comparison of the two methods shows that method (ii) was better as it resulted in higher purities (99.8% vs 87.2%) and yields of iNKT cells (1000 – 2000-fold expansion vs 50 – 500-fold expansion). Although both methods resulted in variable yields of iNKT cells, method (ii) was deemed more reliable as it usually yielded at least two-fold greater expansion than the highest fold increase observed with method (i). Both methods also resulted in polyclonal expansion of iNKT cells, yielding $CD4^+$, $CD8^+$ and DN iNKT cells. The proportions of the iNKT cell subsets after expansion seem to suggest that DN iNKT cells expanded most efficiently while $CD8^+$ iNKT cells were expanded least efficiently. Further experimentation with the culture conditions may lead to optimal protocols for the expansion of $CD8^+$ iNKT cells. Co-cultures of B cells with iNKT cells expanded using these two different methods appeared to be similar in outcome with respect to the iNKT-induced B cell activation and antibody production, suggesting that the method of iNKT cell expansion in our experiments does not affect the function of the iNKT cells.

Interestingly iNKT cells expanded by method (i) produced significant amounts of IL-5 and IL-13 (cytokine CBA results in Appendix, figure 9.1.1), which was 50 – 120-fold greater than iNKT cells expanded by method (ii) (Appendix, figure 9.1.2). This mirrors findings by Sakuishi et al, who observed that enormous amounts of IL-5 were produced by TCR stimulation of iNKT clones with anti-CD3 mAb and IL-2 (Sakuishi et al., 2007). These iNKT clones were initially generated by repeated PHA stimulation in a protocol similar to method (ii) but did not spontaneously secrete IL-5, suggesting that TCR stimulation with anti-CD3 mAb was necessary for selective induction of IL-5 production by the iNKT cells. The same authors also observed that this mimicked the effect of co-culturing $CD1d^+$ antigen-presenting cells with $CD4^+$ iNKT clones and IL-2 in the absence of α-GC. Taken together, our results and Sakuishi et al's paper suggest that method (i) could be used to skew immune responses towards a Th2 response by inducing the iNKT cells to secrete IL-5 and IL-13.

4.4.2 iNKT cells activate B cells.

Our study demonstrates that human iNKT cells can activate B cells *in vitro*, resulting in the upregulation of co-stimulatory and activation markers on B cells, and production of cytokines and immunoglobulins. Flow cytometric analysis on several B cell subsets showed consistent levels of CD1d expression across each subset, suggesting that all the B cell subsets tested have equal ability to present glycolipids to iNKT cells.

The temporal profile of upregulated co-stimulatory and activation markers on B cells as a result of iNKT cell help gives us greater insight into the dynamics of their interaction. CD83 is required for stable expression of MHC class II (Kuwano et al., 2007) and CD86 (Kretschmer et al., 2007) on B cells and is expressed early in B cell activation like CD69. Hence the enhanced expression of CD83 and CD86 with iNKT cell help suggests that iNKT cells are able to activate the B cells and cause them to mature into a phenotype that resembles that of an antigen-presenting cell. CD86 stimulation also has a role in antibody production by B cells. Interaction of CD86 on the B cell with CD28 on the T cell results in upregulation of CD154 expression and secretion of IL-4 by the T cell. Subsequent ligation to CD40 and the IL-4 receptor then induces class switching to IgG_1 production (Kin and Sanders, 2006).

iNKT cell help to B cells also enhanced CD40 expression, which remained upregulated even after 10 days of co-culture. This suggests that the CD40-CD40L interaction between the B cells and iNKT cells is an important conversation in their cross-talk and gives us a glimpse of the possible mechanism underlying their interaction. Interestingly, CD95, also known as the death receptor Fas (Yankee et al., 2001), was upregulated within 3 days of co-culture with iNKT cells, suggesting that activated B cells are quickly primed to undergo apoptosis upon iNKT help. This is perhaps a mechanism of regulation invoked by iNKT cells that prevents the dysregulation of B cell proliferation characteristic of B cell lymphomas (Müschen et al., 2002). Flow cytometric analysis of CD178 (Fas ligand) expression on the iNKT cells and time-lapse analysis of B cell death (using Annexin V and propidium iodide staining) will be instrumental in proving this hypothesis.

4.4.3 The iNKT-B cell interaction is specific.

As a good portion of the experiments were performed in an allogeneic co-culture system, it was possible that the observed activating effects of iNKT cells on B cells could be due to alloreactive recognition of the allogeneic MHC molecules by the iNKT TCR (Matzinger and Bevan, 1977; Nikolich-Žugich, 2007). Hence the effects of iNKT cells on allogeneic B cells were compared against those on autologous B cells.

iNKT cell-induced upregulation of CD40 and CD86 expression on B cells was consistent for both autologous and allogeneic co-cultures. However there was a difference in the temporal profile of upregulation: in allogeneic co-cultures, CD40 and CD86 expression were higher at 3 days than at 10 days, whereas the opposite trend was observed in the autologous co-cultures. In comparison to iNKT cells, non-iNKT cells (or regular T cells) were less proficient at activating B cells in allogeneic co-cultures but more proficient at activating B cells in autologous co-cultures. The profile of iNKT-induced antibody production was similar in both autologous and allogeneic co-cultures. In both cases, iNKT cells induced greater antibody production than non-iNKT cells. These results suggest that the iNKT-induced activation of B cells is a result of specific interaction between the two and that any alloreactivity only serves to enhance the result of the interaction.

4.4.4 α-GC modulates the iNKT-B cell interaction.

Our results are in alignment with Galli et al (Galli et al., 2003) in that the help for antibody production provided by the iNKT cells does not require the iNKT ligand, α-GC. However, addition of α-GC to iNKT-B cell co-cultures did result in a greater increase of CD40 and CD86 expression ($p<0.05$ for both) and cytokine production ($p<0.05$ for TNF-α, IL-4 and IL-13), suggesting that the presence of α-GC causes greater activation of B cells. Paradoxically, the presence of α-GC in the iNKT-B cell co-cultures resulted in decreases in antibody production by the B cells ($p<0.05$ for IgM). Parallel observations in a murine model that spontaneously produces autoreactive antibodies showed that iNKT cell activation by α-GC inhibited autoreactive antibody production (Yang et al., 2011). Our results possibly suggest that when iNKT cells are activated by a potent ligand such as α-GC, it results in reciprocally greater activation of the B cells. This provides feedback to the iNKT cells and causes them to downregulate antibody production by the B cells as a mechanism to keep the immune system in balance.

4.4.5 The effect of iNKT cells on different B cell subsets.

Like iNKT cells, B cells are heterogeneous in nature and although they are known mainly for their effector functions in humoral immunity, studies which show that they have regulatory properties are rapidly emerging. In particular, the regulatory B (Breg) cells, which produce IL-10 and are immunosuppressive in nature, are of great interest in the scientific community (Lo-Man, 2011). There is currently no consensus regarding the phenotype of human Breg cells, with only one study (Blair et al., 2010) providing evidence that $CD24^{hi}CD38^{hi}$ B cells may be Breg cells. Going by current literature which establishes $CD1d^{hi}CD5^{+}$ B cells as Breg cells in mice, we looked at three possible Breg subsets: $CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells.

In our experiments we observed that iNKT cells induced increases in frequencies of $CD1d^{hi}CD5^{+}$ B cells at both 3 days and 10 days, whereas it took 10 days of co-culture for frequencies of $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells to increase. iNKT cells did not induce much change in CD86 expression but induced upregulation of CD40 on $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells. This suggests that the CD40-CD154 interaction, but not the CD86-CD28 interaction, between them is of significance. Cognate CD40-CD154 interactions between B cells and T cells is known to induce regulatory functions in B cells resulting in inhibition of T cell proliferation, while CD86 and CD80 are thought to be required for Breg cells to inhibit Th1 cytokine production from the T cells (Lemoine et al., 2011). Interestingly iNKT cells had an inhibitory effect on CD40 expression on $CD1d^{hi}CD5^{+}$ B cells, which was reversed when α-GC was present in the co-cultures.

Co-culture of iNKT cells with B cells led to increases in the proportion of IgM^{+} unswitched memory B cells at both 3 and 10 days, whereas frequencies of total unswitched memory B cells were only increased at 10 days. Upregulation of CD40 ($p<0.05$ for IgM^{+}) and CD86 was most prominent at 10 days in the presence of α-GC. There has been much controversy about whether circulating $IgM^{+}CD27^{+}IgD^{+}$ B cells are indeed memory B cells (Tangye and Good, 2007). Based on gene expression profiling, they are thought to originate from the splenic marginal zone, independent from the germinal centre which is the site of memory B cell generation (Kruetzmann et al., 2003; Weller et al., 2004). These B cells also possess a mutated immunoglobulin receptor (Weller et al., 2001), suggesting that they do not require cognate interaction with T cells and thus could be responsible for T-independent immune responses. However, there are indications that $IgM^{+}CD27^{+}IgD^{+}$ B cells can also participate in T-dependent immune

responses (Fondere et al., 2003; Tuaillon et al., 2006). This dual function of $IgM^+CD27^+IgD^+$ B cells could perhaps be mediated by iNKT cells, which are able to activate and expand them.

We also observed that iNKT cells induced isotype switching to IgG^+ switched memory B cells, as well as upregulation of CD40 ($p<0.05$) and CD86 on total and IgG^+ switched memory B cells. However, frequencies of total switched memory B cells remained unchanged, suggesting that the iNKT cells only induce isotype switching and not expansion of switched memory B cells. iNKT cells have been observed to induce isotype switching in murine B cells in a contact-dependent manner (Mattner et al., 2008). This effect of iNKT cells on switched memory B cells gives insight into a possible mechanism by which iNKT cells mediate clearance of infection.

Naïve B cells were increased in frequencies at 3 days, then decreased at 10 days with iNKT help. Proportions of IgM^+ naïve B cells were also increased by iNKT cells with simultaneous upregulation of CD40 and CD86 on total and IgM^+ naïve B cells. These data suggest that perhaps iNKT cells cause maturation of naïve B cells into memory B cells, thus explaining their decrease in frequencies at 10 days. This is plausible considering that prolonged CD40 stimulation (>7 days) has been demonstrated to trigger isotype switching to IgG^+ and induce CD27 expression in the absence of somatic hypermutation, the latter of which requires interaction with T cells (Fecteau and Néron, 2003).

Decreased frequencies of total $CD27^-IgD^-$ memory B cells, with no change in frequencies of IgM^+ and IgG^+ were observed with iNKT co-culture. iNKT help induced upregulation of CD40 and CD86 for the IgM^+ and IgG^+ subsets, however the temporal profile of their enhancement was dissimilar. Upregulation of CD40 was most pronounced at 3 days for the IgM^+ subset, thereafter which CD40 expression decreases at 10 days. In contrast, upregulation of CD86 was most pronounced at 10 days for the IgM^+ and IgG^+ subsets. $CD27^-IgD^-$ memory B cells have been of interest in the context of immunosenescence and autoimmunity. $IgG^+CD27^-IgD^-$ memory B cells have been characterised to be increased in healthy elderly and postulated to be responsible for helping them deal effectively with immune challenge in old age (Bulati et al., 2011). $CD27^-IgD^-$ memory B cells are also increased and represent a large fraction of memory B cells in SLE patients (Wei et al., 2007). These patients exhibit high titers of anti-dsDNA antibodies, suggesting that $CD27^-IgD^-$ memory B cells play a role in SLE pathogenesis and autoreactive antibody production. The fact that iNKT cells decrease

their frequencies demonstrates that iNKT cells exert an inhibitory effect on humoral immunity by downregulating a subset of "pathogenic" memory B cells with a propensity for autoreactive antibody production.

4.4.6 iNKT cells may inhibit B cells from presenting antigens to T cells.

Based on the upregulation of activation and co-stimulatory markers observed on B cells with iNKT help, we hypothesized that iNKT cells promote antigen-presentation by the B cells. However, after 3 days of co-culture with iNKT cells, T cell proliferation was reduced in response to B cell antigen presentation, with the exception of SEB. This observation suggests that iNKT cells may inhibit the antigen-presenting function of B cells. However, this conjecture has to be confirmed with further experiments using a more defined antigen such as tetanus toxoid protein as PPD may contain innate ligands in addition to mycobacterium antigens and hence result in non-antigen specific T cell proliferation in response to B cells that have not pre-activated by iNKT cells. SEB is a superantigen that does not undergo intracellular antigen processing (Acharya et al., 1994; Khan, Priya, and Saha, 2009), but binds directly to the alpha chain of MHC class II and is recognised by the Vβ chain of the TCR, resulting in massive T cell activation (Papageorgiou and Acharya, 2000). This suggests that iNKT cells may inhibit B cell intracellular antigen processing. This was observed independent of the presence of α-GC in the co-cultures, suggesting that perhaps this inhibitory effect of iNKT cells on B cell antigen presentation is not mediated through the TCR-CD1d interaction. However, further experiments such as adding peptide fragments to the co-cultures to observe if the T cells proliferate in response to B cell presentation of antigen peptide fragments, are required to ascertain the mechanism of inhibition of B cell antigen presentation by the iNKT cells.

4.4.7 Cell-cell contact is not necessary for B cell activation but is required for antibody production.

Our data demonstrate that cell-cell contact was not required for iNKT-induced upregulation of CD40 and CD86 expression on total B cells but was required to induce antibody production. A separate study by Tonti et al showed that α-GC-activated iNKT cells could induce antibody production by B cells indirectly by activating T helper cells

through activation of other antigen-presenting cells such as dendritic cells (Tonti et al., 2009). Combined with the results from our experiments, it appears that iNKT-induced upregulation of CD40 and CD86 on the B cells could be mediated, although less efficiently, through soluble factors such as cytokines. On the contrary, iNKT-induced antibody production requires cognate iNKT-B or T-B interaction.

Cognate iNKT-B cell interaction appeared to be vital for the expansion of $CD1d^{hi}CD5^{+}$ B cells, which were expanded upon contact with iNKT cells but unchanged in frequencies when contact was removed. iNKT-induced repression of CD40 expression on the $CD1d^{hi}CD5^{+}$ B cells was also relieved when contact between the two cells was prevented. It is difficult to speculate on the implications of this apparent inhibitory effect of iNKT cells on $CD1d^{hi}CD5^{+}$ B cells as we still lack knowledge about the function of these B cells.

The conclusions from this chapter are:

1. iNKT cells activate B cells by upregulating markers such as CD40 and CD86 and inducing antibody production, but may inhibit antigen presentation by the B cells.

2. The outcome of the iNKT-B cell interaction is specific and similar in both allogeneic and autologous co-cultures.

3. The iNKT cell ligand α-GC plays a modulatory role in the iNKT-B cell interaction by enhancing iNKT-induced B cell activation and cytokine production but decreasing antibody production.

4. iNKT cells have different effects on various B cell subsets, resulting in increased frequencies of Breg cells, unswitched memory B cells, isotype-switched memory B cells and naïve B cells but decreased frequencies of $CD27^{-}IgD^{-}$ memory B cells. The number of days of co-culture (3 days or 10 days) also affects the changes in their frequencies, CD40 and CD86 expression.

5. Cell-cell contact between the iNKT cells and B cells is not necessary for iNKT-mediated B cell activation but is required for antibody production.

5 Mechanisms underlying iNKT-B cell interactions.

5.1 Introduction

In the previous chapter, we observed that iNKT cells caused upregulation of CD40 and CD86 expression on B cells and induced antibody production, but inhibited their ability to present antigens to autologous and allogeneic T cells. These suggest that iNKT cells regulate B cell function in many aspects and that perhaps the iNKT-B cell interaction can be targeted in therapy for diseases with excessive B cell function or autoreactive antibody production, such as systemic lupus erythematosus (SLE) (Lipsky, 2001; Sherer et al., 2004). Hence it is crucial to understand the mechanisms that mediate such interactions, such as the surface molecules and cytokines involved, whether a particular iNKT cell subset is vital for B cell help, and signalling mechanisms that are activated or inhibited during the interaction.

5.1.1 iNKT cell subsets may be predisposed towards disparate T helper immune responses.

Like conventional T cells, iNKT cells are heterogeneous and can be separated into $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ (DN) subsets in humans (Lin et al., 2006a). Mice are known to have only the $CD4^+$ and DN iNKT cell subsets (Seino and Taniguchi, 2005) but recent research has hinted at the existence of a small population of $CD8^+$ iNKT cells (Lee et al., 2009). However, unlike conventional T cells, the respective functions of these different iNKT cell subsets are not as well-defined.

There are several studies suggesting that iNKT cell subpopulations differ in their ability to stimulate other immune cells. When activated with PMA and ionomycin, expanded human $CD4^+$ and DN iNKT clones produced both Th1 and Th2 cytokines without any apparent skew in their cytokine profile (Galli et al., 2003). In contrast to Galli's findings, Gumperz et al al observed that freshly isolated human $CD4^+$ iNKT cells produced both Th1 and Th2 cytokines, whereas their $CD4^-$ iNKT (presumably $CD8^+$ plus DN) counterparts were biased towards Th1 cytokine production (Gumperz et al., 2002). Similar results were echoed by Rossignol et al (Rossignol et al., 2007). Stimulation of PBMC with PMA and ionomycin and intracellular cytokine staining showed that the $CD4^+$ iNKT subset significantly upregulated IL-4 production whereas IFN-γ and TNF-α production were not significantly different between the iNKT subsets (Montoya et al., 2007). A fourth study suggests that human $CD4^+$ iNKT cells are predisposed toward Th2

cytokine production whereas DN iNKT cells are predisposed toward Th1 cytokine production (Lee et al., 2002).

Differences in ability to induce antibody production by B cells also seem to exist between the iNKT cell subsets. Expanded $CD4^+$ iNKT cell clones displayed superiority in comparison to DN iNKT cell clones at inducing IgM and IgG_1 production by B cells (Galli et al., 2003). It was also observed that $CD4^+$ iNKT cells, but not DN iNKT cells, were responsible for isotype switching by human B cells (Rossignol et al., 2007). Activated murine $CD8^+$ iNKT cells are also known to be inclined toward Th1 cytokine production (Emoto et al., 2000; Lee et al., 2009) and cytotoxic in nature (Wingender et al., 2006). In a comparative study of the effect of $CD4^+$, $CD8^+$ and DN iNKT subsets on T cells, natural killer cells and B cells, $CD4^+$ iNKT cells displayed substantially greater stimulatory activity on all these cells in comparison to $CD8^+$ and DN iNKT cells (Lin et al., 2006b). These studies suggest that $CD4^+$ iNKT cells may be superior to $CD8^+$ and DN iNKT cells in inducing B cell activation and antibody production.

5.1.2 Surface molecules and soluble factors mediating iNKT-B cell interactions.

Several papers have reported an increase in specific antibody response to co-administered antigen when iNKT cells are activated in vivo with α-GC, which has to be presented on CD1d (Devera et al., 2008; Galli et al., 2007; Lang, Exley, and Lang, 2006). This led to the hypothesis that CD1d is necessary for the iNKT-enhanced antibody production by B cells, and subsequent work done in $CD1d^{-/-}$ mice (Lang, Devera, and Lang, 2008) and with antibodies to block CD1d (Lisbonne et al., 2005) proved that CD1d expression on B cells is absolutely required for such an effect. The requirement for CD1d in iNKT cell help to B cells in humans was also suggested by Galli et al who observed that anti-CD1d mAb could block α-GC-mediated iNKT cell-induced antibody production but not anti-CD3 mAb-mediated iNKT cell-induced antibody production (Galli et al., 2003).

The CD40-CD154 interaction between B cells and T cells is vital for efficient reciprocal activation when the adaptive immune system is activated. CD40 is constitutively expressed on B cells (Bishop and Hostager, 2003) and its expression in human B cells can be further upregulated upon CD40 stimulation (Bishop et al., 2001). CD40 stimulation on its own is sufficient to induce B cell proliferation, although it usually synergizes with signals from the B cell receptor and/or the IL-4 receptor (Brine and Klaus, 1993; Wheeler et al., 1993). CD40 signalling is imperative for most B cell

functions. Work done with murine cells showed that CD40 signalling enhances B cell antigen presentation via increased expression of CD86 and CD80 to augment T cell co-stimulation (Han et al., 1995; Yang and Wilson, 1996), and is essential for isotype switching during antibody production as well as germinal center formation (Kawabe et al., 1994). Indeed, the CD40-CD154 co-stimulatory interaction between B cells and iNKT cells has been shown to be necessary for the B cell proliferation and class-switched antigen-specific antibody production in response to injected antigen-α-GC conjugate (Leadbetter et al., 2008). Blocking the CD40-CD154 interaction inhibits α-GC-induced murine iNKT cell proliferation (Kawano et al., 1997) and cytokine production (Nishimura et al., 2000), suggesting that this co-stimulatory signal is crucial for successful activation of both iNKT cells and B cells.

In the previous chapter we showed that the presence of α-GC in the iNKT-B cell co-cultures resulted in enhanced IL-4 and IL-13 production. IL-4 and IL-13 are cytokines that promote Th2 differentiation and humoral immunity (Lai and Mosmann, 1999). Both have similar effects on human B cells, with IL-4 being more potent than IL-13. IL-13, which is expressed by activated T cells and also iNKT cells, promotes proliferation and isotype switching of activated B cells (Cocks et al., 1993; Defrance et al., 1994). IL-4 or IL-13, in combination with CD40-CD154 signalling, can also synergize to enhance B cell proliferation (Cocks et al., 1993). The role of IL-4 in mediating the iNKT-B cell interaction is unclear. A study by Kitamura et al states that IL-4 secreted by iNKT cells is crucial for early activation (upregulation of CD69 expression) of B cells (Kitamura et al., 2000) whereas in IL-4$^{-/-}$ mice antigen-specific antibody production and ratio of antibody isotypes in response to haptenated α-GC was not impaired by the lack of IL-4 (Leadbetter et al., 2008).

5.1.3 Phosphoinositide-3-kinases (PI3Ks) in lymphocyte function.

Phosphoinositide-3-kinases (PI3Ks) are lipid signalling kinases that are responsible for regulating many cellular processes such as proliferation, survival, adhesion and migration (Cantley, 2002). There are several classes of PI3Ks and the class I PI3K isoforms, p110δ (class IA) and p110γ (class IB) have been documented to be involved in immune responses, such as natural killer cell cytotoxicity and B cell antibody production (Koyasu, 2003). They are also of particular interest as they are expressed exclusively in leukocytes (Vanhaesebroeck et al., 2001). p110δ is activated by receptor tyrosine kinases such as cytokine receptors (IL-1β, IL-4, IL-5, IL-6 and IL-13) (Lee et al.,

2006; Molnarfi et al., 2008), antigen-specific stimuli and costimulatory receptors such as CD28 on T cells (Wu et al., 2005) and CD19 on B cells (Otero, Omori, and Rickert, 2001). p110γ is activated by G-protein-coupled receptors such as chemokine receptors (Sasaki et al., 2000). These two isoforms of PI3K are known to be essential regulators of B and T cell proliferation, survival and function (Rommel, Camps, and Ji, 2007). As such, both have been investigated as potential therapeutic targets in autoimmune inflammatory diseases such as SLE (Ghigo et al., 2010). Blocking of p110γ has been shown to inhibit glomerulonephritis and extend the lifespan of the MRL-*lpr* mouse model of SLE (Barber et al., 2005), while p110δ and p110γ inhibition alleviated joint erosion in a K/BxN serum transfer model of arthritis (Randis et al., 2008). In response to the therapeutic effect seen with inhibition of p110δ and/or p110γ with isoform-specific first generation inhibitors, several second generation inhibitors such as TG100-115 (which inhibits both p110δ and p110γ) have been synthesized and are currently in clinical trials to assess their therapeutic effect in cancer, inflammation and coronary heart disease (Marone et al., 2008).

5.1.3.1 The role of p110δ in B lymphocytes.

The CD19 membrane protein on B cells, when phosphorylated as a consequence of B cell receptor (BCR) signalling, is known to associate with PI3K (Tuveson et al., 1993). The main catalytic isoform acting downstream of the BCR is known to be p110δ (Clayton et al., 2002; Okkenhaug et al., 2002). Activated PI3K generates the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) by phosphorylating phosphatidylinositol 4,5-bisphosphate (PIP_2) (Okkenhaug and Vanhaesebroeck, 2003). PIP_3 contains binding sites for proteins with pleckstrin homology domains to bind to, such as serine/threonine protein kinase B (PKB or Akt). Akt activation promotes survival, proliferation and differentiation in B cells (Herzog, Reth, and Jumaa, 2009; Manning and Cantley, 2007) by phosphorylating and promoting the dissociation of proteins that bind to and inactivate anti-apoptotic proteins. Akt also blocks transcription of genes responsible for cell cycle arrest and apoptosis by phosphorylating the transcription factor (forkhead in rhabdomyosarcoma) and targeting it for proteasomal degradation (Rena et al., 2001). Additionally Akt is known to inactivate glycogen synthase kinase-3β (GSK-3β), which inhibits cell cycle progression (Vivanco and Sawyers, 2002).

In recent years, the advent of isoform-specific PI3K inhibitors has allowed for a surge in studies of the specific PI3K isoforms. A study in which the delta isoform (p110δ) was blocked with the isoform-specific inhibitor, IC87114, has elucidated that p110δ is

essential for murine B1 and marginal zone B cells to respond to BCR clustering, TLR signals (LPS and CpG) and chemoattractants (CXCL13 and sphingosine 1-phosphate), and is an absolute requirement for in vivo production of natural antibodies (Durand et al., 2009). A similar study in p110δ$^{-/-}$ mice confirmed that p110δ is necessary for the B cell response to BCR and co-stimulatory signal (CD40) and protection from apoptosis (Clayton et al., 2002). Many other studies have established that p110δ is essential and nonredundant for B cell response and function (Al-Alwan et al., 2007; Jou et al., 2002; Limon and Fruman, 2010), as well as survival mediated by IL-4 signalling (Bilancio et al., 2006).

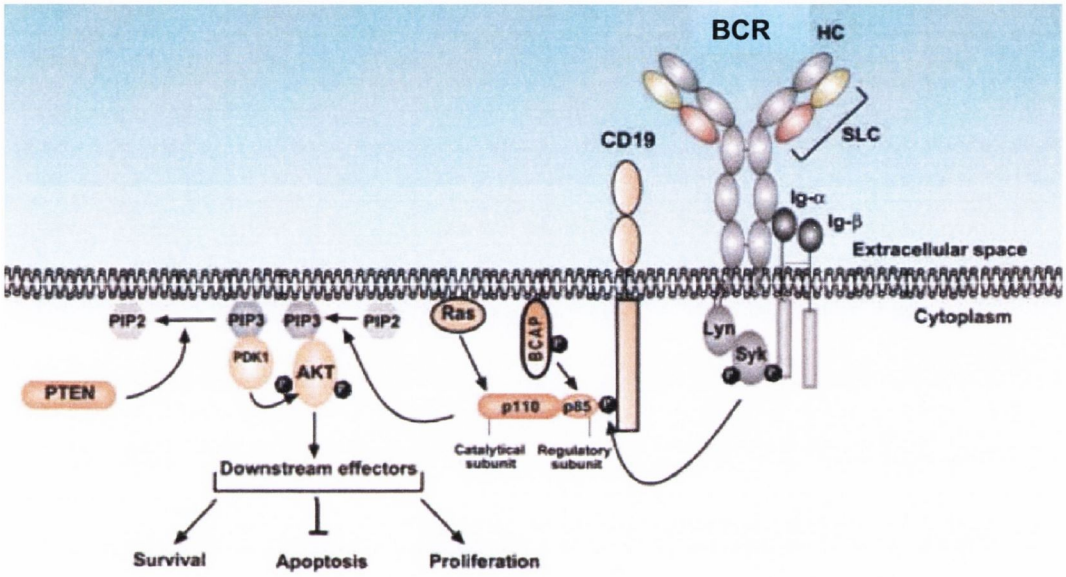

Figure 5.1.1. Overview of PI3K signalling downstream of the B cell receptor (BCR). Taken from (Werner, Hobeika, and Jumaa, 2010). Activation of PI3K by the CD19 co-receptor causes dissociation and activation of the p110 catalytic subunit from the p85 regulatory subunit, allowing it to catalyse the conversion of PIP_2 to PIP_3 on the lipid membrane. Akt/PKB binds to PIP_3, where it is phosphorylated and activated by PDK1. Activated Akt/PKB promotes survival, proliferation and differentiation in B cells.

5.1.3.2 The role of p110δ in iNKT and T lymphocytes.

To date, little work has been done regarding the role of p110δ in iNKT cells. It is known that the PI3K pathway is activated when iNKT cells interact with α-GC-loaded dendritic cells and this is dependent on both p110δ and p110γ. Knockout of either isoform in mice resulted in reduced IFN-γ production upon administration of α-GC and inability to prevent metastasis of melanoma cells to the lung (Kishimoto et al., 2007).

The role of p110δ has been more closely studied in T lymphocytes. p110δ acts downstream of the T cell receptor (TCR) and is required for cytokine production (Soond et al., 2010). TCR activation of p110δ contributes to the migration and localisation of the primed T cells to sites of inflammation (Jarmin et al., 2008). p110δ is also of particular importance in the formation of T follicular helper cells in the germinal centres, which are responsible for the induction of B cell activation, differentiation and antibody production (Rolf et al., 2010). It has been shown that p110δ is critical for the suppressor function of $CD4^+CD25^+Foxp3^+$ Treg cells (Patton et al., 2006) and also controls their expansion and tissue homing (Liu et al., 2009). In addition, p110δ regulates the differentiation of peripheral T helper cells into Th1 and Th2 lineages (Okkenhaug et al., 2006; Patton, Garçon, and Okkenhaug, 2007).

5.1.3.3 Mitogen-activated protein kinases (MAPK)

MAPK are intracellular serine/threonine-specific kinases that regulate cellular differentiation, proliferation and cell death (Pearson et al., 2001). There are 3 major groups of MAPK in mammalian cells, namely the extracellular signal-regulated protein kinases (ERK), the p38 MAPK and the c-Jun NH_2-terminal kinases (Davis, 2000; Schaeffer and Weber, 1999). MAPK are activated by phosphorylation cascades in a typical three-kinase architecture consisting of a MAPK, a MAPK activator (MEK, MKK or MAPK kinase) and a MEK activator (MEK kinase or MAPK kinase kinase) (Schaeffer and Weber, 1999). A schematic diagram of the 3 major groups of MAPK pathways are shown below in **Figure 5.1.2**.

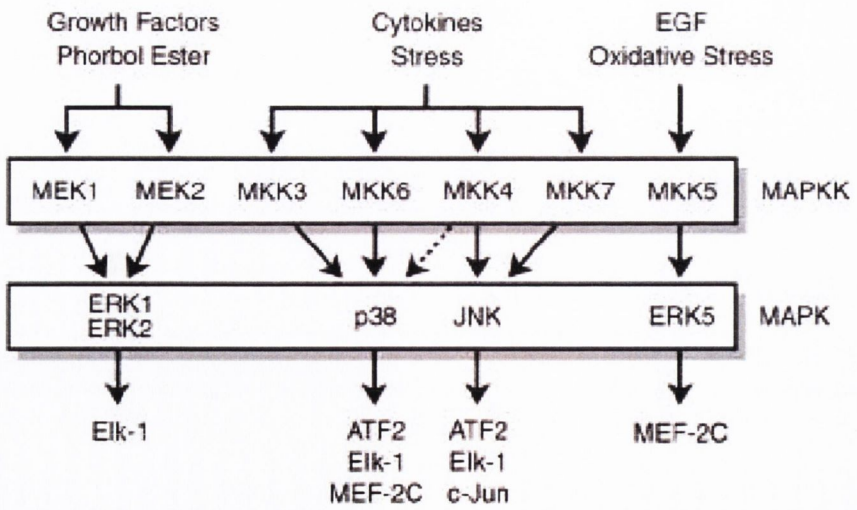

Figure 5.1.2. The 3 major groups of MAPK pathways in mammalian cells. Taken from (Dong, Davis, and Flavell, 2002).

MAPK can be activated by an assortment of external or internal stimuli, and are known to be involved in immune responses. In innate immune responses, Toll-like receptor (TLR) activation by their respective ligands results in signalling cascades that culminate in activation of MAP kinases such as p38 and JNK, leading to the production of inflammatory cytokines such as TNF-α, IL-1 and IL-12 (Chi et al., 2004; Kotlyarov et al., 1999; Lu et al., 1999). The MAPK are also involved in T helper cell differentiation: JNK and p38 are preferentially activated in Th1 cells (Li et al., 2000; Yang et al., 2010) whereas ERK is preferentially activated in Th2 cells (Singh and Zhang, 2004). CD40 signalling, which is crucial for B cell survival and function, results in the selective activation of JNK and p38 but not ERK (Berberich et al., 1996). MAPK also regulate lymphocyte apoptosis, such as activation-induced cell death of $CD8^+$ effector T cells (Merritt et al., 2000).

Both the PI3K pathway and the MAPK pathway can be simultaneously activated during receptor stimulation (Li, Jiang, and Tapping, 2010). **Figure 5.1.3** shows a simplified diagram of the PI3K and MAPK pathways. Crosstalk exists between the PI3K pathway and the MAPK pathway and is complex and not well-understood. Blockade of PI3K phosphorylation has been demonstrated to result in increased phosphorylation of ERK/MAPK (Hausenloy, Mocanu, and Yellon, 2004). Antagonistic inhibition of ERK/MAPK by PI3K/Akt has also been observed in human umbilical cord vein endothelial cells in the control of tissue factor expression for coagulation (Blum et al., 2001) and in human breast cancer cells leading to a shift from cell cycle arrest to proliferation (Zimmermann and Moelling, 1999). However, concurrent inhibition of PI3K and MAPK phosphorylation during ischemia of the heart, followed by concurrent phosphorylation and activation of PI3K and MAPK phosphorylation during heart reperfusion, has been documented (Hausenloy and Yellon, 2004), suggesting that the PI3K and MAPK pathways do not necessary always inhibit each other. The mammalian target of rapamycin (mTOR) is one such mediator that allows crosstalk between the two pathways, as activation of mTOR is known to promote PI3K/Akt activation whereas inhibition of mTOR promotes Ras/MAPK activation (Carracedo et al., 2008).

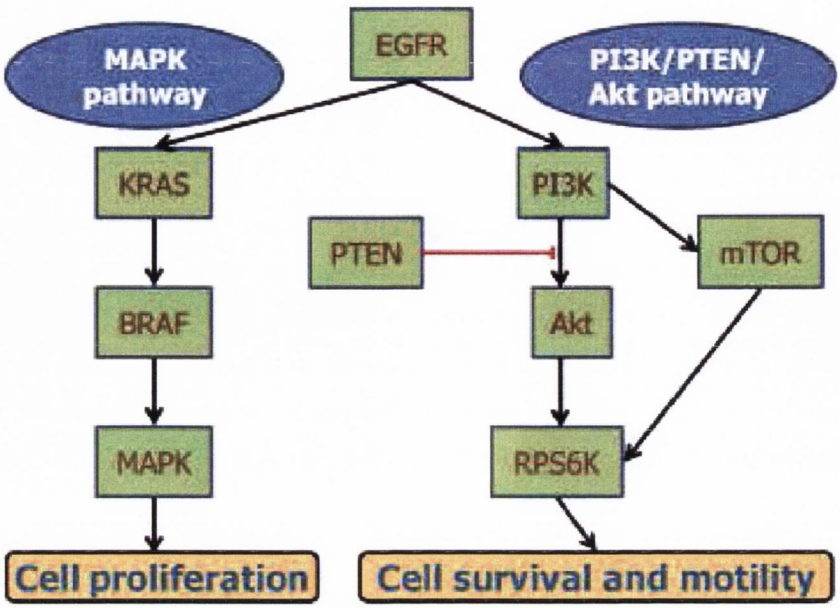

Figure 5.1.3. Simplified schematic of PI3K and MAPK pathways. Taken from (De Hertogh and Geboes, 2010).

Table 5.1.1 lists the 26 intracellular MAP kinases assayed for in the phosphoMAPK array, used in section 5.3.10.

Table 5.1.1. List of the 26 intracellular kinases in the phosphoMAPK array (section 5.3.10).

Kinase	Full Name	Function
Pan Akt	Protein kinase B	Key regulator of signal transduction, inhibits apoptosis and promotes cellular proliferation (Vara et al., 2004), growth, survival and metabolism (Gonzalez and McGraw, 2009). Activated by TCR/CD28 signalling, cytokines and chemokines in T cells (Bauer and Baier, 2001).
Akt1/PKBα	Protein kinase B alpha	Inhibits apoptosis and cell death, promotes angiogenesis (Chen et al., 2001; Cho et al., 2001). Promotes peripheral B cell maturation and survival (Calamito et al., 2010).
Akt2/PKBβ	Protein kinase B beta	Controls glucose metabolism (Garofalo et al., 2003). Promotes peripheral B cell maturation and survival (Calamito et al., 2010).
Akt3/PKBγ	Protein kinase B gamma	Involved in neuronal development (Tschopp et al., 2005).
CREB	cAMP response element-binding protein	Transcription factor. In T and B cells, activation of CREB promotes proliferation and survival and differentially regulates Th1, Th2 and Th17 responses. CREB activation induces IL-10 production and is required for generation and maintenance of regulatory T cells (Wen, Sakamoto, and Miller, 2010).
Erk1	Extracellular signal-regulated protein kinase 1	Activated by T cell receptor signalling, not required for $CD8^+$ T cell activation and proliferation (D'Souza et al., 2008). Deficiency in Erk1 biases the immune system towards a Th1 response (Agrawal et al., 2006). Sustained activation induces T cell anergy (Waiczies et al., 2005).
Erk2	Extracellular signal-regulated protein kinase 2	Activated by T cell receptor signalling, promotes survival of activated $CD8^+$ T cells and required for IL-2 production by them (D'Souza et al., 2008).

Kinase	Full Name	Function
GSK-3α/β	Glycogen synthase kinase 3 alpha/beta	Key enzyme in glycogen metabolism, two isoforms (α and β) exist (Woodgett, 1990). Inactivation of GSK-3 augments anti-inflammatory cytokine production (e.g. IL-10) and suppresses pro-inflammatory cytokine production. CD28 co-stimulation of T cells inactivates GSK-3; GSK-3 activation suppresses IL-2 production and T cell proliferation (Wang, Brown, and Martin, 2011).
GSK-3β	Glycogen synthase kinase 3 beta	Same as GSK-3α/β.
HSP27	Heat shock protein 27	Phosphorylated HSP27 has anti-proliferative and anti-apoptotic effects; causes platelet granule secretion (Kostenko and Moens, 2009). Hyperphosphorylation results in aberrant B cell proliferation (Madsen et al., 1995).
Pan JNK	c-Jun NH_2-terminal kinases	Activated by CD40 and CD27 ligation on B cells, inhibits B cell terminal differentiation and plasma cell generation (Satpathy et al., 2010). Involved in cytokine production from T cells (Bennet et al., 2001) and required for mitogen-stimulated T cell proliferation (Melino et al., 2008). Implicated in progressive chronic disease such as rheumatoid arthritis (Han et al., 2002). Known to phosphorylate p53 (Hu, Qiu, and Wang, 1997).
JNK1	c-Jun NH_2-terminal kinase 1	Negative regulator of Th2 differentiation in vitro (Dong et al., 1998). $JNK1^{-/-}$ $CD8^+$ T cells hypoproliferate and secrete less IL-2 (Conze et al., 2002), and also exhibit decreased effector functions (Gao et al., 2005). Proinflammatory, causes mast cell degranulation and IL-1β production in inflammatory arthritis (Guma et al., 2010).

Kinase	Full Name	Function
JNK2	c-Jun NH_2-terminal kinase 2	Essential for efficient induction of type I IFN and other cytokines in response to viral infection or dsRNA (Chu et al., 1999). Required for IFN-γ production by peripheral $CD4^+$ T cells and subsequent Th1 differentiation in vitro (Yang et al., 1998). $JNK2^{-/-}$ $CD8^+$ T cells hyperproliferate and produce increased amounts of IL-2 (Conze et al., 2002).
JNK3	c-Jun NH_2-terminal kinase 3	Stress-activated, important mediator of caspase 3-dependent neuronal apoptosis induced by excitatory amino acids or cerebral ischemia (Brecht et al., 2005).
MKK3	Mitogen-activated protein kinase (MAPK) kinase 3	Activates p38 pathway (upstream of p38), primary regulators of p38 phosphorylation and activation. MKK3 selectively phosphorylates p38α, γ, and δ whereas MKK6 activates all four p38 isoforms (α, β, γ, and δ) (Enslen, Raingeaud, and Davis, 1998). Implicated in pathogenesis of arthritis (Chabaud-Riou and Firestein, 2004; Yoshizawa et al., 2009).
MKK6	MAPK kinase 6	
MSK2	Mitogen- and stress-activated protein kinase 2	Activated downstream of p38 and Erk1/2, limits production of pro-inflammatory cytokines in response to Toll-like receptor signalling (Ananieva et al., 2008).
p38α	MAP kinase alpha	Negatively regulates iNKT response to α-GC (Stuart et al., 2010). Mediator of LPS signalling in B cells (Han et al., 1994), necessary for normal Th1 function in T cells (Jirmanova et al., 2009). Activated by T cell receptor engagement, resulting in Fas ligand expression and apoptosis (Hsu et al., 1999). Not required for antigen-dependent and non-antigen-dependent B cell and T cell proliferation (Kim et al., 2005).

Kinase	Full Name	Function
p38β	MAP kinase beta	Inhibits apoptosis (Nemoto et al., 1998). Not necessary for LPS-induced cytokine production and progression of autoimmune disease in a $p38\beta^{-/-}$ model was not ameliorated, suggesting it does not have a huge role in immune function (Beardmore et al., 2005).
p38δ	MAP kinase delta	Required for motility and invasion of cholangiocarcinoma cells, postulated to aid in metastasis of cancer cells (Tan et al., 2010).
p38γ	MAP kinase gamma	Prevents chromosomal instability and supports mitotic cell viability during proliferation (Kukkonen-Macchi et al., 2011). Contributes to ability of cells to cope with UV exposure by regulating checkpoint signalling and repair of UV-induced DNA damage (Wu et al., 2010). Essential for G2 checkpoint regulation, induces cell cycle arrest during radiation damage (Wang et al., 2000).
p53		Tumour suppressor. Negatively regulates autoimmunity via suppression of STAT3 phosphorylation and inhibition of Th17 effectors (IL-17, IL-6) in T cells (Zhang et al., 2011b).
p70 S6 kinase (or S6 kinase 1)	p70 S6 kinase	Induces cell apoptosis (Fumarola et al., 2005). Insufficient glucose inhibits p70 S6 kinase phosphorylation and IFN-γ production from $CD8^+$ T cells (Cham and Gajewski, 2005).
RSK1	90 kDa ribosomal S6 kinase 1	Directly phosphorylated by Erk1/2, promotes cell proliferation and protein synthesis (Romeo and Roux, 2011). Rapidly activated by IL-2 and IL-15 in human T cells (Lin, Spolski, and Leonard, 2008).

Kinase	Full Name	Function
RSK2	90 kDa ribosomal S6 kinase 2	Directly phosphorylated by Erk1/2, promotes cell proliferation and protein synthesis (Romeo and Roux, 2011). Promotes cell migration and invasion by phosphorylating HSP27 (Kang and Chen, 2011). Rapidly activated by IL-2 and IL-15 in human T cells. Required for T cell activation and IL-2 production but not Th differentiation (Lin, Spolski, and Leonard, 2008).
TOR (or mTOR)	Mammalian target of rapamycin	Required for maturation and differentiation of multiple immune cell lineages. Involved in T cell homing, homeostasis, memory generation, as well as B cell proliferation, antibody production and migration in response to chemokines (Zhang et al., 2011a).

5.2 Aims and hypotheses

The overall aim of this chapter was to identify key molecular components of the pathways that regulate iNKT-B cell interactions in vitro. Our objective was to characterise the contribution of various co-stimulatory pathways, soluble factors, specific iNKT subsets and intracellular signalling molecules to iNKT-induced B cell activation and antibody production. There were several hypotheses, which are listed out below.

iNKT cells can be divided into three subsets in humans: $CD4^+$, $CD8^+$ and DN (Lin et al., 2006a). To examine if a particular subset is more efficient at activating and enhancing B cell antibody production, expanded polyclonal iNKT cells were sorted into their respective subsets and co-cultured with B cells. As $CD4^+$ iNKT cells exhibit a more Th2-biased cytokine profile compared to $CD8^+$ and DN iNKT cells (Gumperz et al., 2002), we hypothesized that $CD4^+$ iNKT cells would be superior to the other two subsets in providing help to B cells. Intracellular flow cytometry for cytokine production in $CD4^+$ iNKT-B cell co-cultures was also performed to look at the cytokine production profile in both the $CD4^+$ iNKT cells and B cells during co-culture. Results from these experiments would enable us to identify a particular subset that may be targeted in B cell-mediated immunotherapy.

CD1d has been shown to be vital for activation of B cell function by iNKT cells in several murine studies (Lang, Devera, and Lang, 2008; Lisbonne et al., 2005), and other factors such as the CD40-CD154 co-stimulatory pathway, and cytokines such as IL-4 and IL-13 have been shown to be important in promoting B cell activation, proliferation and antibody production (Brine and Klaus, 1993; Cocks et al., 1993; Han et al., 1995). To assess the importance of these co-stimulatory pathways and soluble factors in mediating human iNKT-B cell interactions, antibodies against CD1d, CD40, CD154, IL-4 and IL-13 were added into the iNKT-B cell co-cultures for the purpose of blocking these molecules. Based on the importance of CD1d for iNKT ligand recognition, the CD40-CD154 pathway for iNKT-induced B cell antibody production, and the cytokines IL-4 and IL-13 for B cell isotype switching, we hypothesized that the iNKT-B cell interaction in humans is dependent on CD1d, CD40, CD154, IL-4 and IL-13, and that these molecules could be potential therapeutic targets.

As PI3Kδ (or p110δ) is known to be vital to B and T cell function (Jou et al., 2002; Okkenhaug et al., 2006), we assessed the role of p110δ in mediating iNKT-B cell interactions, with the hypothesis that p110δ is indispensable in the interaction and is another potential candidate for immunotherapy. IC87114 was added to the iNKT-B cell co-cultures to inhibit p110δ. The phosphoMAPK array, which is a multiplex western blot that measures phosphorylation of 26 intracellular MAP kinases (shown in **Table 5.1.1**) simultaneously, was used to profile changes in kinase phosphorylation with co-culture and p110δ inhibition.

5.3 Results

5.3.1 $CD4^+$ iNKT cells most efficiently activate B cells.

To find out if different subsets of iNKT cells had differential effect on activation of various B cell subsets and antibody production, polyclonal iNKT lines expanded from healthy donors were sorted into $CD4^+$, $CD8^+$ and DN iNKT subsets using the cell sorter. The sorted iNKT subsets were then co-cultured with total B cells and changes in CD40 and CD86 expression on various B cell subsets (Breg and memory B cells) were analysed by flow cytometry after 3 days. Supernatants from 3 day and 10 day co-cultures were also analysed by CBA for cytokine and immunoglobulin production respectively.

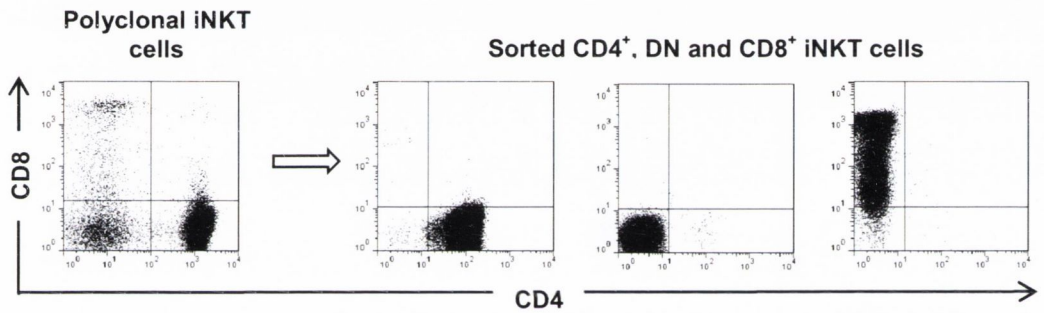

Figure 5.3.1. Sorting of polyclonal iNKT cells into $CD4^+$, DN and $CD8^+$ iNKT cell subsets by flow cytometry. Polyclonal iNKT lines expanded from healthy donors were sorted by the cell sorter into $CD4^+$, DN and $CD8^+$ subsets. Flow cytometry plots are representative of four different experiments.

5.3.1.1 $CD4^+$ iNKT cells were most potent at upregulating CD40 expression on total B cells.

$CD4^+$ iNKT cells, in the presence of α-GC, were most potent at upregulating CD40 expression on total B cells ($p<0.05$). However, they were as potent as $CD8^+$ iNKT cells at upregulating CD86 expression on total B cells. In contrast to $CD4^+$ iNKT cells, $CD8^+$ iNKT cells could upregulate CD86 expression on total B cells without the need for α-GC. (**Figure 5.3.2**)

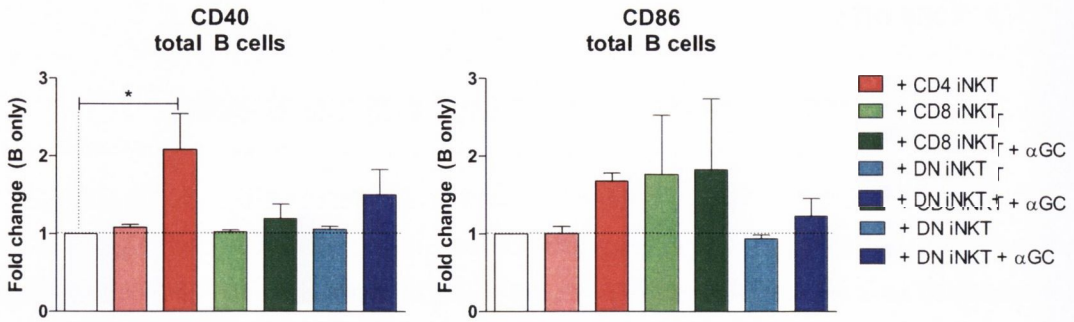

Figure 5.3.2. CD4$^+$ iNKT cells are most potent at upregulating CD40 expression on total B cells. Total B cells were analysed by flow cytometry after three days of B cell co-culture with CD4$^+$, CD8$^+$ or DN iNKT subsets in the absence or presence of α-GC. Graphs show changes in MFI of CD40 and CD86 expression on total B cells. Changes are expressed as fold changes relative to MFI of CD40 or CD86 expression by B cells in medium alone (B only). Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using the Kruskal-Wallis test with post-hoc Dunn's multiple comparison, *p<0.05.

5.3.1.2 CD4$^+$ iNKT cells induce a population of CD1d^{hi}CD5^{hi} B cells.

The CD1d^{hi}CD5$^+$ Breg population comprised less than 1% of unstimulated total B cells and was not a distinctly separate population. However, co-culture with CD4$^+$ iNKT cells induced a distinct population of CD1d^{hi}CD5^{hi} B cells (in 2 out of 3 experiments) which are enhanced in both CD1d and CD5 expression in comparison to the parent population. Co-culture with CD8$^+$ iNKT cells induced a CD5$^+$ population with lower expression of CD1d (in 3 out of 3 experiments). The induced population was enhanced only in CD5 expression but not in CD1d expression in comparison to the parent population. Co-culture with DN iNKT cells induced a population that was not distinctly separate from the total B cell population but was enhanced only in CD5 expression (in 3 out of 4 experiments). Addition of α-GC further enhanced the proportion of CD1d^{hi}CD5^{hi} B cells when co-cultured with CD4$^+$ iNKT cells, decreased the proportion of CD1d^{hi}CD5$^+$ B cells when co-cultured with CD8$^+$ iNKT cells and did not change the proportion of CD1d^{hi}CD5$^+$ B cells when co-cultured with DN iNKT cells. (**Figure 5.3.3**)

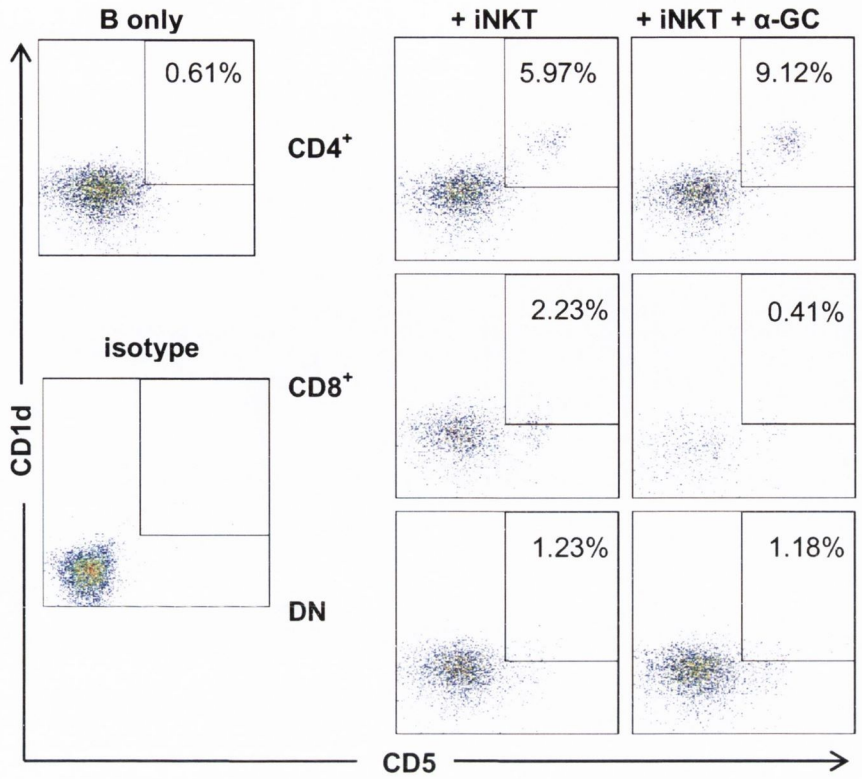

Figure 5.3.3. $CD4^+$ iNKT cells induce a population of $CD1d^{hi}CD5^{hi}$ B cells. Total B cells were co-cultured with $CD4^+$, $CD8^+$ or DN iNKT cells for 3 days in the absence or presence of α-GC and the $CD1d^{hi}CD5^+$ B cells were analysed by flow cytometry after electronically gating on $CD19^+$ lymphocytes. The flow cytometry plots show CD1d and CD5 expression on unstimulated B cells (top left), B cells co-cultured with iNKT subsets (middle) and B cells co-cultured with iNKT subsets in the presence of α-GC (right).

In contrast, none of the iNKT subsets induced much expansion of the $CD24^{hi}CD38^{hi}$ B cell population when co-cultured with B cells in all experiments. (**Figure 5.3.4**A) Analysis of a $CD24^-CD38^{hi}$ B cell population that appeared only upon co-culture with the iNKT cell subsets showed that the particular population was $CD1d^{hi}CD5^{hi}$ when co-cultured with $CD4^+$ iNKT cells but $CD1d^-CD5^{hi}$ when co-cultured with $CD8^+$ or DN iNKT cells, suggesting that this population may be a defined B cell subset that is enhanced in CD1d and/or CD5 expression upon co-culture with iNKT cell subsets. (**Figure 5.3.4**B) Further characterisation of the function, cytokine and antibody production profile of this B cell subset may yield interesting results.

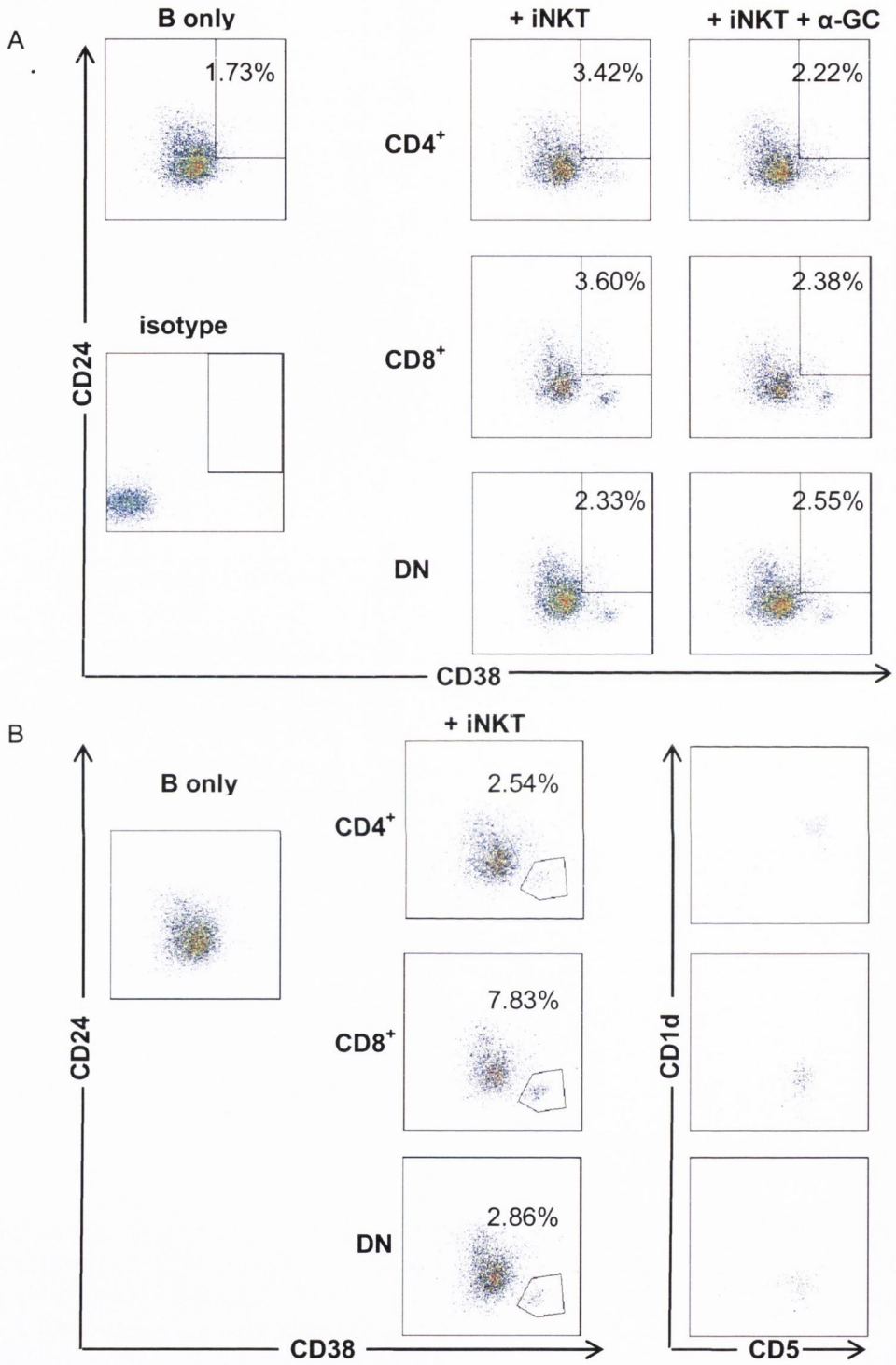

Figure 5.3.4. None of the iNKT subsets induced much expansion of CD24^{hi}CD38^{hi} Breg cells. Total B cells were co-cultured with CD4^{+}, CD8^{+} or DN iNKT cells for 3 days in the absence or presence of α-GC and the CD24^{hi}CD38^{hi} Breg cells were elucidated by flow cytometry. (A) Flow cytometry plots showing CD24 and CD38 expression on unstimulated B cells (top left), iNKT subsets co-cultured with B cells (middle) and iNKT subsets co-cultured with B cells in the presence of α-GC (right). (B) Flow cytometry plots showing CD24 and CD38 expression on unstimulated B cells (top left) and iNKT subsets co-cultured with B cells (middle) with corresponding plots showing CD1d and CD5 expression of the CD38^{hi} gated population (right).

5.3.1.3 Breg cells are expanded most efficiently by $CD4^+$ iNKT cells.

Amongst the three iNKT cell subsets, $CD4^+$ iNKT cells caused the largest expansion of $CD1d^{hi}CD5^+$ and $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ B cells in the absence of α-GC. In the presence of α-GC, $CD4^+$ iNKT cells were also able to induce expansion of $CD1d^{hi}CD5^+$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ B cells whereas $CD8^+$ or DN iNKT cells were not able to do so. (**Figure 5.3.5**A)

$CD4^+$ iNKT help in the presence of α-GC caused increases in CD40 ($p<0.05$ for $CD24^{hi}CD38^{hi}$ B cells) and CD86 expression on all three putative Breg cell subsets and was more efficient at upregulating CD40 expression in comparison to $CD8^+$ and DN iNKT cells. In contrast, $CD8^+$ iNKT help in both the absence and presence of α-GC was most efficient at inducing upregulation of CD86 expression on the B cell subsets. (**Figure 5.3.5**B) $CD4^+$ iNKT help in the presence of α-GC was also most efficient at upregulating CD40 and CD86 on unswitched, switched, $CD27^-IgD^-$ memory B cells and naïve B cells (results in Appendix, 9.14-9.17).

5.3.1.4 Distinct cytokine profiles of $CD4^+$ iNKT cells and B cells in co-culture.

In the previous chapter, it was shown that the expanded iNKT cells were able to produce both Th1 and Th2 cytokines when co-cultured with Hela-CD1d cells. However, when the polyclonal iNKT cells were co-cultured with B cells, there was little change in cytokine secretion observed in the supernatants collected from the co-cultures. As $CD4^+$ iNKT cells appear to be superior at activating B cells, intracellular cytokine staining was performed to examine whether cytokine production could be induced if only $CD4^+$ iNKT cells, rather than polyclonal iNKT cells, were co-cultured with the B cells. Hence $CD4^+$ iNKT cells were co-cultured with B cells for 3 days and cytokine secretion from the cells was inhibited with monensin for 4 hours on the 3rd day of co-culture. Thereafter, flow cytometric analysis of intracellular cytokine (IL-10, IL-13 and IFN-γ) production was performed. As a negative control, unstimulated B cells were left in culture medium for the same duration. Results from this section are preliminary as the experiment has been performed only once.

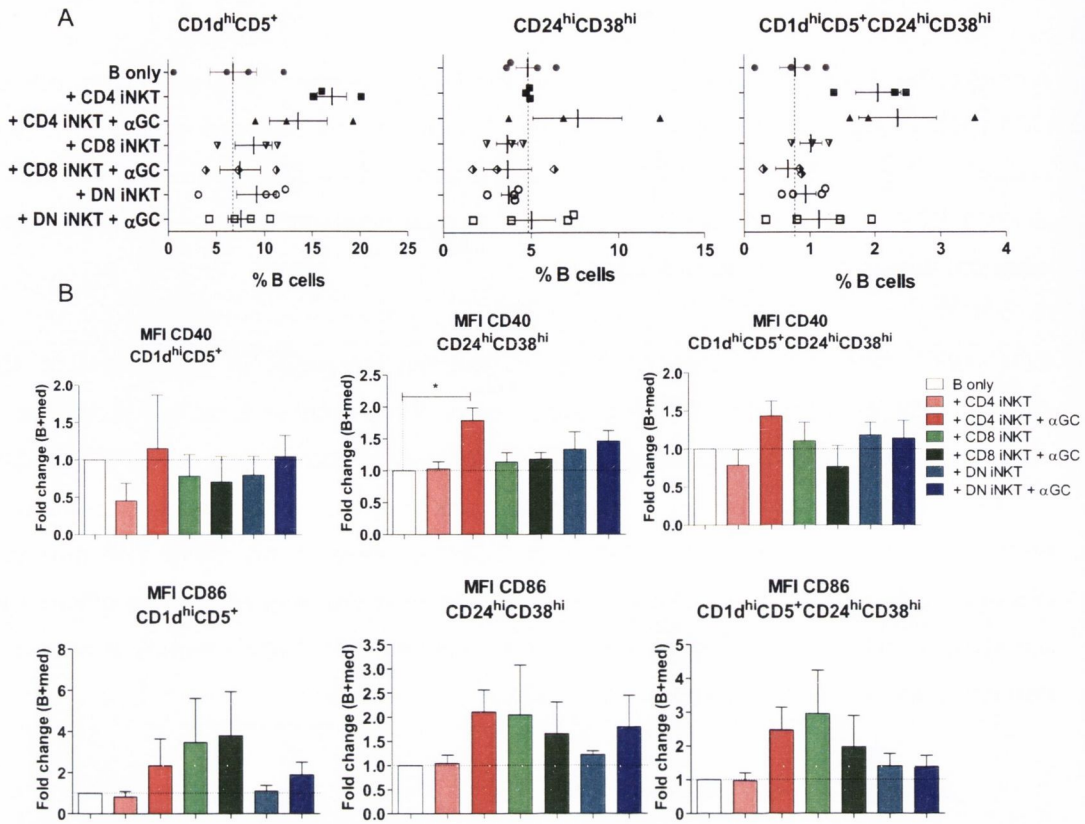

Figure 5.3.5. Breg cells are expanded most efficiently by CD4$^+$ iNKT cells. Three subsets of regulatory B (Breg) cells (CD1d^{hi}CD5$^+$, CD24^{hi}CD38^{hi} and CD1d^{hi}CD5$^+$CD24^{hi}CD38^{hi}) were analysed by flow cytometry after three days of B cell co-culture with CD4$^+$, CD8$^+$ or DN iNKT subsets in the absence or presence of α-GC. (A) Graphs showing changes in proportions of CD1d^{hi}CD5$^+$ (left), CD24^{hi}CD38^{hi} (middle) and CD1d^{hi}CD5$^+$CD24^{hi}CD38^{hi} (right) Breg cells when co-cultured with different iNKT subsets and α-GC. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on CD1d^{hi}CD5$^+$ (left), CD24^{hi}CD38^{hi} (middle) and CD1d^{hi}CD5$^+$CD24^{hi}CD38^{hi} (right) Breg cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments.

In this experiment, flow cytometric analysis of intracellular cytokine production by total B cells showed that co-culture with CD4$^+$ iNKT cells in the absence of α-GC led to increased frequencies of IL-10$^+$ and IL-13$^+$ B cells and decreased frequencies of IFN-γ$^+$ B cells. However, in the presence of α-GC, the frequencies of IL-10$^+$ and IFN-γ$^+$ B cells increased instead while frequencies of IL-13$^+$ B cells decreased. (**Figure 5.3.6**) This suggests that in this experiment, iNKT help in the absence of α-GC induces B cells towards a Th2 cytokine production profile whereas iNKT help in the presence of α-GC shifts it back towards a more balanced Th1/Treg profile.

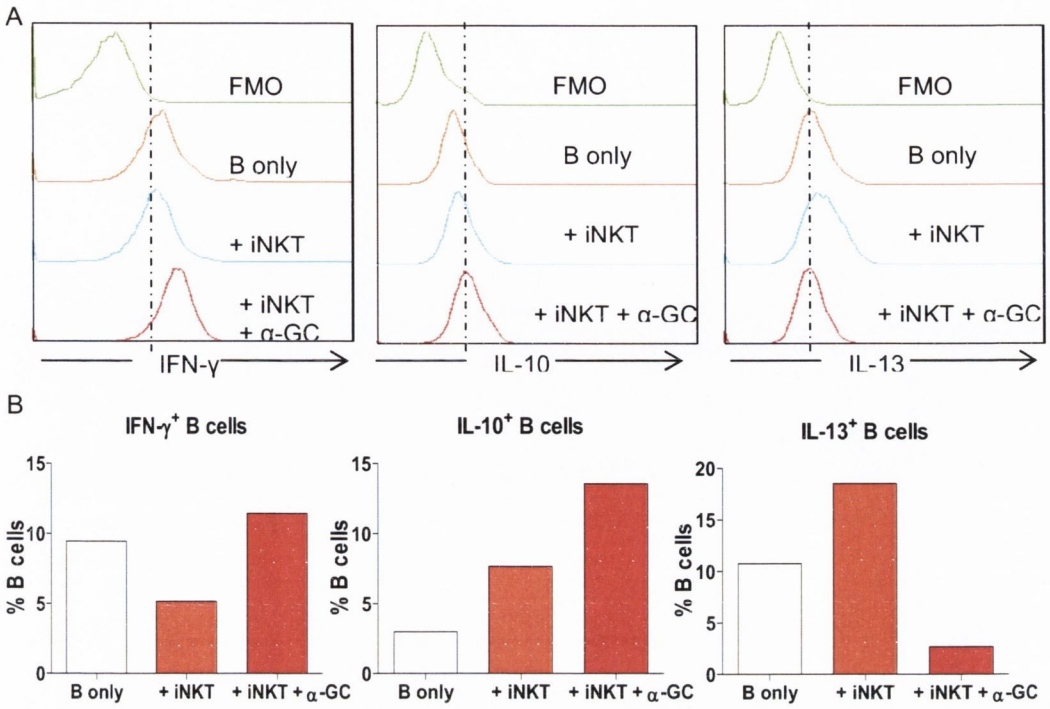

Figure 5.3.6. CD4$^+$ iNKT cells induce cytokine production by B cells. Total B cells were co-cultured with CD4$^+$ iNKT cells for 3 days in the absence or presence of α-GC and total B cells were assayed for intracellular cytokine production using flow cytometry. As negative controls, unstimulated B cells were cultured in medium only for the same duration. (A) Histogram overlays of total B cells expressing IFN-γ, IL-10 and IL-13. (B) The percentage of total B cells expressing IFN-γ, IL-10 and IL-13 are presented as bars. N=1.

In contrast, the expanded CD4$^+$ iNKT cells were largely IFN-γ$^+$, but switched to IL-13 production when co-cultured with B cells in the absence of α-GC. In the presence of α-GC, IL-13 production decreased and the proportion of IL-10$^+$ iNKT cells increased slightly. (**Figure 5.3.7**) In this experiment, we observed that the CD4$^+$ iNKT cells switch from a Th1 to a Th2 cytokine production profile when co-cultured with B cells, and cytokine production decreased in the presence of α-GC.

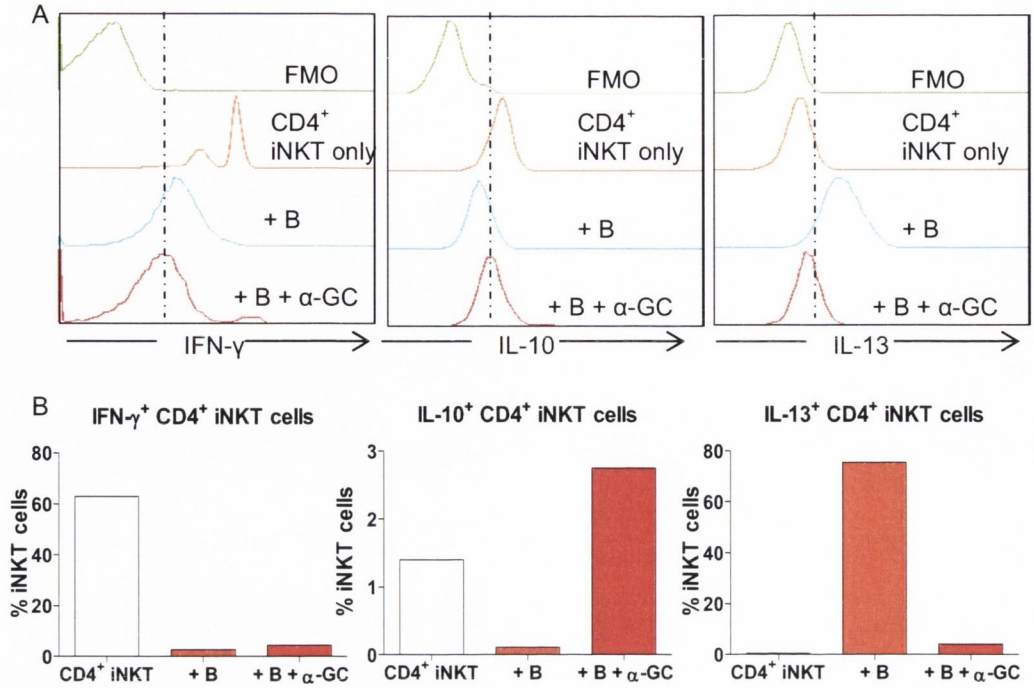

Figure 5.3.7. CD4$^+$ iNKT cells switch from Th1 to Th2 cytokine production when co-cultured with B cells. Total B cells were co-cultured with CD4$^+$ iNKT cells for 3 days in the absence or presence of α-GC and iNKT cells were assayed for intracellular cytokine production using flow cytometry. As negative controls, unstimulated CD4$^+$ iNKT cells were cultured in medium only for the same duration. (A) Histogram overlays of CD4$^+$ iNKT cells expressing IFN-γ, IL-10 and IL-13. (B) The percentage of CD4$^+$ iNKT cells expressing IFN-γ, IL-10 and IL-13 are presented as bars. N=1.

A closer look at the results of this experiment showed that a large proportion (approximately 60%) of the expanded CD4$^+$ iNKT cells consisted of single IFN-γ-producers. Single IL-10$^+$ and double IFN-γ$^+$IL-10$^+$ CD4$^+$ iNKT populations were approximately 1% each of the total CD4$^+$ iNKT population, with the rest not producing any cytokines (36%).

Upon co-culture with B cells in the absence of α-GC, the skew in IFN-γ production by the CD4$^+$ iNKT cells was shifted to IL-13 production (73%) and IL-10 production was lost. A population of double IL-13$^+$IFN-γ$^+$ CD4$^+$ iNKT cells emerged (3%). When α-GC was present, proportions of dual- and single- cytokine producers were all decreased, with a restoration of single IL-10$^+$ (2%) and double IFN-γ$^+$IL-10$^+$ (2%) CD4$^+$ iNKT cells. As a consequence of B cell interaction in the presence of α-GC, 89% of CD4$^+$ iNKT cells became non-cytokine producers in this experiment. (**Figure 5.3.8** and **Table 5.3.1**)

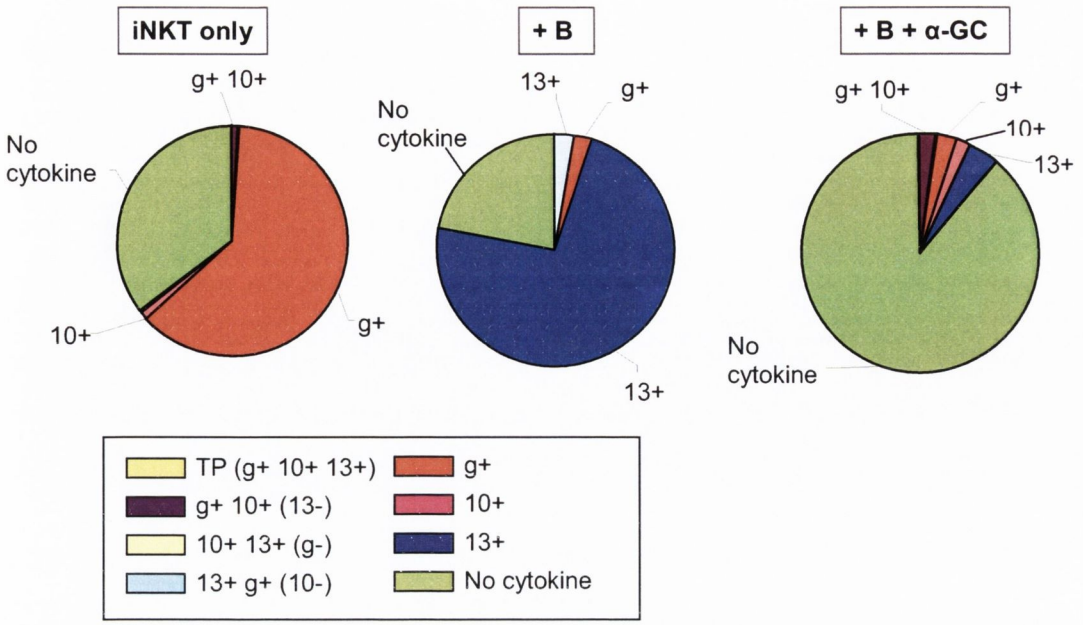

Figure 5.3.8. iNKT cells switch from IFN-γ to IL-13 production when co-cultured with B cells in the absence of α-GC, but downregulate cytokine production in the presence of α-GC. Total B cells were co-cultured with $CD4^+$ iNKT cells for 3 days in the absence (middle) or presence (right) of α-GC and iNKT cells were assayed for intracellular cytokine production using flow cytometry. As negative controls, $CD4^+$ iNKT cells were cultured in medium only for the same duration (left). The percentage of $CD4^+$ iNKT cells expressing one cytokine, two cytokines and three cytokines simultaneously are depicted as pie charts. Key in legend: "g" represents IFN-γ, "10" represents IL-10 and "13" represents IL-13, "TP" represents 'triple-positive', i.e. IFN-γ^+IL-10^+ IL-13^+ $CD4^+$ iNKT cells. N=1.

Table 5.3.1. The proportions of iNKT cells expressing triple cytokines, dual cytokines, single cytokines and no cytokines. N=1.

	Triple cytokine	Dual cytokine			Single cytokine			
% of total iNKT cells	IFN-γ^+ IL-10^+ IL-13^+	IFN-γ^+ IL-10^+ IL-13^-	IL-10^+ IL-13^+ IFN-γ^-	IL-13^+ IFN-γ^+ IL-10^-	IFN-γ^+	IL-10^+	IL-13^+	No cytokine
iNKT only	0.18	1.00	0.04	0.11	61.79	1.18	0.16	35.54
+ B	0.06	0.00	0.04	2.60	2.71	0.01	72.76	21.83
+ B + α-GC	0.07	2.15	0.35	0.04	2.31	2.34	4.01	88.73

Proportions are expressed as a percentage of total iNKT cells.

5.3.1.5 $CD4^+$ iNKT cells switch $CD1d^{hi}CD5^+$ and $CD24^{hi}CD38^{hi}$ B cells from a Th2 to Th1/Treg cytokine profile in the presence of α-GC.

As $CD4^+$ iNKT cells were previously shown to be able to induce a distinct $CD1d^{hi}CD5^{hi}$ B cell population and work by others indicate that $CD4^+$ iNKT cells may predispose the immune system towards a Th2 response, it was hypothesized that they may also assist Breg cells in their function (such as IL-10 production). Hence intracellular cytokine (IL-10, IL-13 and IFN-γ) production by $CD1d^{hi}CD5^+$ and $CD24^{hi}CD38^{hi}$ B cells in the $CD4^+$ iNKT-B cell co-culture was also analysed by flow cytometry. Findings from this section are also preliminary as the experiment was performed only once.

Similar to results seen previously with total B cells, co-culture with $CD4^+$ iNKT cells in the absence of α-GC decreased the frequencies of IFN-γ-producing $CD1d^{hi}CD5^+$ B cells but increased frequencies of IL-10- and IL-13-producing $CD1d^{hi}CD5^+$ B cells. In this experiment the presence of α-GC increased the frequencies of IFN-γ- and IL-10-producing $CD1d^{hi}CD5^+$ B cells but decreased frequencies of IL-13-producing $CD1d^{hi}CD5^+$ B cells, suggesting α-GC causes a shift from Th2 to Th1/Treg cytokine profile in the $CD1d^{hi}CD5^+$ B cells. (**Figure 5.3.9**A and B)

Subsequently we took a closer look at the combinations of triple-, dual- and single-cytokine-producing $CD1d^{hi}CD5^+$ B cells in the experiment. Unstimulated $CD1d^{hi}CD5^+$ B cells expressed a mix of triple, dual and single cytokines, with most of them being single $IL\text{-}13^+$ or $IFN\text{-}\gamma^+$. 20% of the unstimulated $CD1d^{hi}CD5^+$ B cells did not produce any cytokines. When co-cultured with $CD4^+$ iNKT cells in the absence of α-GC, the proportion of $IL\text{-}10^+$ and $IL\text{-}13^+$ $CD1d^{hi}CD5^+$ B cells doubled and $IL\text{-}10^+IL\text{-}13^+$ $CD1d^{hi}CD5^+$ B cells tripled. However, proportions of $IFN\text{-}\gamma^+IL\text{-}10^+IL\text{-}13^+$, $IFN\text{-}\gamma^+$ $IL\text{-}13^+$ and $IFN\text{-}\gamma^+$ $CD1d^{hi}CD5^+$ B cells decreased. (**Figure 5.3.9**C and **Table 5.3.2**)

When α-GC was added to the $CD4^+$ iNKT-B cell co-cultures, proportions of triple-expressing ($IFN\text{-}\gamma^+IL\text{-}10^+IL\text{-}13^+$), dual-expressing ($IFN\text{-}\gamma^+IL\text{-}10^+$ and $IFN\text{-}\gamma^+IL\text{-}13^+$) and single-expressing ($IFN\text{-}\gamma^+$ and $IL\text{-}10^+$) $CD1d^{hi}CD5^+$ B cells increased with the exception of $IL\text{-}10^+IL\text{-}13^+$ and $IL\text{-}13^+$ $CD1d^{hi}CD5^+$ B cells, which decreased by 1.3-fold and 3-fold respectively. (**Figure 5.3.9**C and **Table 5.3.2**) These data suggest that in this experiment, $CD4^+$ iNKT cells, in the absence of α-GC, prime $CD1d^{hi}CD5^+$ B cells towards a Th2/Treg response. However in the presence of α-GC, the $CD1d^{hi}CD5^+$ B cells revert back to a Th1/Treg response.

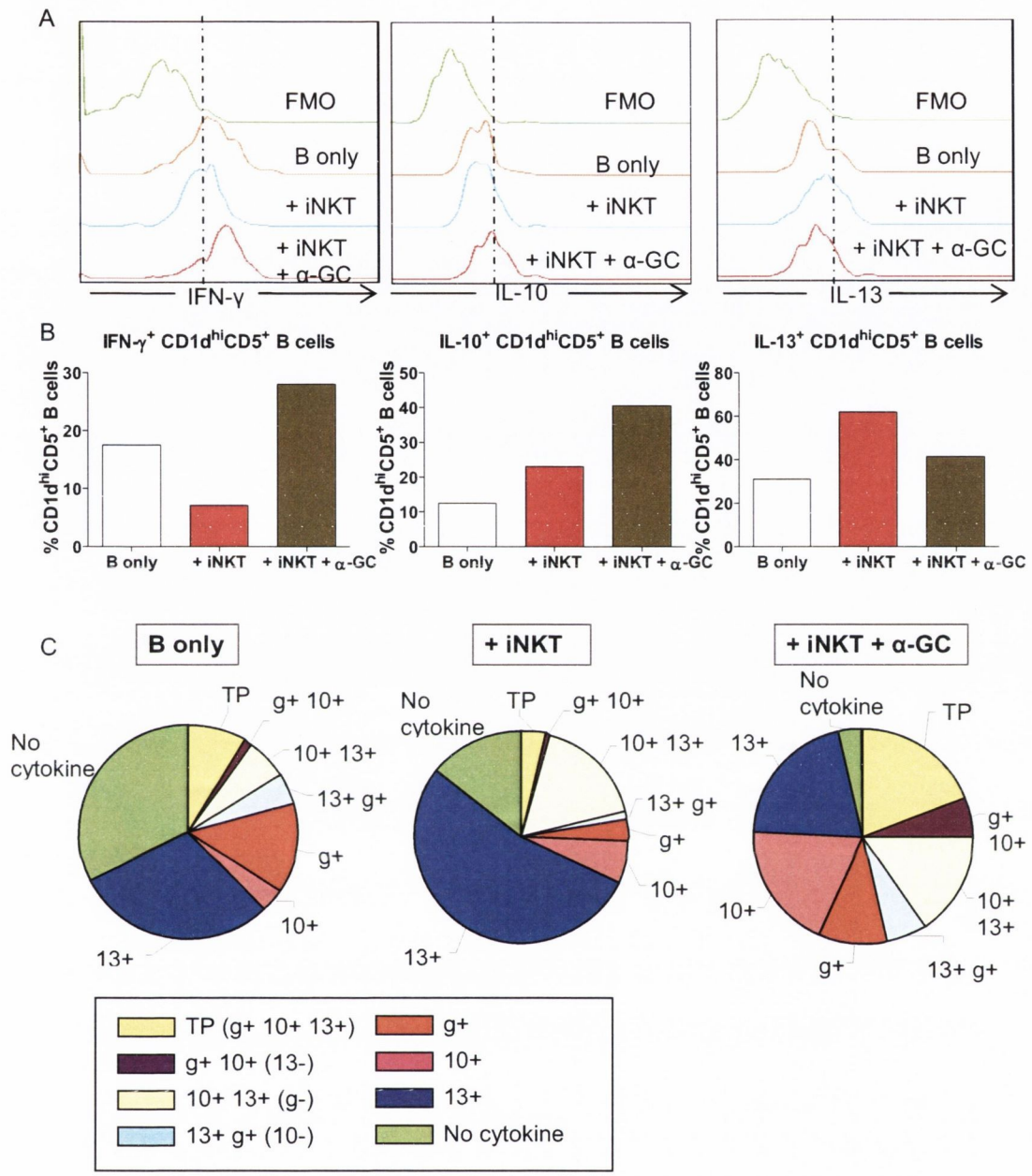

Figure 5.3.9. CD4$^+$ iNKT cells switched CD1d^{hi}CD5$^+$ B cells from a Th2 to a Th1/Treg cytokine profile in the presence of α-GC. Total B cells were co-cultured with CD4$^+$ iNKT cells for 3 days in the absence or presence of α-GC and CD1d^{hi}CD5$^+$ B cells were assayed for intracellular cytokine production using flow cytometry. As negative controls, unstimulated B cells were cultured in medium only for the same duration. (A) Histogram overlays of CD1d^{hi}CD5$^+$ B cells expressing IFN-γ, IL-10 and IL-13. (B) Bar charts depicting the percentages of IFN-γ$^+$, IL-10$^+$ and IL-13$^+$ CD1d^{hi}CD5$^+$ B cells. (C) The percentage of CD1d^{hi}CD5$^+$ B cells expressing one cytokine, two cytokines and three cytokines simultaneously are depicted as pie charts. Key in legend: "g" represents IFN-γ, "10" represents IL-10 and "13" represents IL-13, "TP" represents 'triple-positive', i.e. IFN-γ$^+$IL-10$^+$ IL-13$^+$ CD1d^{hi}CD5$^+$ B cells. N=1.

Table 5.3.2. The proportions of $CD1d^{hi}CD5^+$ B cells expressing IFN-γ, IL-13, IL-10 in different combinations as a result of $CD4^+$ iNKT help (with or without α-GC). N=1.

	Triple cytokine	Dual cytokine			Single cytokine			
% of total $CD1d^{hi}CD5^+$ B cells	IFN-γ^+ IL-10^+ IL-13^+	IFN-γ^+ IL-10^+	IL-10^+ IL-13^+	IL-13^+ IFN-γ^+	IFN-γ^+	IL-10^+	IL-13^+	No cytokine
B only	5.65	0.56	3.95	2.82	8.47	2.26	18.64	20.34
+ iNKT	3.05	0.68	13.90	1.02	2.37	5.42	44.07	11.86
+ iNKT + α-GC	13.00	4.00	10.50	4.00	7.00	13.00	14.00	2.50

Proportions are expressed as a percentage of total $CD1d^{hi}CD5^+$ B cells.

In this experiment, $CD24^{hi}CD38^{hi}$ B cells were similar to $CD1d^{hi}CD5^+$ B cells in that $CD4^+$ iNKT cells in the absence of α-GC increased frequencies of IL-10- and IL-13-producing cells but decreased frequencies of IFN-γ-producing cells. However in the presence of α-GC, IL-13-producing cells were diminished, IL-10-producing cells increased whereas IFN-γ-producing cells were unchanged. (**Figure 5.3.10**A and B)

A closer look at the results of this experiment showed that in comparison to $CD1d^{hi}CD5^+$ B cells, a large proportion (77%) of unstimulated $CD24^{hi}CD38^{hi}$ B cells did not produce any cytokine, and co-culture with $CD4^+$ iNKT cells did not result in much fluctuation in the proportion of non-cytokine producers regardless of the presence of α-GC. Co-culture with $CD4^+$ iNKT cells in the absence of α-GC increased the proportions of IL-10^+IL-13^+ and IL-13^+ $CD24^{hi}CD38^{hi}$ B cells by 3-fold and 2-fold respectively, whilst decreasing proportions of all other cytokine-producing $CD24^{hi}CD38^{hi}$ B cells. The presence of α-GC diminished all combinations of triple-, double- and single-cytokine-expressing $CD24^{hi}CD38^{hi}$ B cells containing IL-13. Only IFN-γ^+IL-10^+ and IL-10^+ $CD24^{hi}CD38^{hi}$ B cells were increased by 6-fold and 4-fold respectively. (**Figure 5.3.10**C and **Table 5.3.3**) Similar to $CD1d^{hi}CD5^+$ B cells, these data suggest that in this experiment $CD4^+$ iNKT cells prime $CD24^{hi}CD38^{hi}$ B cells towards a Th2 response in the absence of α-GC, but revert them back to a Th1/Th2 response in the presence of α-GC.

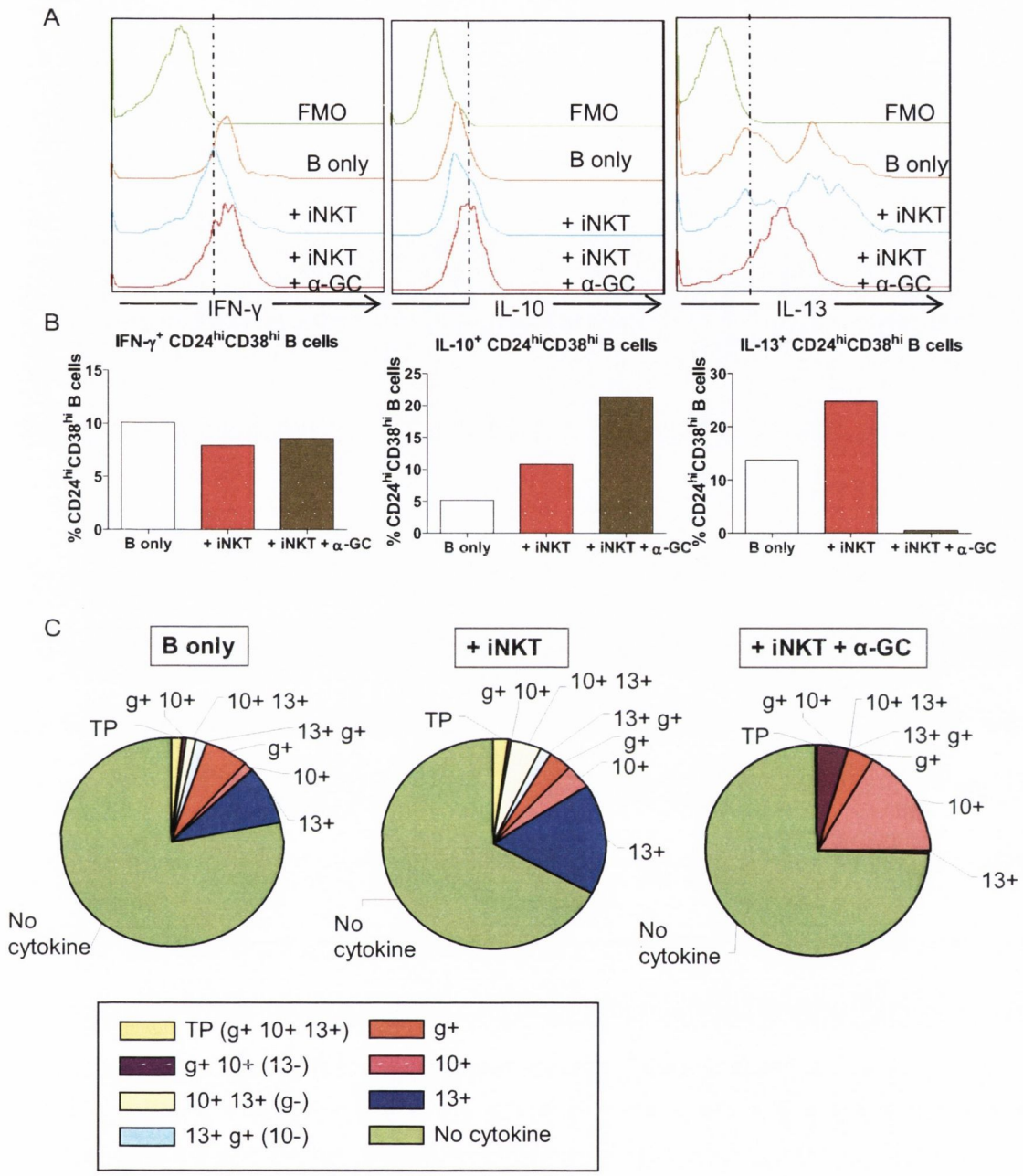

Figure 5.3.10. CD4$^+$ iNKT cells switched CD24^{hi}CD38^{hi} B cells from a Th2 to a Th1/Th2 cytokine profile in the presence of α-GC. Total B cells were co-cultured with CD4$^+$ iNKT cells for 3 days in the absence or presence of α-GC and CD24^{hi}CD38^{hi} B cells were assayed for intracellular cytokine production using flow cytometry. As negative controls, unstimulated B cells were cultured in medium only for the same duration. (A) Histogram overlays of CD24^{hi}CD38^{hi} B cells expressing IFN-γ, IL-10 and IL-13. (B) Bar charts depicting the percentages of IFN-γ$^+$, IL-10$^+$ and IL-13$^+$ CD24^{hi}CD38^{hi} B cells. (C) The percentage of CD24^{hi}CD38^{hi} B cells expressing one cytokine, two cytokines and three cytokines simultaneously are depicted as pie charts. Key in legend: "g" represents IFN-γ, "10" represents IL-10 and "13" represents IL-13, "TP" represents 'triple-positive', i.e. IFN-γ$^+$IL-10$^+$ IL-13$^+$ CD24^{hi}CD38^{hi} B cells. N=1.

Table 5.3.3. The proportions of $CD24^{hi}CD38^{hi}$ Breg cells expressing IFN-γ, IL-13, IL-10 in different combinations as a result of $CD4^+$ iNKT help (with or without α-GC). N=1.

	Triple cytokine	Dual cytokine			Single cytokine			
% of total $CD24^{hi}CD38^{hi}$ B cells	IFN-γ^+ IL-10^+ IL-13^+	IFN-γ^+ IL-10^+	IL-10^+ IL-13^+	IL-13^+ IFN-γ^+	IFN-γ^+	IL-10^+	IL-13^+	No cytokine
B only	1.72	0.43	1.50	1.50	6.44	1.50	9.01	77.90
+ iNKT	2.19	0.69	4.39	1.51	3.57	3.57	16.74	67.35
+ iNKT + α-GC	0.23	4.38	0.06	0.12	3.86	16.72	0.12	74.51

Proportions are expressed as a percentage of total $CD24^{hi}CD38^{hi}$ B cells.

5.3.2 Co-culture of B cells with $CD4^+$ or DN iNKT but not $CD8^+$ iNKT results in production of IL-4, TNF-α, IFN-γ, IL-5 and IL-13.

To examine whether the iNKT cell subsets, when co-cultured with B cells, would result in a bias in cytokine production, supernatants from 3-day co-cultures of $CD4^+$, $CD8^+$ or DN iNKT cells with B cells (in the absence or presence of α-GC) were analysed by multiplex cytometric bead arrays for secretion of IFN-γ, IL-2, IL-4, TNF-α, IL-5, IL-10 and IL-13. Supernatants of negative controls (B cells or iNKT cells in medium alone) were also analysed.

Basal amounts of IL-2 were produced by all three iNKT subsets as well as by B cells, and the amount of IL-2 remained unchanged when the iNKT cells were co-cultured with B cells and when α-GC was present. Levels of IL-4, TNF-α, IFN-γ, IL-5 and IL-13 released in co-cultures of B cells with $CD4^+$, $CD8^+$ or DN iNKT cells but without α-GC were similar to those in supernatants of B cells or iNKT cells only. However, when α-GC was present in co-cultures of B cells with $CD4^+$ iNKT cells or DN iNKT cells, levels of IL-4, TNF-α, IFN-γ, IL-5 and IL-13 production were enhanced. These cytokines were produced only at very low levels when $CD8^+$ iNKT cells were co-cultured with B cells in the presence of α-GC. IL-10 production was not observed for all three iNKT subsets, except for DN iNKT cells in an individual experiment (figure not shown). (**Figure 5.3.11**)

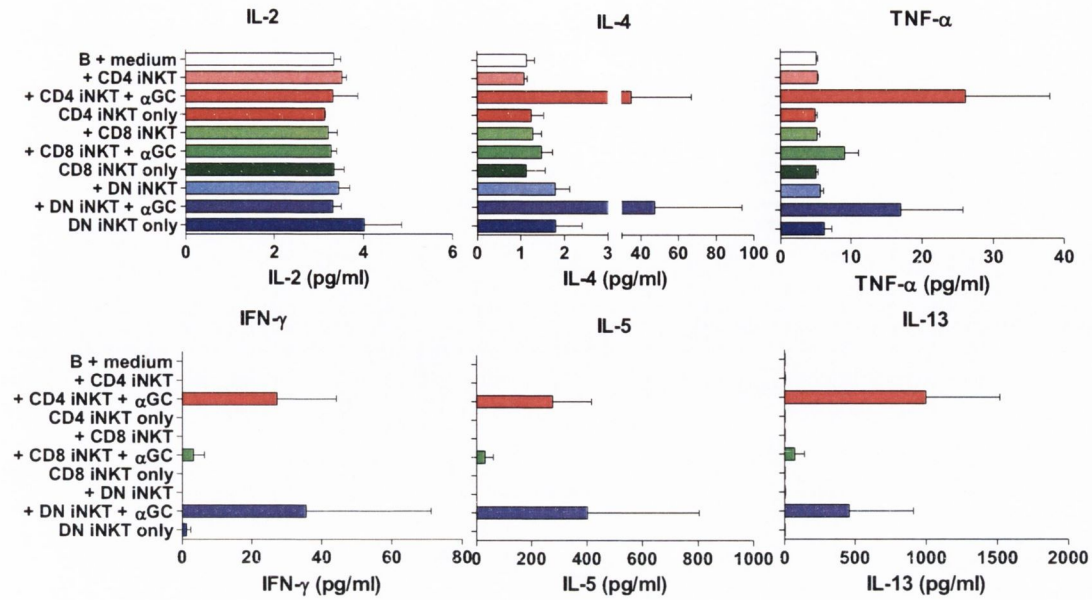

Figure 5.3.11. Co-culture of B cells with CD4$^+$ or DN, but not CD8$^+$ iNKT cells resulted in copious production of IL-4, TNF-α, IFN-γ, IL-5 and IL-13. Total B cells were co-cultured with CD4$^+$, CD8$^+$ or DN iNKT cells for 3 days in the absence or presence of α-GC. As negative controls, B cells, CD4$^+$, CD8$^+$ and DN iNKT cells were each cultured separately in medium only for the same duration. Supernatants were analysed by multiplex CBA for IL-2, IL-4, TNF-α, IFN-γ, IL-5, IL-13 and IL-10. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Kruskal-Wallis test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

5.3.3 CD4$^+$, CD8$^+$ and DN iNKT cells in the absence of α-GC can induce IgM, IgA and IgG production by B cells.

In the previous chapter we showed that polyclonal iNKT cells can induce IgM, IgA and IgG production by B cells. To examine if co-cultures of iNKT cell subsets with B cells would induce similar antibody production or selectively induce particular antibody isotypes, supernatants from 10-day co-cultures of CD4$^+$, CD8$^+$ or DN iNKT cells with B cells (in the absence or presence of α-GC) were analysed by multiplex cytometric bead arrays for secretion of IgM, IgA, total IgG and IgG_2. Supernatants of negative controls (B cells or iNKT cells in medium alone) were also analysed.

CD4$^+$, CD8$^+$ and DN iNKT cells induced the production of IgM, IgA, total IgG and IgG_2 production by B cells. However the CD8$^+$ iNKT cells were most efficient at inducing IgG_2 production. Addition of α-GC reduced levels of antibody production induced by all three iNKT cell subsets, with the exception of total IgG and IgG_2 production induced by CD4$^+$

iNKT cells. α-GC had a markedly potent effect on reducing antibody production induced by $CD8^+$ iNKT cells. (**Figure 5.3.12**)

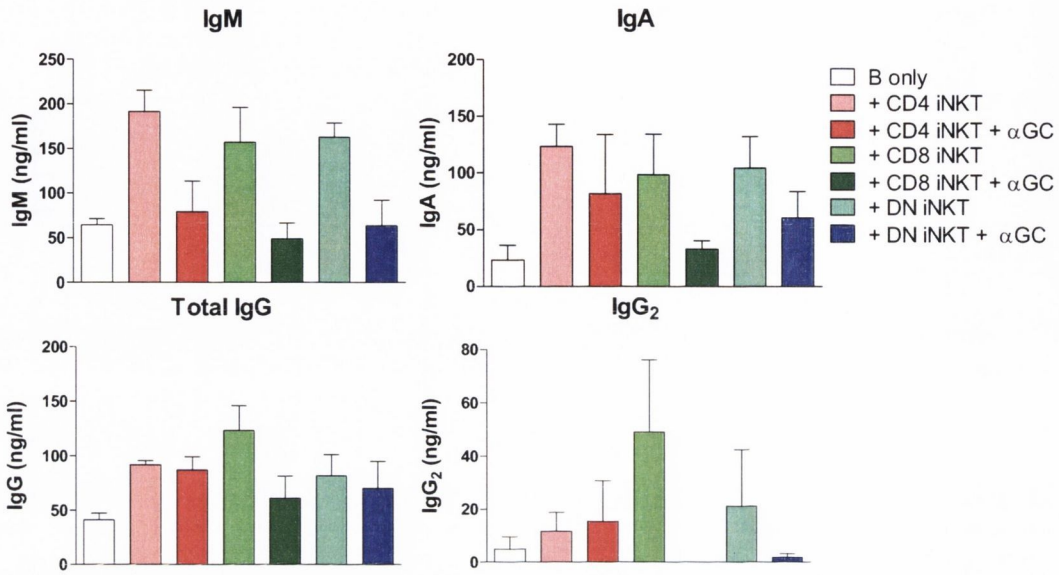

Figure 5.3.12. $CD4^+$, $CD8^+$ and DN iNKT cells in the absence of α-GC can induce IgM, IgA and IgG production by B cells. Total B cells were co-cultured with $CD4^+$, $CD8^+$ or DN iNKT cells for 3 days in the absence or presence of α-GC. As negative controls, B cells, $CD4^+$, $CD8^+$ and DN iNKT cells were each cultured separately in medium only for the same duration. Supernatants were analysed by multiplex CBA for IgM, IgA, total IgG and IgG_2. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test.

5.3.4 Investigation of the role of CD1d, IL-4, IL-13, and the CD40-CD154 interaction in iNKT help to B cells.

In the previous chapter we observed that iNKT cells upregulated activation markers (such as CD40 and CD86) and induced antibody production by B cells. The presence of α-GC enhanced expression of activation markers but decreased iNKT-induced B cell antibody production. To examine the mechanics underlying the interaction between the iNKT cells and B cells, the role of CD1d, IL-4, IL-13, CD40 and CD154 (also known as CD40 ligand) was investigated by using antibodies against these molecules to block them.

B cells and expanded iNKT cells were co-cultured in equal numbers for 3 days and 10 days and 10 μg/ml of antibodies against CD1d, IL-4, IL-13, CD40 or CD154 were added to the iNKT – B cell co-cultures in independent experiments. As negative controls, iNKT cells and B cells were separately cultured in medium only for the same number of days. iNKT and B cells were also separately cultured in medium with 10 μg/ml of blocking

antibodies as an additional control for any effect that the antibodies may exert on the cells. After 3 days, flow cytometric analysis of changes in mean fluorescence intensity (MFI) of activation and co-stimulatory molecules on B cells was performed. Supernatants from the co-cultures were also removed at 3 days and 10 days and assayed for cytokine and immunoglobulin production respectively using multiplex cytometric bead arrays.

5.3.5 iNKT cell help to B cells is CD1d-dependent.

5.3.5.1 Anti-CD1d mAb abrogated iNKT-mediated increases in CD40 and CD83 expression on B cells.

Similar to previous results (section 4.3.3), we observed an upregulation of CD40 and CD83 expression on B cells when they were co-cultured with iNKT cells as compared to B cells cultured in medium alone. When anti-CD1d mAb was added, there was a downregulation of CD40 and CD83, relative to iNKT – B cell co-cultures. The reduced detection of CD1d expression by B cells when anti-CD1d mAb were added showed that CD1d was blocked by the antibodies (**Figure 5.3.13**).

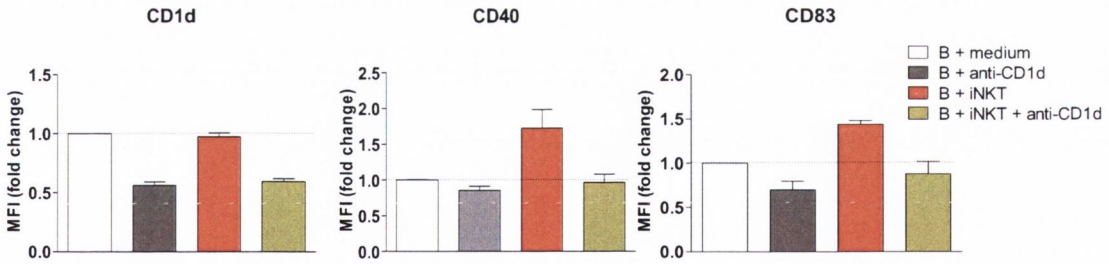

Figure 5.3.13. Anti-CD1d mAb abrogated iNKT-mediated increases in CD40 and CD83 expression on B cells. Flow cytometric analysis of MFI of activation and co-stimulatory markers on unstimulated B cells (white bars), B cells incubated with anti-CD1d mAb (grey bars), B cells incubated with iNKT cells (orange bars) and B cells incubated with both iNKT cells and anti-CD1d mAb (olive bars). Bars are plotted as fold change relative to unstimulated B cells. Cells were incubated for three days. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

5.3.5.2 Blocking CD1d inhibits IgG_1, IgM and IgA production but not cytokine production in iNKT help to B cells.

Supernatants from 10-day co-cultures were also analysed for IgG_1, IgG_2, IgM and IgA production. We observed that the iNKT cell-mediated increase in IgG_1, IgM and IgA production was inhibited when anti-CD1d mAb were added. There was no IgG_2 production in any of the co-cultures except in one experiment. (**Figure 5.3.14**) Anti-CD1d mAb had no effect on cytokine production in the co-cultures (Appendix 9.7). These results suggest that CD1d is required for iNKT-induced antibody production.

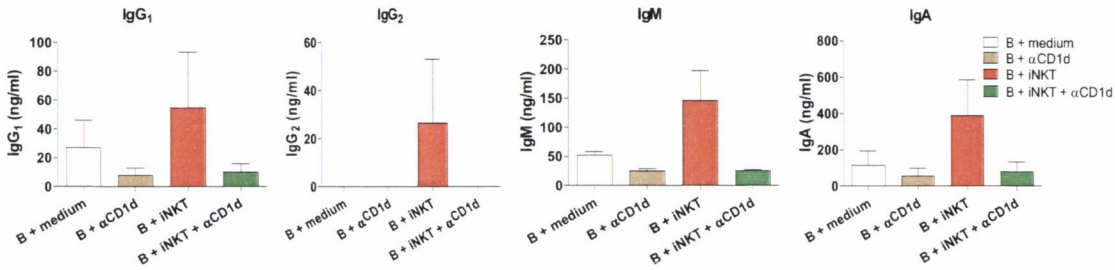

Figure 5.3.14. Addition of anti-CD1d mAb inhibited IgG_1, IgM and IgA production in iNKT – B cell co-cultures. Supernatants collected after ten days from the iNKT-B cell co-cultures were analysed using multiplex cytometric bead arrays for IgG_1, IgG_2, IgM and IgA. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

5.3.6 iNKT – B cell help is not dependent on IL-4.

5.3.6.1 Anti-IL-4 mAb did not inhibit the iNKT-induced upregulation of CD86, CD40 and CD83 expression on B cells.

B cells were also co-cultured with iNKT cells in the presence of anti-IL-4 mAb and flow cytometric analysis of activation markers on B cells was carried out after 3 days. Co-culturing B cells with iNKT cells resulted in upregulation of CD40, CD83 and CD86 expression on the B cells. However, addition of anti-IL-4 mAb had no significant effect on the expression of any of these markers. (**Figure 5.3.15**)

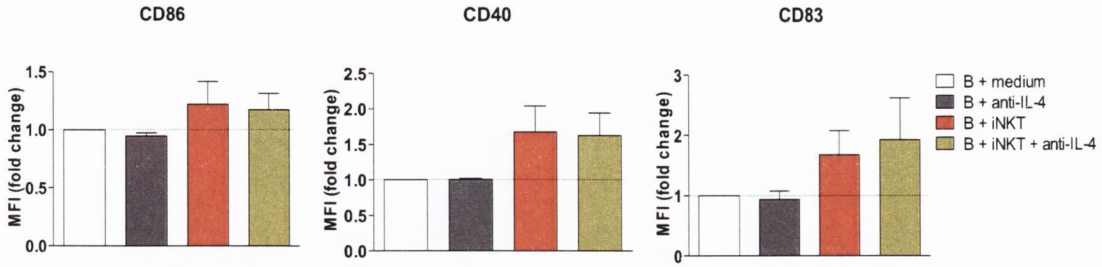

Figure 5.3.15. Anti-IL-4 mAb did not inhibit the iNKT-induced upregulation of CD86, CD40 and CD83 on B cells. Flow cytometric analysis of MFI of activation and co-stimulatory markers on unstimulated B cells (white bars), B cells incubated with anti-IL-4 mAb (grey bars), B cells incubated with iNKT cells (orange bars) and B cells incubated with both iNKT cells and anti-IL-4 mAb (olive bars). Cells were incubated for three days. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using the Friedman's test with post-hoc Dunn's multiple comparison test. No significant changes in expression of CD86, CD40 and CD83 were observed when IL-4 was blocked during iNKT – B cell incubations.

5.3.6.2 Anti-IL-4 mAb had no significant effect on iNKT-induced antibody or cytokine production by B cells.

Addition of anti-IL-4 mAb to iNKT-B cell co-cultures did not lead to changes in IgG_1, IgG_2, IgM and IgA production as compared to iNKT-B cell co-cultures without anti-IL-4 mAb. (**Figure 5.3.16**) Anti-IL-4 mAb did not influence cytokine production in the co-cultures (Appendix 9.8).

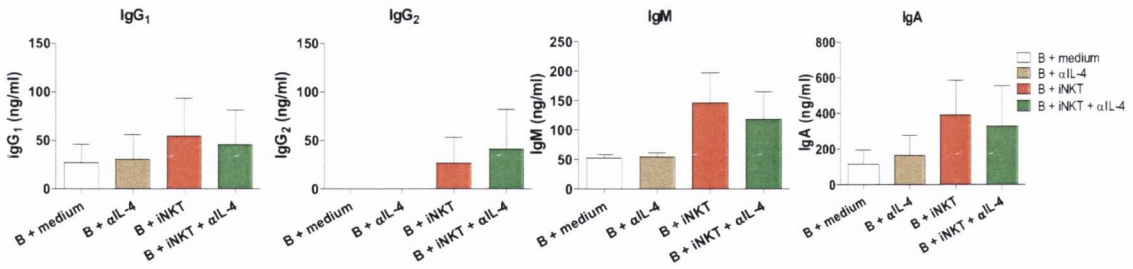

Figure 5.3.16. Addition of anti-IL-4 mAb had no significant effect on antibody production in iNKT – B cell co-cultures. Supernatants collected after ten days from the iNKT-B cell co-cultures were analysed using multiplex cytometric bead arrays for IgG_1, IgG_2, IgM and IgA. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test. No significant changes in antibody production were observed when IL-4 was blocked during iNKT – B cell incubations.

5.3.7 iNKT – B cell help is dependent on IL-13.

5.3.7.1 Anti-IL-13 mAb inhibited the iNKT-induced upregulation of CD86, CD40 and CD83 expression on B cells.

B cells were co-cultured with iNKT cells in the presence of anti-IL-13 mAb and flow cytometric analysis of activation markers on the B cells was carried out after 3 days. iNKT help to B cells resulted in slight upregulation of CD86, CD40 and CD83, all of which were downregulated when anti-IL-13 mAb was added to the co-cultures. (**Figure 5.3.17**)

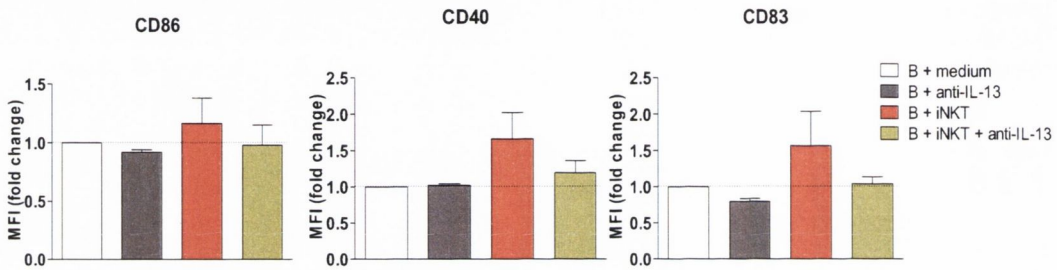

Figure 5.3.17. Anti-IL-13 mAb downregulated the iNKT-induced upregulation of CD86, CD40 and CD83 expression on B cells. Flow cytometric analysis of MFI of activation and co-stimulatory markers on unstimulated B cells (white bars), B cells incubated with anti-IL-13 mAb (grey bars), B cells incubated with iNKT cells (orange bars) and B cells incubated with both iNKT cells and anti-IL-13 mAb (olive bars). Cells were incubated for three days. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

5.3.7.2 Anti-IL-13 mAb had no effect on antibody production but slightly decreased IL-6 production in iNKT help to B cells.

Addition of anti-IL-13 mAb to iNKT-B cell co-cultures did not inhibit antibody production by B cells as a consequence of iNKT help. (**Figure 5.3.18**) Anti-IL-13 mAb moderately decreased the enhanced IL-6 production in the co-cultures (Appendix 9.9).

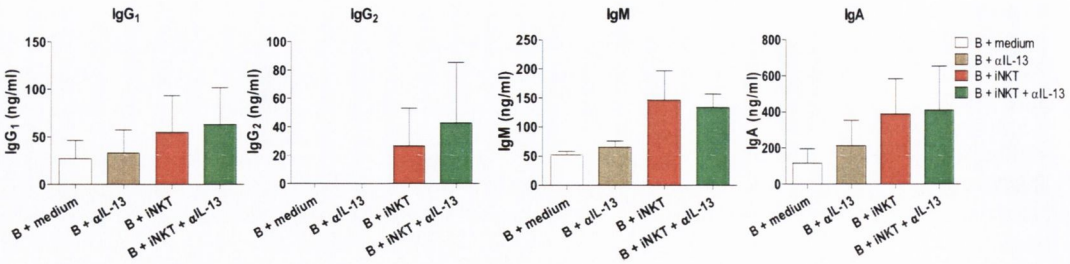

Figure 5.3.18. Anti-IL-13 mAb had no effect on antibody production in the iNKT – B cell co-cultures. Supernatants collected after ten days from the iNKT-B cell co-cultures were analysed using multiplex cytometric bead arrays for IgG_1, IgG_2, IgM and IgA. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

5.3.8 iNKT – B cell help is dependent on CD40-CD154 interaction between the cells.

To investigate the role of the CD40-CD154 interaction between the B cell and the iNKT cell, we used monoclonal antibodies targeted against CD40 and CD154 to block CD40 on the B cells and CD154 on the iNKT cells separately.

5.3.8.1 Anti-CD40 mAb upregulated iNKT-induced CD86 expression whereas anti-CD154 mAb downregulated CD40 and CD86 expression on total B cells.

iNKT cells in the presence of α-GC induced upregulation of CD40 and CD86 expression by B cells. The iNKT-enhanced CD40 expression on total B cells was partially inhibited when either anti-CD40 or anti-CD154 mAb were added to iNKT-B cell co-cultures. However, anti-CD154 mAb but not anti-CD40 mAb partially inhibited iNKT-enhanced CD86 expression. Reduced CD40 expression by B cells in the presence of anti-CD40 mAb could be due to blocking of secondary antibody binding. As CD40 signalling in B cells results in upregulation of CD86 expression (Yang and Wilson, 1996), this suggests that unlike the anti-CD154 mAb, the anti-CD40 mAb had an agonistic effect and stimulated the B cells instead of inhibiting the CD40-CD154 interaction between the B cells and iNKT cells. (**Figure 5.3.19**)

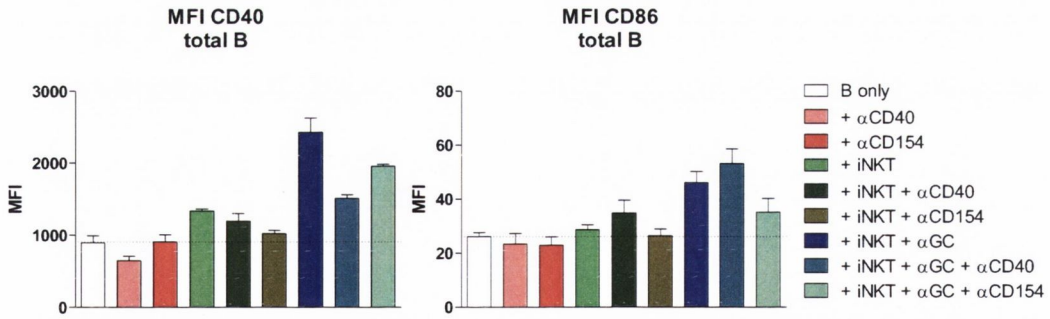

Figure 5.3.19. Anti-CD40 mAb upregulated iNKT-induced CD86 expression whereas anti-CD154 mAb downregulated iNKT-induced CD40 and CD86 expression on B cells. Total B cells were analysed by flow cytometry after three days of B cell co-culture with iNKT cells and blocking antibodies. Graphs show changes in MFI of CD40 and CD86 expression on total B cells. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

5.3.8.2 Anti-CD154 mAb dampened the iNKT-induced upregulation of CD40 and CD86 expression by regulatory B (Breg) cells, whereas anti-CD40 mAb further upregulated iNKT-induced CD86 expression.

Addition of anti-CD40 or anti-CD154 mAb to the iNKT-B cell co-cultures did not result in any significant changes in frequencies of $CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells. (**Figure 5.3.20**A)

iNKT cells in the presence of α-GC upregulated CD40 expression on all three Breg cell subsets. Similar results were seen for CD86 expression on the three Breg cell subsets. When anti-CD154 mAb were added to the co-cultures, the enhanced CD40 and CD86 expression was downregulated. However, when anti-CD40 mAb were added, CD86 expression was elevated across all three Breg cell subsets, indicating that the anti-CD40 mAb had an activating effect instead of an inhibitory effect. (**Figure 5.3.20**B) These data suggest that anti-CD154 mAb blocked iNKT-mediated Breg cell activation whereas anti-CD40 mAb enhanced iNKT-mediated Breg cell activation.

Changes in frequencies, CD40 and CD86 expression by naïve and memory B cells (unswitched, switched and $CD27^{-}IgD^{-}$) were also analysed by flow cytometry in the above blocking experiments. Similar results were seen with gated naïve and memory B cells. The frequencies of unswitched memory B cells were unchanged following incubation with anti-CD154 mAb but CD40 and CD86 expression were decreased. The frequencies and levels of CD86 expression by unswitched memory B cells were increased by adding anti-CD40 mAb to the co-cultures (Appendix 9.10). Adding anti-CD154 and anti-CD40 mAb to the co-cultures had unremarkable effects on switched memory B cells (Appendix 9.11). Anti-CD154 mAb inhibited iNKT-enhanced IgM, CD40 and CD86 expression by naïve B cells and $CD27^{-}IgD^{-}$ memory B cells (Appendix 9.12-9.13).

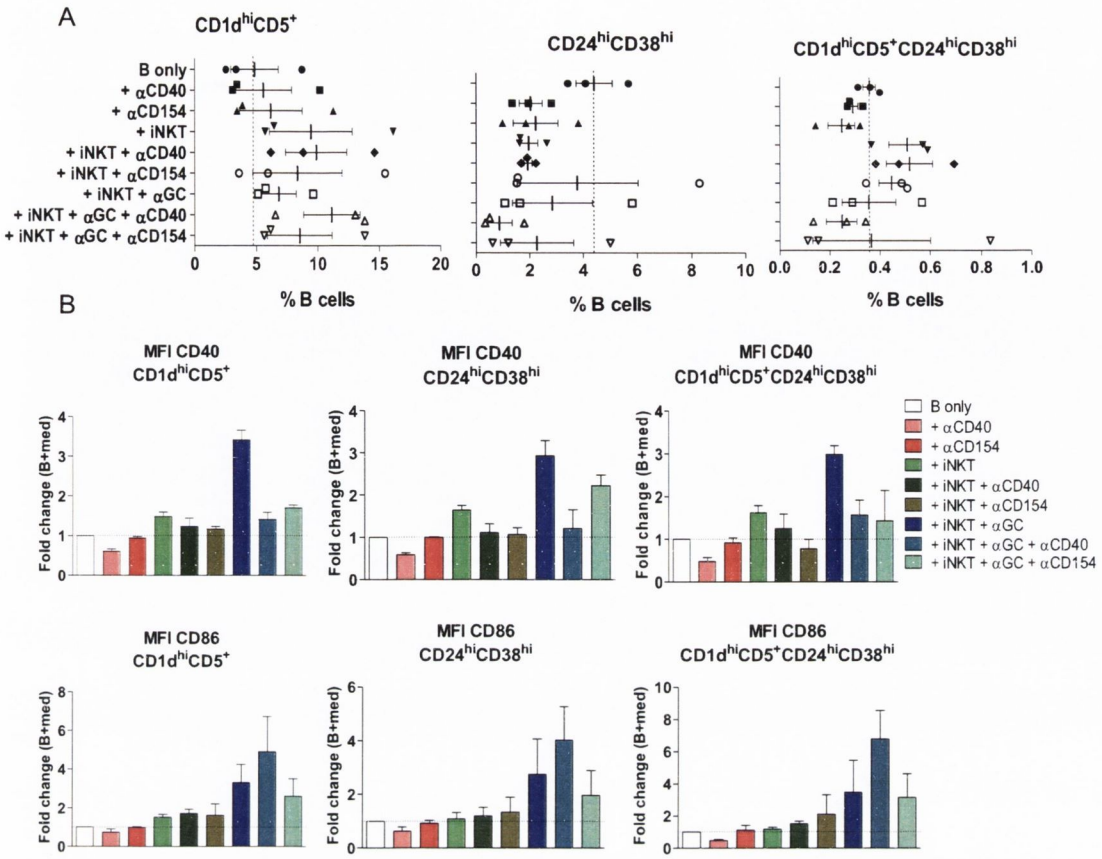

Figure 5.3.20. Anti-CD154 mAb inhibits iNKT-induced CD40 and CD86 upregulation by Breg cells, whereas anti-CD40 mAb enhances iNKT-induced CD86 upregulation on them. Three subsets of regulatory B (Breg) cells ($CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$) were analysed by flow cytometry after three days of B cell co-culture with iNKT cells and blocking antibodies. (A) Graphs showing changes in proportions of $CD1d^{hi}CD5^{+}$ (left), $CD24^{hi}CD38^{hi}$ (middle) and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ (right) Breg cells with different treatments. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on $CD1d^{hi}CD5^{+}$ (left), $CD24^{hi}CD38^{hi}$ (middle) and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ (right) Breg cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

5.3.8.3 Anti-CD154 mAb did not inhibit antibody production whereas anti-CD40 mAb enhanced antibody production in iNKT – B cell co-cultures.

Supernatants from 10-day co-cultures were also analysed for total IgG, IgG_2, IgM and IgA production. We observed that total IgG, IgG_2, IgM and IgA production by iNKT-B cell co-cultures were relatively unchanged when anti-CD154 mAb were added, except for an observed decrease in total IgG in iNKT-B cell co-cultures with α-GC. Interestingly, anti-CD40 mAb alone were sufficient to enhance antibody production from the B cells, and

this effect was more potent than that observed with iNKT help. When anti-CD40 mAb was added to iNKT-B cell co-cultures, enhanced total IgG production was observed. However, when α-GC was present, addition of anti-CD40 mAb resulted in decreases in antibody production. (**Figure 5.3.21**) The graphs suggest that anti-CD154 mAb did not inhibit antibody production, whereas anti-CD40 mAb were agonistic and enhanced antibody production.

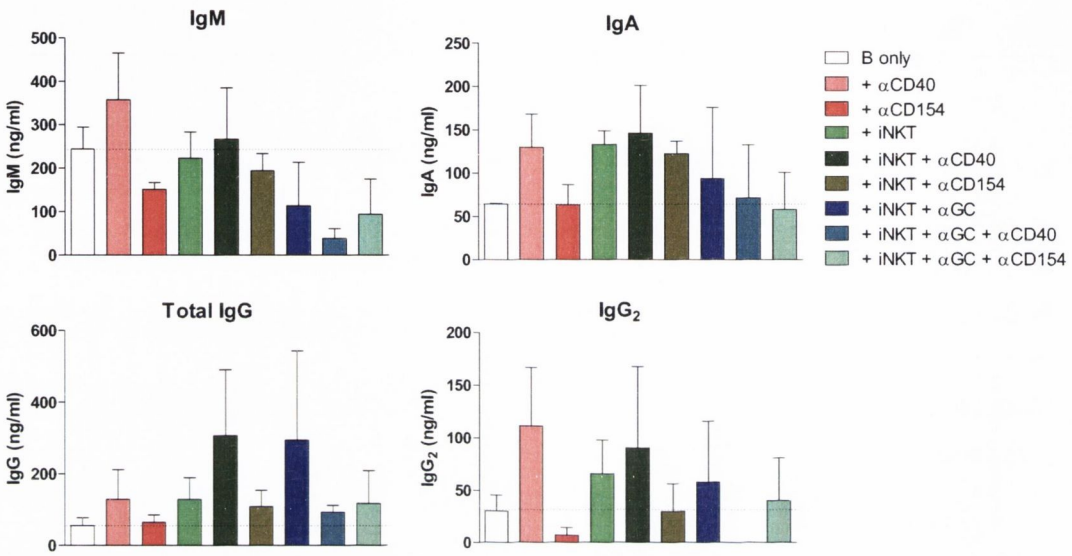

Figure 5.3.21. Anti-CD154 mAb did not inhibit antibody production, whereas anti-CD40 mAb enhanced antibody production. Supernatants collected after ten days from the iNKT-B cell co-cultures with blocking antibodies against CD154 or CD40 were analysed using multiplex cytometric bead arrays for total IgG, IgG_2, IgM and IgA. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

5.3.9 Investigating the role of the delta isoform of phosphoinositide 3-kinase (PI3Kδ) in mediating iNKT-B cell interactions.

As the delta isoform of PI3K (PI3Kδ) is known to be vital for B cell development, maintenance and proliferation, we investigated the role of PI3Kδ in mediating iNKT-B cell interactions by using a PI3Kδ-specific inhibitor, IC87114 to inhibit the function of PI3Kδ during iNKT-B cell co-culture. iNKT cells and B cells were co-cultured for 3 days either in the absence of α-GC, in presence of α-GC, or in the presence of both α-GC and IC87114. As negative controls, iNKT cells and B cells were cultured separately in medium only for the same duration. B cells were subsequently analysed by flow cytometry for changes in CD40 and CD86 expression whereas iNKT cells were

analysed for changes in CD154 expression. To look at changes in phosphorylation levels of various kinases in the B cells and iNKT cells, co-cultured cells were separated by magnetic bead separation and lysed. Cell lysates were blotted onto the phosphoMAPK array and relative changes in phosphorylation of various kinases were determined by comparing the intensities of the spots on the arrays relative to negative controls using the Photoshop image analysis software. Results from the following sections are preliminary findings as the experiments have been performed only once.

5.3.9.1 Determining IC50 of the PI3Kδ inhibitor, IC87114.

In order to determine the appropriate concentration of IC87114 to use in our experiments, freshly-isolated B cells were stimulated for 3 days with PMA and ionomycin (in replacement of activation by iNKT cells) and treated with varying concentrations of IC87114 in the range of 0 – 300 μM. The B cells were immobilized on the culture plate using poly-L-lysine and after 3 days of treatment they were fixed and permeabilised. Cell-based ELISA assaying for phosphorylated and total Akt protein was performed simultaneously on the permeabilised cells and the resulting intensity of fluorescence from each treatment was read using a fluorospectrophotometer. The fluorescence intensities of phosphorylated Akt in the cells were normalized to the corresponding fluorescence intensities of total Akt in the same cells and an inhibition curve was plotted with the GraphPad Prism software to obtain the IC50 value of IC87114. (**Figure 5.3.22**) The IC50 of IC87114 was determined to be 0.157 μM.

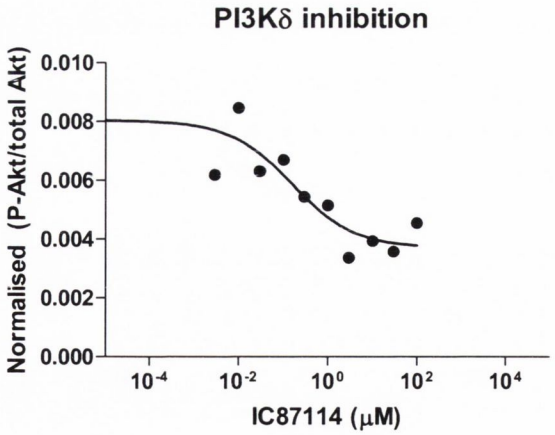

Figure 5.3.22. Inhibition curve drawn using normalized values of phosphorylated Akt against total Akt. IC50 of the PI3Kδ inhibitor, IC87114, was determined to be 0.157 μM. B cells were stimulated for 3 days with PMA/ionomycin and treated with IC87114 in the range of 0 – 300 μM. Cell-based ELISA was performed on the B cells and fluorescence intensities of phosphorylated Akt and total Akt in the cells were obtained.

5.3.9.2 Flow cytometry analysis of changes in surface markers and B cell subset proportions with PI3Kδ inhibition.

To examine the effect of inhibiting PI3Kδ on the expression of surface markers on the B cells during iNKT-B cell co-culture, flow cytometric analysis of CD40 and CD86 was carried out on the B cells after three days of co-culture with IC87114 (in the absence or presence of α-GC). Changes in the frequencies of the various B cell subsets were also evaluated. Results discussed in the following sections are preliminary findings since the experiment was carried out once.

5.3.9.3 IC87114 inhibits iNKT-induced upregulation of CD40 and CD86 expression on B cells.

Flow cytometry was performed on total B cells in this experiment to examine the effect of IC87114 on iNKT-enhanced CD40 and CD86 expression. As seen previously, co-culture with iNKT cells in the presence of α-GC induced upregulation of CD40 and CD86 on total B cells. This enhancing effect of iNKT cells was abrogated when IC87114 was added to the co-cultures. (**Figure 5.3.23**)

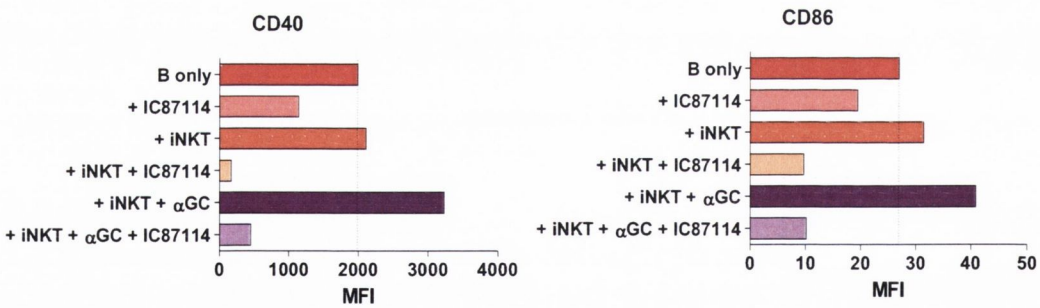

Figure 5.3.23. IC87114 inhibits iNKT-induced upregulation of CD40 and CD86 on B cells. B cells were co-cultured with iNKT cells for 3 days in the absence or presence of α-GC, with or without 1 μM IC87114. As negative controls, B cells were cultured in medium only or with IC87114. Flow cytometric analysis of changes in CD40 and CD86 expression was performed on total B cells. N=1.

5.3.9.4 IC87114 enhances iNKT-induced upregulation of Breg cell frequencies.

Frequencies of Breg cells, naïve and memory B cells were analysed by flow cytometry in the same experiment to study the effect of IC87114 on these B cell subsets in the iNKT-B cell co-cultures. Frequencies of $CD1d^{hi}CD5^{+}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells were slightly increased whereas frequencies of $CD24^{hi}CD38^{hi}$ Breg cells were similar with iNKT cell co-culture. Addition of IC87114 resulted in higher frequencies of $CD1d^{hi}CD5^{+}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells but did not result in any change in frequencies of $CD24^{hi}CD38^{hi}$ B cells. When α-GC was present the enhancing effect of IC87114 was diminished compared to when α-GC was absent. (**Figure 5.3.24**) These results might indicate that PI3Kδ prevents iNKT cells from excessive expansion of Breg cells, which might result in oversuppression of the immune system and increased susceptibility to infection.

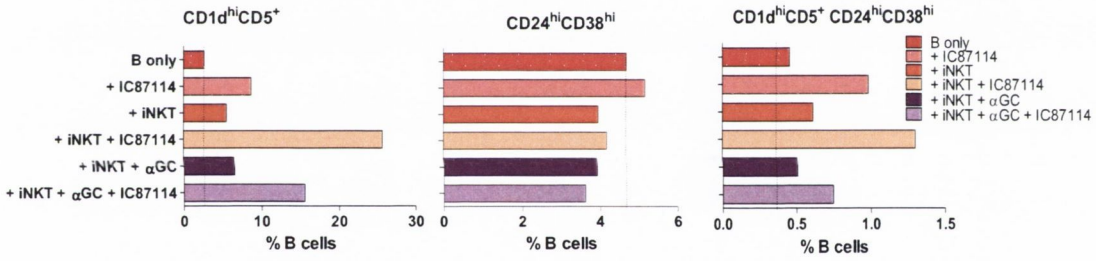

Figure 5.3.24. iNKT-induced upregulation of Breg cell frequencies are enhanced by IC87114. B cells were co-cultured with iNKT cells for 3 days in the absence or presence of α-GC, with or without 1 μM IC87114. As negative controls, B cells were cultured in medium only or with IC87114. Flow cytometric analysis of changes in proportion of $CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells was performed. N=1.

Addition of IC87114 to the iNKT-B cell co-cultures also increased frequencies of unswitched, switched and $CD27^{-}IgD^{-}$ memory B cells but decreased frequencies of naïve B cells (results in Appendix, 9.19 – 9.20).

5.3.10 Changes in phosphorylation levels of intracellular kinases in B cells and iNKT cells as a result of interaction (phosphoMAPK array).

Cell lysates of B cells or iNKT cells were obtained from the following treatments after 3 days of co-cultures: B cells in medium only, iNKT cells in medium only, B + iNKT, B + iNKT + α-GC, and B + iNKT + α-GC + IC87114. After 3 days of co-culture, B cells and iNKT cells were separated from each other using magnetic bead separation before cell lysis. Flow cytometric analysis showed that the separated cells were >70% pure B cells or iNKT cells. The cell lysates were incubated with membranes (from the phosphoMAPK array) spotted with antibodies against the phosphorylated form of 26 different intracellular kinases (**Table 5.1.1**) and detected with chemiluminescent reagent. Each kinase was spotted in duplicate on each membrane. The membranes were exposed to film for 30 minutes and the intensity of the spots on the developed film was analysed by Photoshop. The three pairs of positive control spots are found on the upper left corner, upper right corner and lower left corner of the membranes and they correspond to the maximal intensity on the blots. Signal intensities of negative control spots (PBS) were subtracted from the averaged signal intensity from the pair of spots corresponding to each kinase to account for differences in background signal intensities between membranes. Corresponding signals on each array were then compared to determine relative changes in kinase phosphorylation between treatments.

Kinase phosphorylation was observed in unstimulated B cells but there was very little phosphorylated kinases in unstimulated iNKT cells (**Figure 5.3.25**, top row). Co-culture of iNKT cells with B cells in the absence of α-GC downregulated kinase phosphorylation in the B cells but upregulated phosphorylation of a few kinases in the iNKT cells (**Figure 5.3.25**, second row). When α-GC was added into the iNKT-B cell co-cultures, the profile of kinase phosphorylation in the B cells was unchanged in comparison to co-cultures without α-GC, whereas the previously upregulated phosphorylated kinases in the iNKT cells were now downregulated in phosphorylation (**Figure 5.3.25**, third row). Addition of IC87114 into co-cultures with α-GC appeared to completely eradicate kinase phosphorylation in the B cells but relieved the inhibitory effect that B cells exerted on kinase phosphorylation in the iNKT cells through the presentation of α-GC (**Figure 5.3.25**, last row).

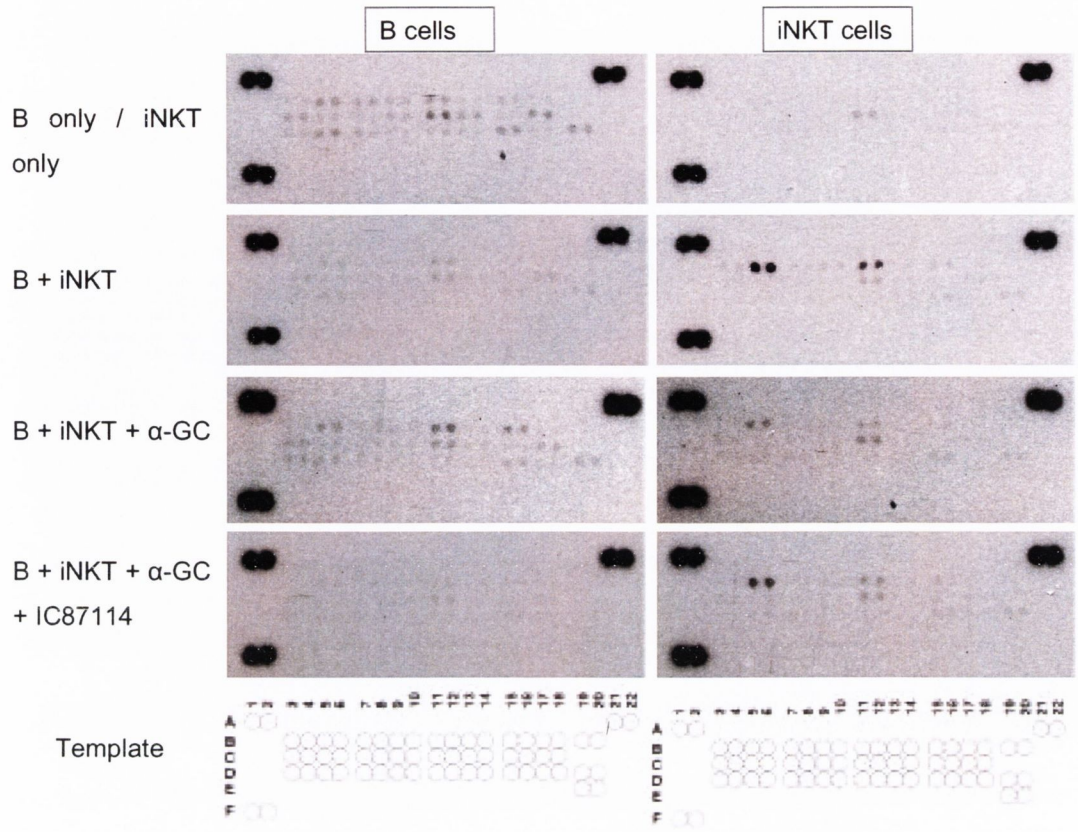

Figure 5.3.25. Western blots from the phosphoMAPK array. Membranes containing spotted antibodies against the phosphorylated form of 26 different intracellular kinases were incubated with cell lysates from iNKT-B cell co-cultures in the absence or presence of α-GC, with or without IC87114. Chemiluminescent detection of signal from the membranes was performed by exposure to film and intensity of the spots on the developed film was analysed with Photoshop. Three pairs of positive control spots are found on the upper left corner, upper right corner and lower left corner of the membranes. Each kinase is spotted in duplicate. Bottom row: template for array coordinate reference to respective MAPK (refer to Table 5.3.4).

Analysis of signal intensities from the membranes showed that in this experiment, co-culture of iNKT cells and B cells in the absence of α-GC downregulates phosphorylation of all the 26 intracellular kinases assayed for in the B cells, relative to B cells that were cultured in medium only (B only). In the presence of α-GC, phosphorylation of Erk2, Erk1, CREB, Akt1/PKBα, JNK1, HSP27, p38γ and p38α was upregulated relative to unstimulated B cells and B cells co-cultured with iNKT cells in the absence of α-GC. However, although phosphorylation of pan Akt, Akt3/PKBγ, Akt2/PKBβ, JNK3, p38δ, p38β, MKK6, MKK3, p70 S6 kinase and p53 were upregulated in the B cells relative to co-cultures without α-GC, they were still downregulated relative to unstimulated B cells. In contrast, phosphorylation of pan JNK, JNK2, GSK-3β, GSK-3α/β, MSK2, TOR, RSK2 and RSK1 remained unchanged relative to co-cultures without α-GC. When IC87114

was added, phosphorylation of all 26 intracellular kinases in the B cells was downregulated relative to iNKT-B cell co-cultures with α-GC while phosphorylation of Erk2, Erk1, GSK-3α/β, MKK6 and MKK3 was totally abrogated. (**Figure 5.3.26**)

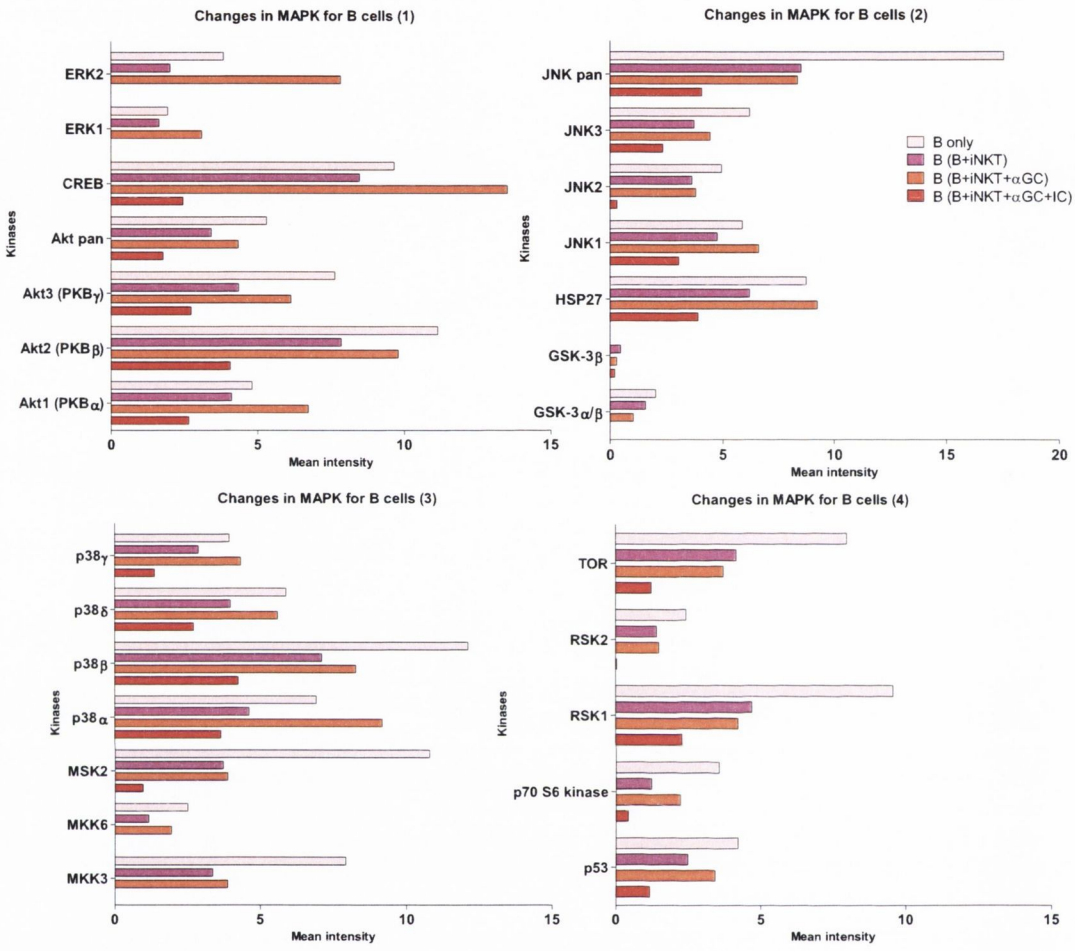

Figure 5.3.26. Changes in phosphorylation levels of MAP kinases in B cells co-cultured with iNKT cells in the absence or presence of α-GC and IC87114. Averaged signal intensities of spots (from which background signal was subtracted) corresponding to each kinase were plotted as bars. B cells were cultured in medium only (pink), with iNKT cells (magenta), with iNKT cells and α-GC (orange) and with iNKT cells, α-GC and IC87114 (red). N=1.

In the absence of α-GC, B cell co-culture with iNKT cells resulted in upregulation of phosphorylation in intracellular kinases in the iNKT cells: we observed massive increases in phosphorylation of CREB and Akt2/PKBβ and slight increases in phosphorylation of Erk1, Erk2, pan Akt, Akt1/PKBα, Akt3/PKBγ, JNK1, HSP27, p38β, MSK2, MKK3, TOR, RSK1 and p70 S6 kinase.

In the presence of α-GC, the massive upregulation in phosphorylation of CREB and Akt2/PKBβ was decreased but still remained higher relative to unstimulated iNKT cells.

Phosphorylation of Erk1, Erk2, pan Akt, Akt3/PKBγ, JNK3, MKK3, TOR, RSK1 and RSK2 were decreased in the iNKT cells. In contrast, phosphorylation of JNK1, JNK2, HSP27, p38α, p38β and p38δ in the iNKT cells were all increased. GSK-3β was phosphorylated only when α-GC was present in the co-cultures, whereas phosphorylation of GSK-3α/β (in unstimulated iNKT cells and co-cultures without α-GC) was eliminated. Phosphorylation of p53 did not change much between treatments.

Addition of IC87114 to the iNKT-B cell co-cultures in the presence of α-GC resulted in decreases in phosphorylation of Erk1, Akt1/PKBα, JNK1, JNK2, HSP27, GSK-3β, p38α, p38β, MSK2 and p70 S6 kinase in the iNKT cells relative to co-cultures containing α-GC but not IC87114. Conversely, IC87114 resulted in increases in phosphorylation of Erk2, CREB, pan Akt, Akt2/PKBβ, JNK3, p38δ, MKK3, TOR and RSK2 relative to co-cultures containing α-GC but not IC87114. Phosphorylation of Akt3/PKBγ, pan JNK, p38γ, MKK6 and RSK1 in the iNKT cells was unchanged by the presence of IC87114. (**Figure 5.3.27**)

Overall the preliminary results from this experiment suggest that B cells and iNKT cells reciprocally inhibit MAPK signalling in each other and that α-GC and PI3Kδ have modulatory roles in this reciprocal inhibition. In the absence of α-GC presentation, B cells activate Akt2 and CREB signalling in iNKT cells while iNKT cells suppress B cell signalling. When α-GC is presented by B cells, it suppresses iNKT cell signalling and relieves iNKT-mediated inhibition of B cell signalling. Use of the inhibitor IC87114 showed that PI3Kδ inhibits B cell signalling to a greater extent than iNKT cell signalling and relieves B cell-mediated inhibition of iNKT cell signalling.

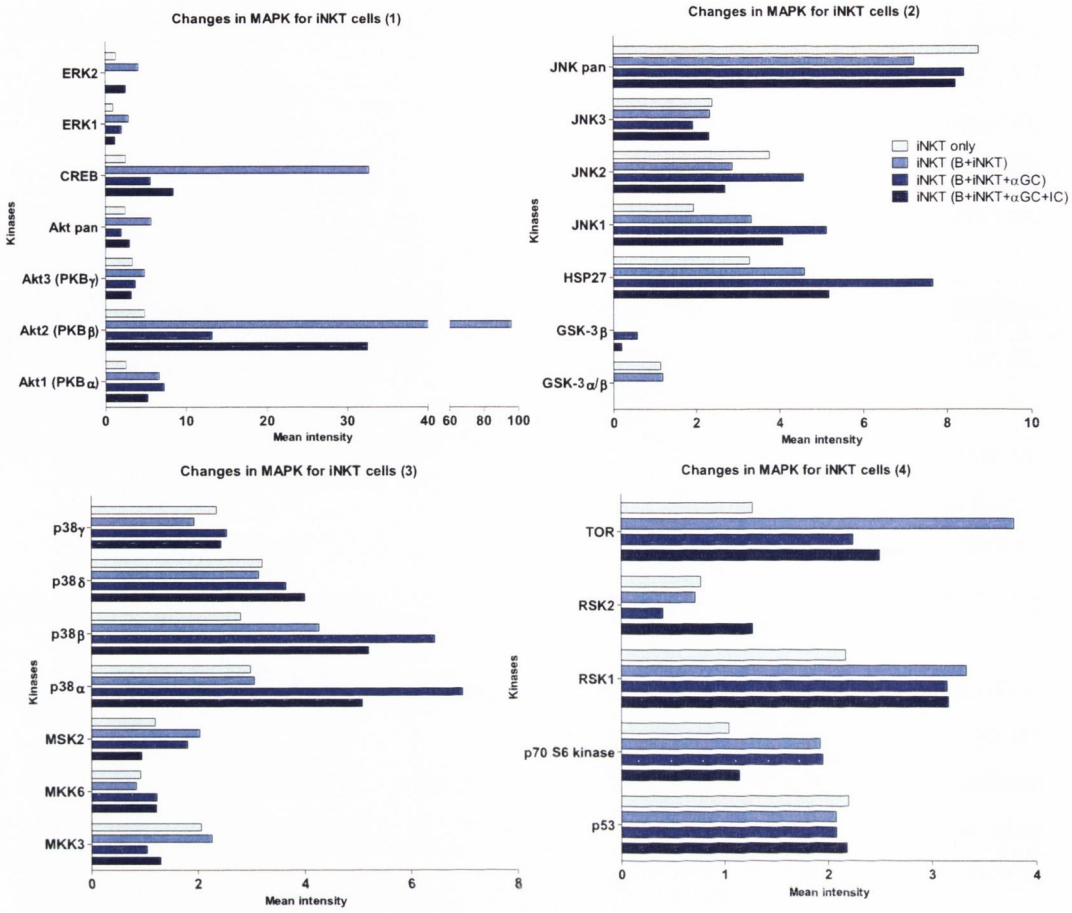

Figure 5.3.27. Changes in phosphorylation levels of MAP kinases in iNKT cells co-cultured with B cells in the absence or presence of α-GC and IC87114. Averaged signal intensities of spots (from which background signal was subtracted) corresponding to each kinase were plotted as bars. iNKT cells were cultured in medium only (light blue), with B cells (turquoise), with iNKT cells and α-GC (blue) and with iNKT cells, α-GC and IC87114 (navy). N=1.

Table 5.3.4. PhosphoMAPK array coordinates for each MAPK. Taken from manufacturer's website.

Coordinate	Target/Control	Alternate Nomenclature	Phosphorylation Site Detected
A1, A2	Positive Control	Control (+)	—
A21, A22	Positive Control	Control (+)	—
B3, B4	Akt1	PKBα, RACα	S473
B5, B6	Akt2	PKBβ, RACβ	S474
B7, B8	Akt3	PKBγ, RACγ	S472
B9, B10	Akt pan	—	S473, S474, S472
B11, B12	CREB	—	S133
B13, B14	ERK1	MAPK3, p44 MAPK	T202/Y204
B15, B16	ERK2	MAPK1, p42 MAPK	T185/Y187
B17, B18	GSK-3α/β	GSK3A/GSK3B	S21/S9
B19, B20	GSK-3β	GSK3B	S9
C3, C4	HSP27	HSPB1, SRP27	S78/S82
C5, C6	JNK1	MAPK8, SAPK1γ	T183/Y185
C7, C8	JNK2	MAPK9, SAPK1α	T183/Y185
C9, C10	JNK3	MAPK10, SAPK1β	T221/Y223
C11, C12	JNK pan	—	T183/Y185, T221/Y223
C13, C14	MKK3	MEK3, MAP2K3	S218/T222
C15, C16	MKK6	MEK6, MAP2K6	S207/T211
C17, C18	MSK2	RSKβ, RPS6KA4	S360
D3, D4	p38α	MAPK14, SAPK2A, CSBP1	T180/Y182
D5, D6	p38β	MAPK11, SAPK2B, p38-2	T180/Y182
D7, D8	p38δ	MAPK13, SAPK4	T180/Y182
D9, D10	p38γ	MAPK12, SAPK3, ERK6	T183/Y185
D11, D12	p53	—	S46
D13, D14	p70 S6 Kinase	S6K1, p70α, RPS6KB1	T421/S424
D15, D16	RSK1	MAPKAPK1α, RPS6KA1	S380
D17, D18	RSK2	ISPK-1, RPS6KA3	S386
D19, D20	TOR	—	S2448
E19, E20	PBS	Control (-)	—
F1, F2	Positive Control	Control (+)	—

5.4 Discussion

5.4.1 $CD4^+$ iNKT cells are not superior to $CD8^+$ and DN iNKT cells in providing B cell help.

5.4.1.1 Effects of $CD4^+$, $CD8^+$ and DN iNKT cells on B cell expression of CD40 and CD86, cytokine and antibody production.

DN and $CD8^+$ iNKT cells are thought to be proinflammatory cells and effectors of tumour and viral surveillance, whereas the $CD4^+$ subset has been strongly associated with immunoregulatory function of these cells (Konishi et al., 2004; Montoya et al., 2007). Decreases in the $CD4^+$ iNKT subset in expanded iNKT clones from type 1 diabetes patients was correlated with a Th1 shift in the cytokine production profile towards IFN-γ production (Kis et al., 2007). $CD4^+$ iNKT cells exhibit significant Th2 cytokine production in comparison to DN and $CD8^+$ iNKT cells (Lee et al., 2002; O'Reilly et al., 2011; Rossignol et al., 2007) and display superiority in activating B cells (Lin et al., 2006b) and inducing antibody production from B cells (Rossignol et al., 2007). Hence based on current literature and previous observations (in section 4) that iNKT cells regulate B cell function, we hypothesized that $CD4^+$ iNKT cells would be the major iNKT subset exerting effects on B cells. In our experiments we found that $CD4^+$ iNKT cells were most efficient at upregulating CD40 ($p<0.05$) expression on total B cells in comparison to $CD8^+$ and DN iNKT cells, suggesting that there was greater CD40-CD154 co-stimulatory interaction between the B cells and the $CD4^+$ iNKT cells. This is significant in the context of disease: high levels of CD154 expression have been characterised in SLE patients, which trigger excessive B cell activation and production of autoreactive antibodies (Toubi and Shoenfeld, 2004). Since $CD4^+$ iNKT cells have such potent effects on B cells, it would be beneficial to target this subset specifically in immunotherapy for autoimmune diseases.

However, when it came to cytokine production, both $CD4^+$ and DN iNKT cells were equally potent and much better than $CD8^+$ iNKT cells at inducing cytokine production when co-cultured with B cells. Our results are aligned with Galli et al's observations (Galli et al., 2003), where unlike freshly isolated iNKT cells, expanded $CD4^+$ and DN iNKT cells were not biased towards a Th1 profile but induced both Th1 (TNF-α and IFN-γ) and Th2 (IL-4, IL-5 and IL-13) cytokines when co-cultured with B cells. Hence it appears that expansion induces a degree of tolerance in the DN iNKT subset. Co-culture of iNKT subsets with a different immune cell type from B cells, such as dendritic cells, induces different cytokine production phenotypes. In co-cultures of human $CD4^+$

iNKT cells or DN iNKT cells with dendritic cells, the $CD4^+$ iNKT cells produced IL-4, IL-13 and IFN-γ which induced IL-12 production (Th1 phenotype) by the dendritic cells. However, DN iNKT cells exhibited cytolytic activity towards the dendritic cells and downregulated Th1 cytokine production, inducing IL-10 production by the dendritic cells instead (Liu et al., 2008).

Detectable changes in production of TNF-α, IFN-γ, IL-4, IL-5 and IL-13 were observed when the iNKT subsets were individually co-cultured with B cells, unlike in previous total iNKT-B cell co-cultures (containing polyclonal iNKT cells with all three subsets) where changes in cytokine production were minimal. This may be due to mutual inhibition by the different iNKT subsets, which cancels out the polarizing effects that each individual subset has on the T helper response when present together in the interaction. This reinforces the idea that harnessing the effect of a specific iNKT subset, rather than total iNKT cells, may have a better immunotherapeutic outcome. Interestingly the presence of α-GC was necessary to detect cytokine production in these co-cultures, suggesting that a glycolipid agonist is required for such polarizing effects to be observed with the iNKT subsets.

Although other studies report that $CD4^+$ iNKT cells are superior in inducing antibody production (Galli et al., 2003) and isotype switching by B cells (Rossignol et al., 2007), we observed otherwise and in our experiments the three iNKT subsets did not appear to differ significantly in their ability to induce antibody production nor specific isotypes of antibody production from the B cells. However $CD8^+$ iNKT cells were most efficient at inducing IgG_2 production by B cells, which has been linked to Th1 responses (O'Brien-Simpson et al., 2000) and correlates well with their reported Th1 cytokine production phenotype (Takahashi et al., 2002). The differences observed between our study and that of Galli's and Rossignol's could be due to the use of α-GC and differences in methods of expanding the iNKT cells. In Galli's study, polyclonal iNKT cells were expanded using α-GC, followed by sorting into their respective subsets and clonal expansion with PHA (Galli et al., 2003). In Rossignol's study, α-GC-loaded B cells were co-cultured with freshly-isolated iNKT subsets which resulted in expansion of the iNKT cells and also antibody production (Rossignol et al., 2007). In contrast, our iNKT lines were expanded in a polyclonal manner using mitogen stimulation rather than stimulation with α-GC. α-GC may predispose $CD4^+$ iNKT cells to interact optimally with B cells, as we observed greater upregulation of CD40 and cytokine production in co-cultures of $CD4^+$ iNKT cells with B cells and α-GC.

Based on the results of our experiments and others', we conclude that $CD4^+$ iNKT cells are superior to $CD8^+$ and DN iNKT cells in activating B cells (by upregulation of CD40 expression). However, in our experiments $CD4^+$ iNKT cells were not superior to $CD8^+$ and DN iNKT cells at inducing antibody production or selectively inducing a particular antibody isotype.

5.4.1.2 $CD4^+$ iNKT cells may induce regulatory B cells.

In the intracellular cytokine staining experiment, we observed that $CD4^+$ iNKT cells increased IL-10 production by B cells during co-culture, suggesting that they may induce tolerance perhaps through induction of regulatory B cells (Bouaziz et al., 2010). Interestingly in the absence of α-GC, iNKT cells shifted the B cells from an initial Th1/Th2 profile (producing IFN-γ, IL-10 and IL-13) to a Th2 profile (enhanced IL-10 and IL-13 production, reduced IFN-γ production), which explains the initial enhancement of antibody production observed when iNKT cell help is provided to B cells in the absence of α-GC. However when α-GC was present in the co-cultures, the B cells were reverted back to a Th1/Th2 profile (diminished IL-13 production, enhanced IFN-γ and IL-10 production) corresponding with observed decreases in antibody production in section 4.3.5, suggesting that α-GC is required for the immunoregulatory effect of $CD4^+$ iNKT cells. The B cells also exerted polarizing effects on the $CD4^+$ iNKT cells, causing them to switch from IFN-γ to IL-13 production in the absence of α-GC, and then a shutdown in intracellular cytokine production in the presence of α-GC, apart from a slight increase in IL-10-producing iNKT cells. (However, cytokines were detected in the supernatants by cytometric bead arrays, which may be residual cytokines secreted from the previous days of co-culture.) This may be due to the induction of a possible subset of Breg cells ($CD1d^{hi}CD5^+$ B cells) seen in **Figure 5.3.3** as Breg cells have been known to induce IL-10 and regulatory properties in effector T cells (Gray et al., 2007). However these conclusions are preliminary as they are based on a single experiment.

Breg cells are not well-defined in humans and apart from IL-10 production there is still a lack of consensus with regards to their distinct phenotype (Gray and Gray, 2010). We observed that although $CD4^+$ iNKT cells did not cause an expansion of $CD24^{hi}CD38^{hi}$ B cells, the only published subset of human Breg cells (Blair et al., 2010), they did cause upregulation of CD40 ($p<0.05$) and CD86 expression on these cells with the aid of α-GC, as well as a fourfold increase in IL-10-producing $CD24^{hi}CD38^{hi}$ B cells. In addition, $CD4^+$ iNKT cells induced the expansion of $CD1d^{hi}CD5^+$ and $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ B cells

(section 5.3.1.2), the former being a well-characterised Breg subset in mice (Yanaba et al., 2008) and the latter being previously observed in the $CD24^{hi}CD38^{hi}$ Breg subset (Blair et al., 2010). $CD4^{+}$ iNKT cells also increased proportions of IL-10-producing $CD1d^{hi}CD5^{+}$ and $CD24^{hi}CD38^{hi}$ B cells, suggesting that they have the ability to expand Breg cells.

When looking at the cytokine production profile of $CD1d^{hi}CD5^{+}$ and $CD24^{hi}CD38^{hi}$ B cells, IL-10-producing cells constitute only 10% and 5% of these B cell subsets respectively, suggesting that there is further heterogeneity within these B cell subsets and perhaps not all of the B cells in these subsets have regulatory capacities. Adding to the complexity, these subsets are extremely heterogeneous in their cytokine production profile, with some exhibiting Treg (single IL-10-producers), Th2 (single IL-13-producers), Th1 (single IFN-γ-producers) and even Th1/Th2 (dual IFN-γ- and IL-10-producers, dual IFN-γ- and IL-13-producers and triple IFN-γ-, IL-13- and IL-10-producing) profiles. This highlights the need for a re-assessment of Breg classification as IL-10 producing B cells, as our experiments have shown that these B cells can produce other cytokines in conjunction with IL-10.

5.4.2 The iNKT-B cell interaction is dependent on CD1d, IL-13 and the CD40-CD154 interaction.

In the series of experiments where we blocked CD1d, CD40, CD154, IL-4 and IL-13 in the iNKT-B cell co-cultures to investigate their roles in mediating the iNKT-B cell interaction, we showed that the activating effect that iNKT cells exert on interaction with B cells is dependent on CD1d, IL-13 and the CD40-CD154 interaction, but not IL-4.

Antibodies against CD1d, CD154 and IL-13 had an inhibitory effect on iNKT-enhanced B cell activation, suggesting that these antibodies were successful in blocking these molecules. In contrast, antibodies against CD40 resulted in increased B cell activation, suggesting that the antibodies were agonistic instead of blocking the interaction. Amongst the three, only CD1d was necessary for iNKT-induced antibody production by the B cells whereas CD154 and IL-13 were not necessary, suggesting that targeting CD1d but not IL-13 or the CD40-CD154 interaction may be valuable in inhibiting unwanted or excessive antibody responses caused by iNKT help to B cells. The importance of CD1d in the iNKT-B cell interaction in mice has been confirmed by several groups (Lang, Devera, and Lang, 2008; Leadbetter et al., 2008), who using healthy $CD1d^{-/-}$ mice, found that B cell expression of CD1d is absolutely necessary for

the iNKT-enhanced antibody response to administered antigen. However, work done in an SLE murine model suggests that CD1d deficiency contributes to autoreactive antibody production instead (Yang et al., 2011; Yang et al., 2007). Further work is needed in this aspect to ascertain the role of CD1d in human iNKT-B cell interactions in the context of autoimmune disease pathogenesis and autoreactive antibody production.

Blocking the CD40-CD154 interaction with anti-CD154 mAb diminished iNKT-enhanced CD40 and CD86 expression on total B cells, $CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells. This suggests that the CD40-CD154 interaction between the B cells and iNKT cells is crucial for activation of these B cell subsets. However, blocking CD154 did not affect iNKT-induced B cell antibody production even though the CD40 co-stimulation on B cells is known to be important for isotype switching and antibody production during normal B-T cell interaction (Bishop and Hostager, 2003). This suggests that the iNKT cells do not use the conventional CD40-CD154 pathway to stimulate B cell antibody production but perhaps through another pathway, such as the CD1d-TCR interaction.

IL-13 and IL-4 have been described as anti-inflammatory cytokines that promote Th2 immune responses and decreases iNKT-mediated antitumour immunity in mice (Renukaradhya et al., 2008; Terabe et al., 2000). Despite the importance of IL-4 and IL-13 for antibody isotype switching and B cell proliferation, attempts to block IL-4 and IL-13 with antibodies targeted against them showed that IL-4 was not required for iNKT-mediated B cell activation and antibody production whereas IL-13 was required for iNKT-induced B cell activation but not antibody production. This lack of effect of anti-IL-4 and anti-IL-13 mAb was also reported by Galli et al, who observed that adding either anti-IL-4 mAb or anti-IL-13 mAb to iNKT-B cell co-cultures did not inhibit iNKT-induced B cell proliferation (Galli et al., 2003). These data suggest that IL-4 and IL-13 have overlapping functions in mediating iNKT-induced B cell antibody production and can compensate for each other when one is lacking. However, IL-4 does not appear to be able to compensate for the function of IL-13 in mediating B cell activation.

5.4.3 Intracellular changes in B cells and iNKT cells during interaction.

The results for this section are preliminary as the experiments have been conducted only once. The purpose of this work was to fish for signalling molecules downstream of the surface receptors which might be of interest for targeting the iNKT-B cell interaction

in disease. The phosphoMAPK array, which assays for phosphorylation of 26 different intracellular kinases, was used to simultaneously detect changes in kinase phosphorylation during the different co-culture conditions.

5.4.3.1 Kinase phosphorylation decreases in B cells but increases in iNKT cells during iNKT-B cell co-culture.

Co-culture of B cells with iNKT cells in the absence of α-GC decreased kinase phosphorylation in B cells, suggesting that iNKT cells downregulated B cell signalling and function. This agrees with previous observations that iNKT cells inhibit the antigen-presentation function of B cells (in section 4.3.6). Conversely, intracellular kinase phosphorylation was enhanced in iNKT cells, in particular Akt2/PKBβ and CREB. Akt/PKB in T cells is known to be activated by cytokines, chemokines, TCR and the CD28 co-stimulatory signal, leading to T cell fate determination and survival (Bauer and Baier, 2002). However the specific role of Akt2 in immune cells, particularly in iNKT cells is largely unknown. Plasmin-stimulated human dendritic cells have been documented to induce Th1 differentiation of $CD4^+$ T cells through Akt2 signalling (Li et al., 2010). In our previous experiments looking at intracellular cytokine production, iNKT cells were observed to switch from a Th1 (IFN-γ) to a Th2 (IL-13) profile when co-cultured with B cells in the absence of α-GC (section 5.3.1.4). Perhaps then it is plausible that B cell-induced Akt2 signalling in iNKT cells polarizes the iNKT cells towards a Th2 profile, resulting in enhanced IL-13 production. Further experiments are necessary to elucidate the effect of Akt2 signalling on iNKT effector and/or immunoregulatory functions.

CREB is a nuclear transcription factor that is activated by Akt phosphorylation (Du and Montminy, 1998). Phosphorylation of serine 133 on CREB causes it to interact with its coactivator, CREB-binding protein (CBP) to activate transcription of CREB-responsive genes (Shaywitz and Greenberg, 1999), one of which is IL-10 production (Ananieva et al., 2008). Glycolipids have been shown to activate TCR signalling in human iNKT cells, resulting in the phosphorylation of CREB (Chang et al., 2007). However, it is interesting that B cells are able to mediate this effect in iNKT cells without α-GC being present. In fact, in the presence of α-GC, kinase phosphorylation is restored in B cells but decreased in iNKT cells instead. This suggests that B cells reciprocally inhibit iNKT signalling and function when they present α-GC to the iNKT cells. The Akt2-CREB signalling pathway may be important in iNKT cells for mediating enhanced antibody production from the B cells, as phosphorylation of Akt2 and CREB are both upregulated when iNKT cells enhance antibody production in the absence of α-GC, but

downregulated when the iNKT-enhanced antibody production is reduced in the presence of α-GC.

5.4.3.2 Inhibiting PI3Kδ abrogates kinase phosphorylation in B cells but restores kinase phosphorylation in iNKT cells.

Inhibiting PI3Kδ in the iNKT-B cell co-cultures abrogated kinase phosphorylation in B cells, which falls within our expectations as PI3Kδ is the main isoform of PI3K acting downstream of the BCR (Okkenhaug et al., 2002). Inhibiting PI3Kδ also partially relieved the B cell-mediated inhibition of Akt2 and CREB phosphorylation in the iNKT cells, lending further support to the observation that B cells reciprocally inhibit iNKT cells in the presence of α-GC.

Flow cytometric analysis of changes in B cells showed inhibition of CD40 and CD86 expression on total B cells when PI3Kδ was inhibited in the co-cultures. However, frequencies of $CD1d^{hi}CD5^{+}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells (but not $CD24^{hi}CD38^{hi}$ B cells) were upregulated when PI3Kδ was inhibited, suggesting an expansion of these B cell subsets. Thus, targeting PI3Kδ to expand Breg cells might be a viable strategy in immunotherapy for autoimmune diseases where a Th2 bias in the immune microenvironment might be beneficial for downregulating excessive Th1-mediated inflammatory responses. The therapeutic effect of inhibiting PI3Kδ has been demonstrated in vivo, whereby oral administration of the PI3Kδ inhibitor IC87114 in mice injected with arthritogenic serum was observed to be successful in reducing inflammation, bone and cartilage erosion (Randis et al., 2008). However, with evidence emerging that suggests an interdependent cooperativity exists between PI3Kδ and PI3Kγ in immune function (Rommel, 2010), the role of PI3Kγ in iNKT-B cell interactions should also be elucidated to determine if dual inhibition of both PI3K isoforms would be more valuable in treatment of autoimmune inflammatory diseases.

The conclusions from this chapter are:

1. $CD4^+$ iNKT cells may be more efficient than $CD8^+$ and DN iNKT cells at activating B cells but not inducing antibody production. $CD4^+$ iNKT cells were observed to induce a population of $CD1d^{hi}CD5^+$ B cells, which may be Breg cells. $CD4^+$ iNKT cells also enhanced IL-10 production in an intracellular cytokine staining experiment.

2. CD1d, CD40-CD154 co-stimulatory signalling and IL-13 are required for iNKT-induced B cell activation. However, only CD1d is required for iNKT-induced B cell antibody production.

3. During a single iNKT-B cell co-culture experiment, B cell signalling (kinase phosphorylation) decreased while iNKT cell signalling increased in the absence of α-GC. However in the presence of α-GC, B cell signalling was restored while iNKT cell signalling was inhibited, suggesting reciprocal regulation between the iNKT cells and B cells. Inhibiting PI3Kδ inhibited B cell signalling but relieved the inhibition on iNKT cell signalling, giving insight into the possible role of PI3Kδ in the iNKT-B cell interaction. The role of PI3Kδ in iNKT-B cell interactions should be further investigated through the use of other methods to inhibit its function (such as siRNA knockdown) and/or augment its function (such as overexpression) to complement this preliminary finding.

6 iNKT-B cell interactions in SLE.

6.1 Introduction

6.1.1 Systemic Lupus Erythematosus (SLE).

SLE is a systemic, chronic autoimmune disease that is difficult to diagnose because of its highly variable and diverse clinical manifestations (Kotzin, 1996). In SLE the immune system can attack multiple organs simultaneously, such as the heart, kidneys and the brain (Kotzin, 1996). There seems to be a preponderance towards females, with the female to male ratio being approximately 9:1 (Somers et al., 2009). The SLE disease activity index (SLEDAI) is commonly used to assign a numerical score corresponding to disease activity. Based on 24 variables (or clinical manifestations) that were identified as important factors in disease activity, physicians rate a score of 0 – 3 for none to severe disease for each of these variables which are weighted based on the organ system involved. These are then added up to produce the SLEDAI score (Bombardier et al., 1992). The cause of SLE is largely unknown, and several factors have been associated with the pathogenesis of SLE.

6.1.1.1 Etiology of SLE.

Following from the observation that SLE runs in families, several genes have been associated with increased susceptibility to SLE, such as HLA class I and II (Eroglu and Kohler, 2002; Martens et al., 2009). Environmental factors such as exposure to sun (ultra-violet light) (Jönsen et al., 2007), drugs (Borchers, Keen, and Gershwin, 2007), elevated levels of prolactin and estrogen hormones (Cohen-Solal et al., 2006; Grimaldi, 2006) and viral infection (Trapani, Ermini, and Falcini, 1999; Verdolini et al., 2002) have also been connected to the triggering of SLE in genetically susceptible individuals.

A hallmark of autoimmune disease development is the development of pathogenic IgG autoantibodies (Mietzner et al., 2008). These autoantibodies are targeted against nuclear antigens such as dsDNA, U1 and Sm small ribonucleoprotein (snRNP) complexes, Ro/SSA and La/SSB RNP complexes, which are collectively termed as "anti-nuclear antibodies (ANA)" (Racanelli et al., 2011; Tan, 1989). However, the mechanisms of disease pathogenesis caused by autoantibodies are unclear. The commonly accepted theory is that these autoantibodies cross-link self or non-self antigens to form multivalent immune complexes, which deposit in organs and result in

inflammation and tissue damage due to Fc-mediated complement activation and/or Fc receptor-mediated activation of myeloid and lymphoid lineage cells (Foster, Cizman, and Madaio, 1993; Townsend, Monroe, and Chan, 2010). Hence markers of inflammation, such as elevated erythrocyte sedimentation rate (ESR), lowered C reactive protein (CRP), as well as decreased C3 and C4 levels which indicate complement activation, are often assayed for as indicators of disease activity (Egner, 2000). Impaired clearance of apoptotic cells has been observed in lymph nodes and skin biopsies of SLE patients (Gaipl et al., 2006; Shao and Cohen, 2011), adding weight to the notion that defective clearance of apoptotic bodies may trigger autoimmune disease (Rosen and Casciola-Rosen, 2001).

Due to the multifactorial and complex nature of the disease, there is as yet no cure for SLE and prescribed treatments such as steroids only treat disease symptoms and help reduce inflammation. However, this has the knock-on side effect of increased susceptibility to infections and associated risk of mortality (Barber, Gold, and Fortin, 2011). Hence there has been a plethora of research looking at ways in which disease mechanisms can be targeted with biologicals.

6.1.1.2 iNKT cells and B cells in pathogenesis of SLE and therapeutic applications.

B cells are considered central to the pathogenesis of SLE, mediating disease through (i) production of autoantibodies that cause inflammation and tissue damage and (ii) antibody-independent functions (Chan, Madaio, and Shlomchik, 1999). Antibody-independent functions include inhibition of regulatory T cells, activation of autoreactive memory T cells, pathogenic effector Th1 and Th17 cells, activation of dendritic cells and T follicular helper cells. These functions are achieved primarily through antigen presentation, co-stimulatory signalling (CD40, CD86 and CD80) and pro-inflammatory cytokine production (IL-6, IFN-γ) (Calero and Sanz, 2010; Chan, Madaio, and Shlomchik, 1999; Townsend, Monroe, and Chan, 2010). In view of their manifold roles in disease pathogenesis, depletion of B cells has been explored in the treatment of SLE using anti-CD20 mAb (also known as rituximab). However, two phase III randomised placebo-controlled trials utilising rituximab in the treatment of moderately active non-renal SLE (EXPLORER trial) (Merrill et al., 2011) and class III/IV lupus nephritis (LUNAR trial) (Furie et al., 2009) showed that rituximab was not superior to placebo or conventional immunosuppressive therapy (via steroids). As a result, alternative strategies to target B cells have been developed, such as anti-BAFF mAb (belimumab) and anti-CD22 mAb (epratuzumab). Of the two, belimumab, which targets the B cell activating factor, has

been approved for use by the US Federal Drug Agency in SLE therapy (Sanz, Yasothan, and Kirkpatrick, 2011) due to its promising success in achieving clinical superiority over placebo in the BLISS-52 (Navarra et al., 2011) and BLISS-76 (Furie et al., 2010) phase III clinical trials. Epratuzumab targets the B cell inhibitory receptor CD22 and has been shown to improve disease activity in a 12-week phase IIb trial (Kalunian et al., 2010).

Human $V\alpha24^{+}V\beta11^{+}$ iNKT cells have been reported to be deficient in SLE patients in comparison to healthy controls (Kojo et al., 2001; van der Vliet et al., 2001). Elevated levels of DN iNKT cells have also been reported in SLE patients, however administration of steroids restored their circulating frequencies back to normal (Oishi et al., 2001). iNKT cells are thought to be dysfunctional in SLE patients due to their discrepancy in response to the iNKT cell ligand α-GC: in a cohort of 10 patients, 5 proliferated in response to α-GC whereas the other 5 did not (Kojo et al., 2001). High levels of anti-dsDNA IgG autoantibodies have been associated with the low iNKT cell numbers in SLE patients (Green et al., 2007). However, the authors in this paper classified iNKT cells as $CD56^{+}CD3^{+}$ T cells, which is not strictly specific for iNKT cells.

The role of iNKT cells in SLE pathogenesis has been explored using various murine models and the results garnered thus far have been conflicting. Studies have been conducted with the (New Zealand black x New Zealand white) F_1 (NZB/W F_1) hybrid murine model, a hereditary model of SLE that develops lupus with age and resembles human SLE in autoantibody production and development of glomerulonephritis (Gabriel, Morley, and Rogers, 2009). iNKT cells are deemed to be pathogenic in the NZB/W F_1 (NZB/W F_1) hybrid mice, with observations that they expand as the mice age and become phenotypically and functionally hyperactive, such that activation with α-GC results in increasing pro-inflammatory cytokine (IFN-γ) production with advancing age and disease progression (Forestier et al., 2005). Isotype switching from IgM to IgG autoantibody production has been associated with development of glomerulonephritis in the NZB/W F_1 mice at 6 months of age. Such help was observed to be provided by iNKT cells, which increased spontaneous secretion of IgM and IgM anti-dsDNA antibodies by B-1 B cells and marginal zone B cells, and also facilitated production of IgG anti-dsDNA antibodies by B-1 B cells. Interestingly, conventional T cells were not able to provide such potent T helper activity to the B cells (Takahashi and Strober, 2008). Despite these studies that show iNKT cells are pathogenic in NZB/W F_1 mice, germline deletion of CD1d in this strain of mice exacerbates the lupus-like disease with observations of enhanced anti-DNA autoantibody production and more severe nephritis (Yang et al., 2007).

In contrast, iNKT cells are deemed to be protective in several other murine models. The MRL/lpr-lpr murine model is a hereditary model of SLE caused by a mutation in the Fas receptor resulting in defective apoptosis (Gabriel, Morley, and Rogers, 2009) and is thought to be very similar to human SLE. They are deficient in iNKT cell numbers and function (Godó, Sessler, and Hamar, 2008), and are characterised by massive production of IgM and IgG autoantibodies (Cohen and Eisenberg, 1992). In the MRL/lpr-lpr murine model, iNKT cells are significantly reduced in numbers prior to the onset of autoimmune disease, and depletion of Vα14$^+$ cells by antibodies targeted against them resulted in early onset of disease and increased titers of autoantibodies (Mieza et al., 1996). Repeated administration of α-GC expanded the iNKT cells and alleviated lupus-associated inflammatory dermatitis in the mice (Yang et al., 2003). In mice that were injected with apoptotic cells to simulate autoantibody production caused by an increased load of circulating apoptotic cells, iNKT cells were observed to play a protective role in suppressing autoreactive B cell activation by limiting the formation of germinal centers and thus autoantibody production (Wermeling et al., 2010). Since current literature and our own findings show that iNKT cells have immunoregulatory effects on B cell function, it is important to clear up the confounding issues regarding the role of iNKT cells in SLE. In particular, it is especially essential to characterise their role in the context of human SLE.

6.2 Aims and hypotheses

The overall aim of this chapter was to characterise the role of iNKT cells in regulating B cell function in human SLE patients. Based on current knowledge from both *in vivo* and *in vitro* studies that iNKT cells can enhance antibody production by B cells and that iNKT cells are deficient in SLE, our hypothesis was that human iNKT cells are dysfunctional in SLE and are able to hyper-activate B cells, which may account for the characteristically increased levels of autoantibodies in SLE patients.

Phenotyping of various iNKT cell (CD4$^+$, CD8$^+$ and DN) and B cell subsets (regulatory B cells, memory B cells and naïve B cells) was performed using flow cytometry to compare their frequencies in SLE patients and healthy controls. Flow cytometric analysis of CD154 on the iNKT cell subsets, and CD40 and CD80 expression on B cell subsets, was also carried out to compare activation states of these subsets between SLE patients and healthy controls.

In order to test the ability of iNKT cells from SLE patients to stimulate/regulate B cells, the iNKT cells were sorted from their PBMC and expanded using anti-CD3 mAb/IL-2 stimulation and culture with irradiated feeder cells. The expanded iNKT cells were then co-cultured with healthy B cells and compared with healthy iNKT-B cell co-cultures. Flow cytometric analysis of CD154 on the iNKT cell subsets, and CD40 and CD80 expression on B cell subsets, was performed after 3 days. Cytokine and immunoglobulin production was assayed from supernatants of 3 and 10 day co-cultures respectively using cytometric bead arrays.

6.3 Results

6.3.1 Frequencies and activation status of iNKT cells and B cell subsets in SLE patients and healthy controls.

PBMCs were obtained from the peripheral blood of 15 SLE patients and 20 healthy controls, and phenotyped by flow cytometry for frequencies of iNKT cells, Breg cells, memory B cells and naïve B cells. The various subsets were also phenotyped for CD154 (on iNKT cells), CD40 and CD80 (on B cells) expression to compare activation state of the cells between healthy controls and SLE patients.

6.3.1.1 $CD4^+$ iNKT cell frequencies are higher and increased in CD154 expression in SLE patients compared to healthy controls.

There were no significant differences observed in frequency and CD154 expression of total iNKT cells ($6B11^+CD3^+$) between SLE patients and healthy controls. However, the proportion of $CD4^+$ iNKT cells was higher in SLE patients (p=0.0339), as well as the MFI of CD154 expression on $CD4^+$ iNKT cells (p=0.0403) in comparison to healthy controls. No significant differences in frequencies or intensities of CD154 expression were observed for $CD8^+$ iNKT and DN iNKT cells. (**Figure 6.3.1**)

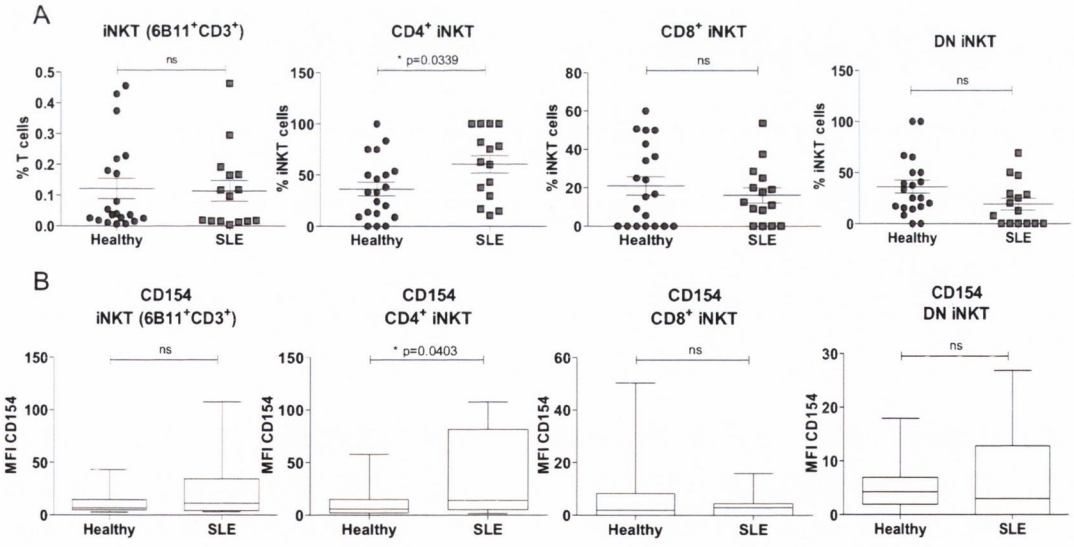

Figure 6.3.1. $CD4^+$ iNKT cell frequencies and levels of CD154 expression are increased in SLE patients compared to healthy controls. Flow cytometric analysis was performed on freshly-isolated PBMCs obtained from healthy donors (n=20) and SLE patients (n=15). (A) Frequencies of total ($6B11^+CD3^+$), $CD4^+$, $CD8^+$ and DN iNKT cells. Horizontal lines represent mean ± SEM. (B) MFI of CD154 expression on the various iNKT subsets. Floating bars represent minimum to maximum with horizontal line at mean, error bars correspond to SEM. Statistical analysis was performed using the Mann-Whitney U test, *p<0.05.

6.3.1.2 Frequencies of naïve B cells are increased whereas frequencies of memory B cells and regulatory B cells are decreased in SLE patients compared to healthy controls.

PBMCs were obtained from the peripheral blood of 15 SLE patients and 20 healthy controls, and phenotyped by flow cytometry for frequencies of naïve B cells, memory B cells (unswitched, switched and $CD27^-IgD^-$) and Breg cells. The various B cell subsets were also phenotyped for CD40 and CD80 expression to compare activation state of the cells between healthy controls and SLE patients.

The frequencies of naïve B cells ($CD27^-IgD^+$) as a proportion of total B cells and IgM^- naïve B cells as a proportion of total naïve B cells were increased in SLE patients (p=0.0081 and 0.0224 respectively) whereas IgM^+ naïve B cells as a proportion of total naïve B cells were decreased (p=0.0245). (**Figure 6.3.2**A)

Frequencies of total unswitched memory ($CD27^+IgD^+$) B cells (p=0.0007) as proportions of total B cells and IgM^+ unswitched memory B cells (p=0.0098) as proportions of total unswitched memory B cells were significantly decreased in SLE patients compared to healthy controls. Conversely, frequencies of IgM^- unswitched memory B cells (p=0.0098) were significantly increased in SLE patients compared to healthy controls. (**Figure 6.3.2**B and **Figure 6.3.3**)

Frequencies of total switched memory ($CD27^+IgD^-$) B cells were significantly decreased (p=0.0081) in SLE patients in comparison to healthy controls. Frequencies of IgM^+ and IgG^+ switched memory B cells, as proportions of total switched memory B cells, were also reduced in SLE patients but these differences were not statistically significant. (**Figure 6.3.2**C)

In comparison to healthy controls, the frequencies of $CD27^-IgD^-$ memory B cells that expressed IgM were decreased in SLE patients (p=0.0437). Frequencies of total and IgG^+ $CD27^-IgD^-$ memory B cells appeared to be slightly lower in SLE patients than in healthy controls but the differences were not statistically significant. (**Figure 6.3.2**D)

In comparison to healthy controls, the frequencies of $CD1d^{hi}CD5^+$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ B cells were significantly decreased in SLE patients. (Figure 6.3.2E)

In summary, frequencies of naïve B cells are increased whereas frequencies of memory B cells and Breg cells are decreased in SLE patients in comparison to healthy controls.

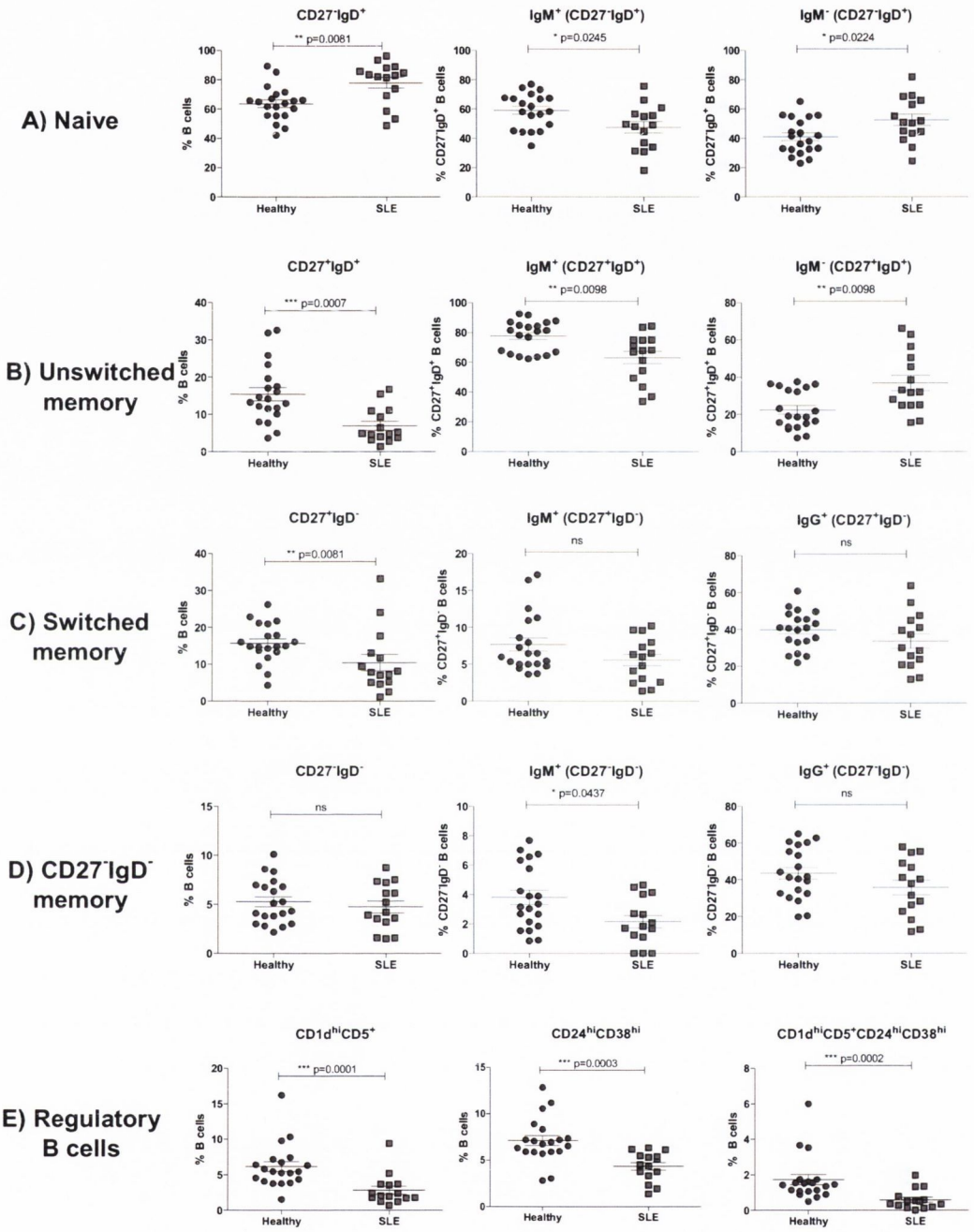

Figure 6.3.2. Frequencies of naïve B cells are increased whereas frequencies of memory B cells and Breg cells are decreased in frequency in SLE patients compared to healthy controls. Flow cytometric analysis was performed on freshly-isolated PBMCs obtained from healthy donors (n=20) and SLE patients (n=15). Dots (healthy controls) and squares (SLE patients) represent frequencies of the various B cell subsets. Horizontal lines represent mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test, *p<0.05, **p<0.01, ***p<0.001.

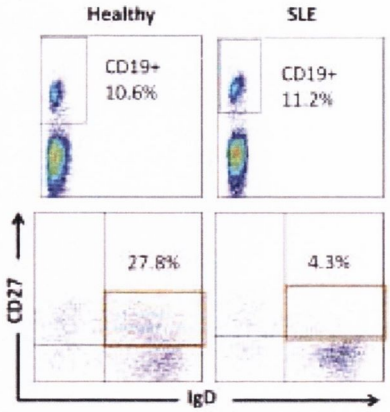

Figure 6.3.3. Frequencies of naïve B cells are increased whereas frequencies of memory B cells are decreased in SLE patients. Flow cytometric analysis was performed on freshly-isolated PBMCs obtained from healthy donors (n=20) and SLE patients (n=15). Representative flow cytometry plots showing decreased frequency of unswitched memory B cells (orange box) in SLE patients compared to healthy controls.

6.3.1.3 CD40 expression is enhanced on naïve and memory B cells in SLE patients.

Flow cytometric analysis of CD40 expression was performed on naïve B cells, memory B cells and Breg cells from freshly-isolated PBMCs of SLE patients and healthy controls to compare their activation states.

CD40 expression was increased on all three subsets of naïve B cells (p=0.0007, p=0.0003 and p=0.0002 for total, IgM^+ and IgM^- naïve B cells respectively). (**Figure 6.3.4**A)

Similar to naïve B cells, CD40 expression was also enhanced on memory B cells in SLE patients. CD40 expression on total unswitched memory B cells (p=0.0343), IgM^+ (p=0.066) and IgM^- unswitched memory B cells (p=0.0437) were increased in SLE patients. CD40 expression was also augmented on total switched memory B cells (p=0.0343), IgM^+ (p=0.0098) and IgG^+ (p=0.0224) switched memory B cells, as well as on all three subsets of $CD27^-IgD^-$ memory B cells (p=0.0316, p=0.0264 and ns for total, IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells respectively). (**Figure 6.3.4**B, C and D)

In SLE patients, CD40 expression was slightly higher on $CD1d^{hi}CD5^+$ and $CD24^{hi}CD38^{hi}$ B cells but slightly lower on $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ B cells. However these differences were not statistically significant. (**Figure 6.3.4**E)

In summary, CD40 expression was enhanced on naïve and memory B cells but not Breg cells.

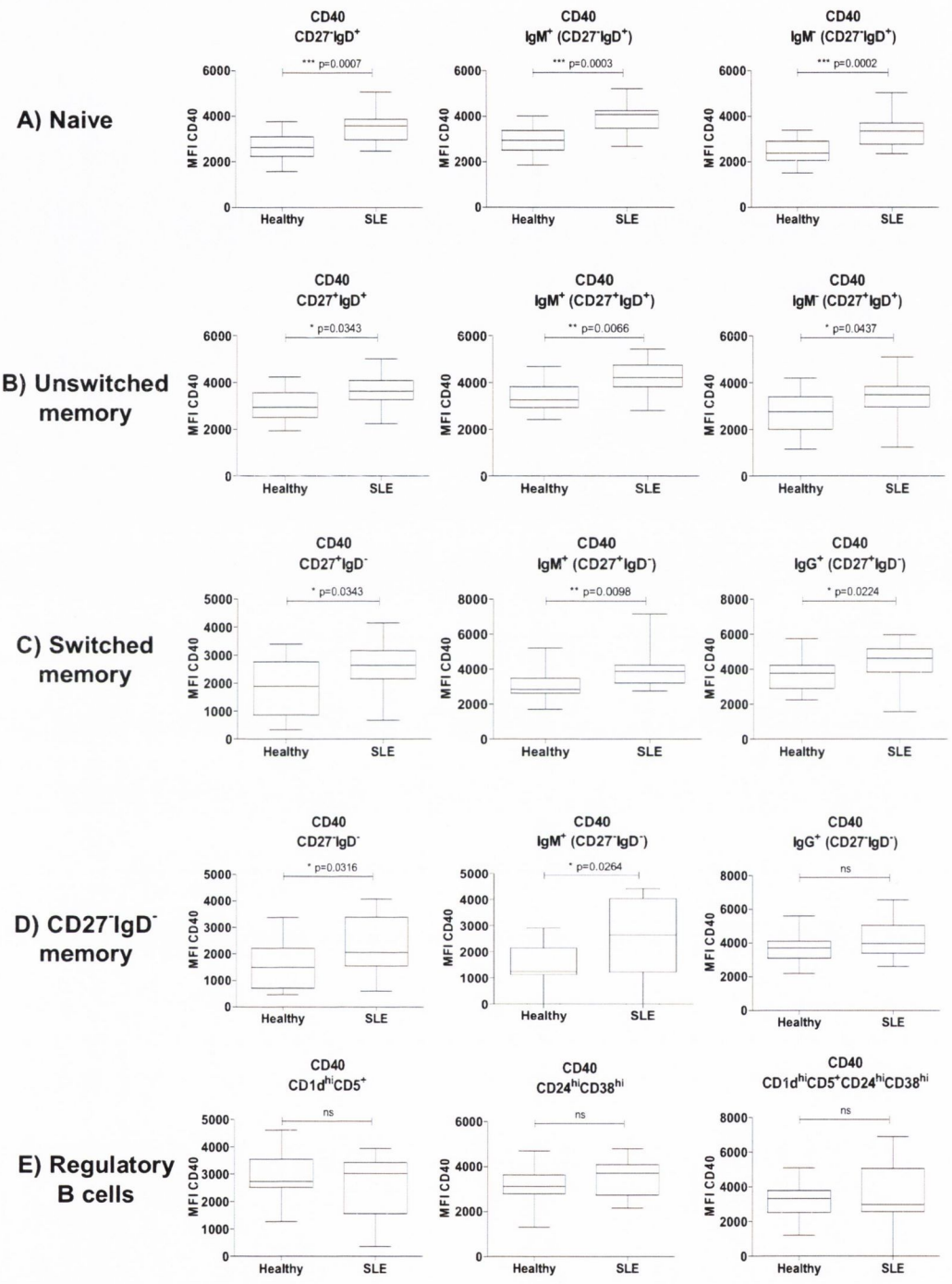

Figure 6.3.4 CD40 expression is enhanced on naïve and memory B cells in SLE patients. Flow cytometric analysis was performed on freshly-isolated PBMCs obtained from healthy donors (n=20) and SLE patients (n=15). MFI of CD40 expression on unswitched, switched and $CD27^-IgD^-$ memory B cells, and their respective IgM^+, IgM^- or IgG^+ subsets was analysed. Floating bars represent minimum to maximum with horizontal line at mean, error bars correspond to SEM. Statistical analysis was performed using the Mann-Whitney U test, *p<0.05, **p<0.01.

6.3.1.4 CD80 expression is increased on Breg, switched and $CD27^-$ IgD^- memory B cells but decreased on unswitched memory B cells in SLE patients.

Flow cytometric analysis of CD80 expression was performed on naïve B cells, memory B cells and Breg cells from freshly-isolated PBMCs of SLE patients and healthy controls to compare their activation states.

CD80 expression between SLE patients and healthy controls was not significantly different on total, IgM^+ and IgM^- naïve B cells. (**Figure 6.3.5**A)

CD80 expression on total (p=0.0172), IgM^+ (p=0.029) and IgM^- (ns) unswitched memory B cells were decreased. In contrast, CD80 expression was slightly enhanced on total switched memory B cells (ns) and IgG^+ switched (p=0.0473) memory B cells but not for IgM^+ switched memory B cells. CD80 expression was enhanced for total (ns) and IgG^+ (p=0.0066) $CD27^-IgD^-$ memory B cells, but lowered for IgM^+ $CD27^-IgD^-$ memory B cells (p=0.0203). (**Figure 6.3.5**B, C and D)

CD80 expression was significantly higher for $CD1d^{hi}CD5^+$ (p=0.0157) and $CD24^{hi}CD38^{hi}$ (p=0.0023) B cells and slightly higher for $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ (ns) B cells. (**Figure 6.3.5**E)

In summary CD80 expression was increased on Breg cells, switched and $CD27^-IgD^-$ memory B cells but decreased on unswitched memory B cells in SLE patients. There was no difference in CD80 expression on naïve B cells between SLE patients and healthy controls.

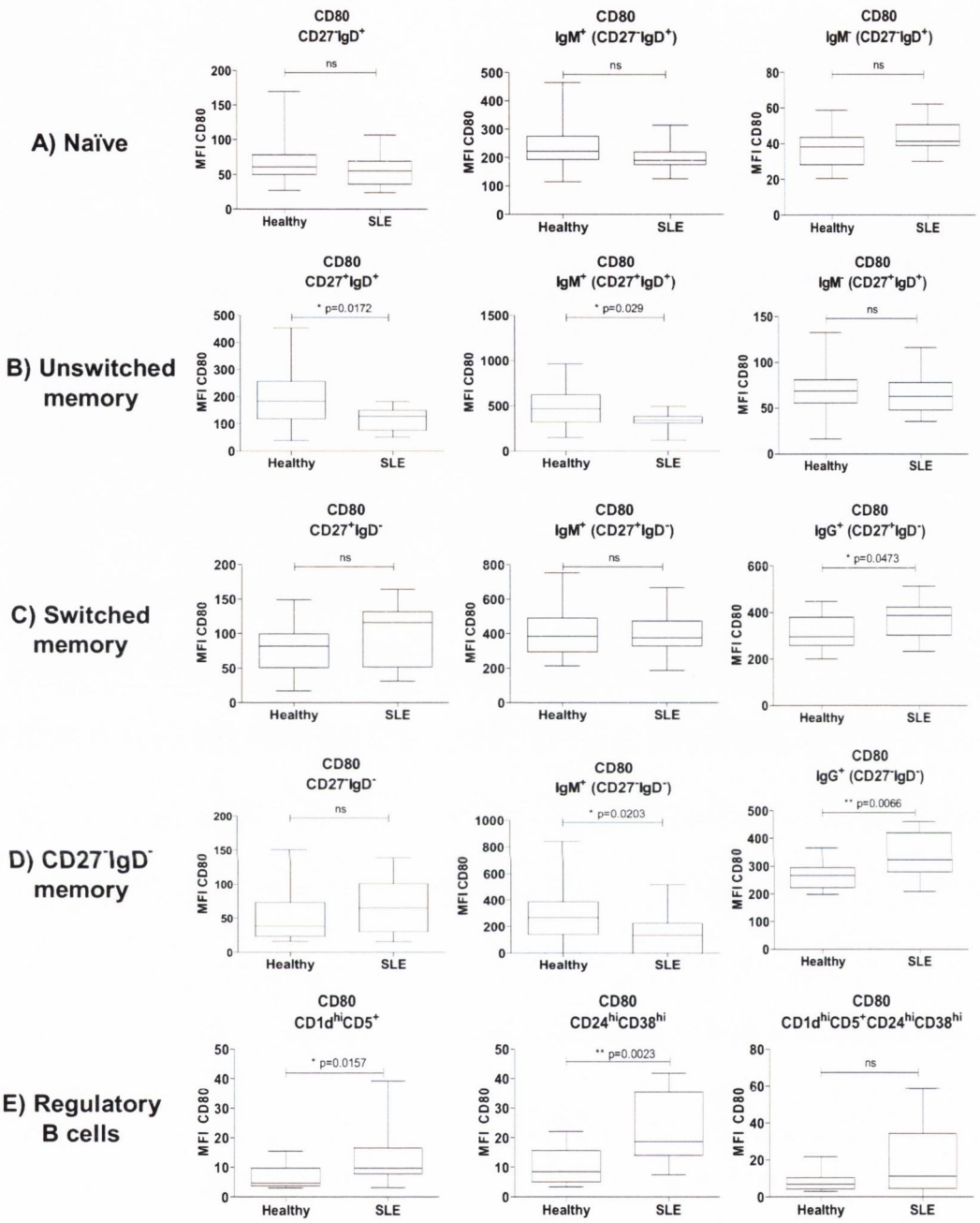

Figure 6.3.5. CD80 expression is increased on Breg, switched and CD27$^-$IgD$^-$ memory B cells but decreased on unswitched memory B cells in SLE patients. Flow cytometric analysis was performed on freshly-isolated PBMCs obtained from healthy donors (n=20) and SLE patients (n=15). MFI of CD40 expression on unswitched, switched and CD27$^-$IgD$^-$ memory B cells, and their respective IgM$^+$, IgM$^-$ or IgG$^+$ subsets was analysed. Floating bars represent minimum to maximum with horizontal line at mean, error bars correspond to SEM. Statistical analysis was performed using the Mann-Whitney U test, *p<0.05, **p<0.01.

6.3.2 Culture of iNKT lines from SLE patients

6.3.2.1 Phenotype of in vitro expanded iNKT cells

iNKT cells enriched from PBMC by magnetic bead separation were stimulated *in vitro* by stimulation with purified anti-human CD3 antibody, irradiated allogeneic feeders and IL-2. Flow cytometric analysis was performed on the PBMC before separation and on expanded iNKT lines after 4 weeks of culture to assess the frequency of iNKT cells (Figure 6.3.6A, left). As starting cell numbers were very small, flow cytometric analysis on magnetic bead-purified fractions was not carried out. In comparison to starting cell numbers (counted after enrichment by magnetic bead separation), iNKT frequencies were enhanced approximately 20 – 600 fold after 4 weeks of culture, and purity of iNKT lines ($6B11^+CD3^+$) was 5.76 ± 5.7% (mean ± SD, n=4). iNKT lines were resorted using magnetic bead separation (Figure 6.3.6A, middle) and re-stimulated with anti-human CD3 antibody and IL-2 for a further 4 weeks (Figure 6.3.6A, right). Subsequent flow cytometric analysis of re-stimulated lines indicated their purities to be 24.1 ± 21.6% (mean ± SD, n=4). The iNKT lines were also phenotyped for expression of CD4 and CD8 prior to use in co-cultures to elucidate proportions of $CD4^+$, DN and $CD8^+$ iNKT cells. The mean percentage of $CD4^+$ iNKT subset is 65.1 ± 23.9%; DN subset is 30.0 ± 23.7%; and $CD8^+$ subset is 3.0 ± 4.0% (Figure 6.3.6B). Hence it appears that the $CD4^+$ subset is the most predominant population in the expanded SLE iNKT lines, followed by the DN subset and the $CD8^+$ subset (Table 6.3.1).

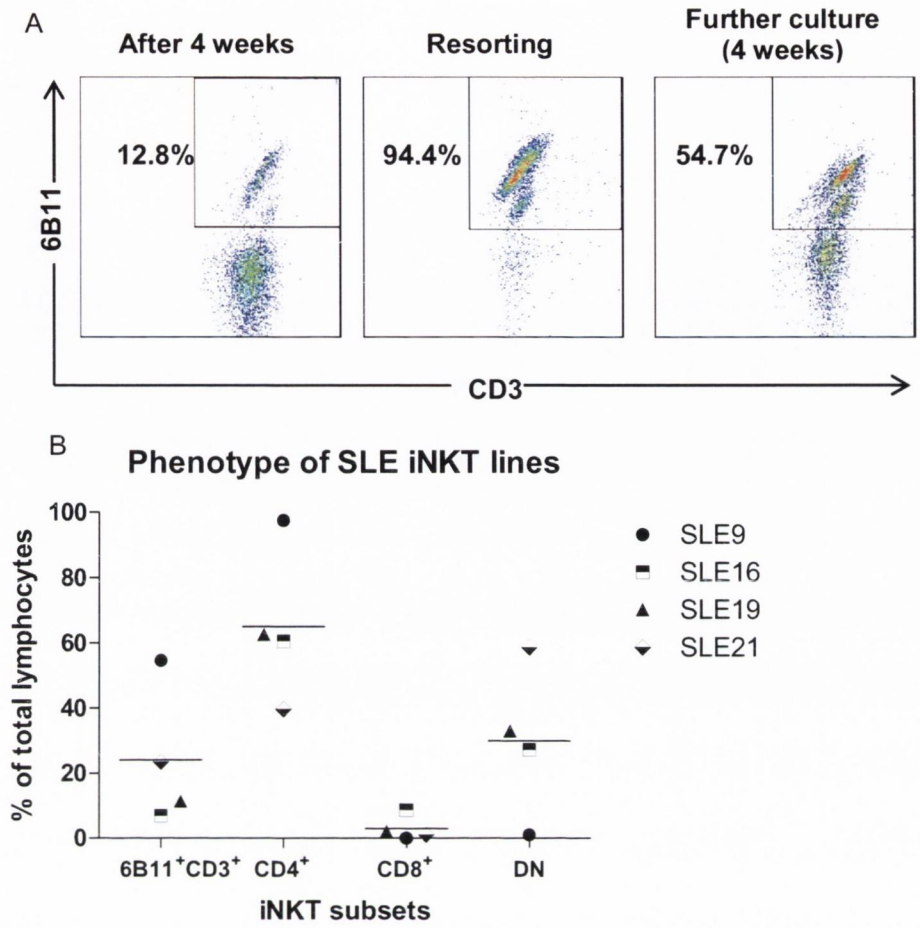

Figure 6.3.6. Flow cytometric assessment of iNKT cell and subset frequencies in iNKT lines.

(A) Flow cytometric analysis of CD3 and Vα24Jα18 TCR (6B11) expression by iNKT lines after 4 weeks of initial culture with anti-CD3, feeders and IL-2 (left), re-sorted because of low purity (middle), and after a further four weeks in culture with anti-CD3 and IL-2 stimulation (right). Cells were stained with antibodies against the iNKT receptor CDR3 loop (6B11) and CD3. Flow cytometry plots shown are representative of four different iNKT lines set up from SLE patients.

(B) Percentage of $CD4^+$, DN and $CD8^+$ iNKT cell subsets in 6 iNKT lines. Purity of iNKT ($6B11^+CD3^+$) is 24.1 ± 21.6% (mean ± sd). Mean percentages of $CD4^+$, DN and $CD8^+$ iNKT cells are 65.1 ± 23.9%, 30.0 ± 23.7% and 3.0 ± 4.0%.

Table 6.3.1. Purity and phenotype of 4 lines (after 8 weeks' expansion) set up from SLE patients.

iNKT subsets	$6B11^+CD3^+$	$CD4^+$	$CD8^+$	DN
SLE9	54.7	97.5	0.026	1.01
SLE16	6.89	60.6	8.73	27.3
SLE19	11.4	62.6	2.14	33
SLE21	23.5	39.8	1.03	58.7

6.3.3 Evaluating the ability of iNKT lines from SLE patients to activate healthy B cells.

To determine whether the iNKT cells from SLE patients are defective in immunoregulatory function, iNKT lines cultured from SLE patients were co-cultured with B cells obtained from healthy donors for 3 days and 10 days (henceforth denoted as "SLE iNKT-B cell co-cultures" or "SLE co-cultures"). As negative controls, iNKT cells or B cells were individually cultured in medium only for the same duration. Flow cytometric analysis of changes in CD154 expression on the iNKT cells, and CD40 and CD80 expression on various B cell subsets was performed after 3 days. Cytokine and immunoglobulin production were evaluated by CBA after 3 and 10 days respectively. These were compared to similar data obtained in co-cultures of healthy B cells with healthy iNKT lines (henceforth denoted as "healthy iNKT-B cell co-cultures" or "healthy co-cultures") which were carried out simultaneously.

6.3.3.1 $CD4^+$ iNKT cells from SLE patients, but not healthy controls, upregulate CD154 expression when co-cultured with healthy B cells in the absence of α-GC.

Similar to healthy controls, the frequencies of total iNKT cells in SLE iNKT-B cell co-cultures remained unchanged when co-cultured with B cells in the absence or presence of α-GC. However, in contrast to healthy controls, the proportions of $CD4^+$ iNKT cells decreased slightly while $CD8^+$ and DN iNKT cells increased slightly when SLE iNKT cells were co-cultured with B cells. These changes were observed both in the presence and absence of α-GC for $CD4^+$ and DN iNKT cells, but only in the presence of α-GC for $CD8^+$ iNKT cells. (**Figure 6.3.7**A)

In healthy iNKT-B cell co-cultures, no significant changes (relative to negative controls) in frequencies or CD154 expression were observed for all of the iNKT cell subsets. This was consistent both in the absence and presence of α-GC. (**Figure 6.3.7**A and B)

Dissimilar results were observed when SLE iNKT cells were co-cultured with healthy B cells. Moderately enhanced CD154 expression was observed across all the iNKT subsets, in particular for $CD4^+$ iNKT cells ($p<0.05$) when the iNKT cells were co-cultured with B cells in the absence of α-GC. However, when α-GC was present, CD154 expression for all the iNKT subsets was reduced akin to healthy iNKT-B co-cultures. (**Figure 6.3.7**B)

These results suggest that in contrast to iNKT cells from healthy individuals, iNKT cells from SLE patients appear to be more activated when co-cultured with B cells in the absence of α-GC, implying that the iNKT cells in SLE patients may be hyper-responsive to B cell contact.

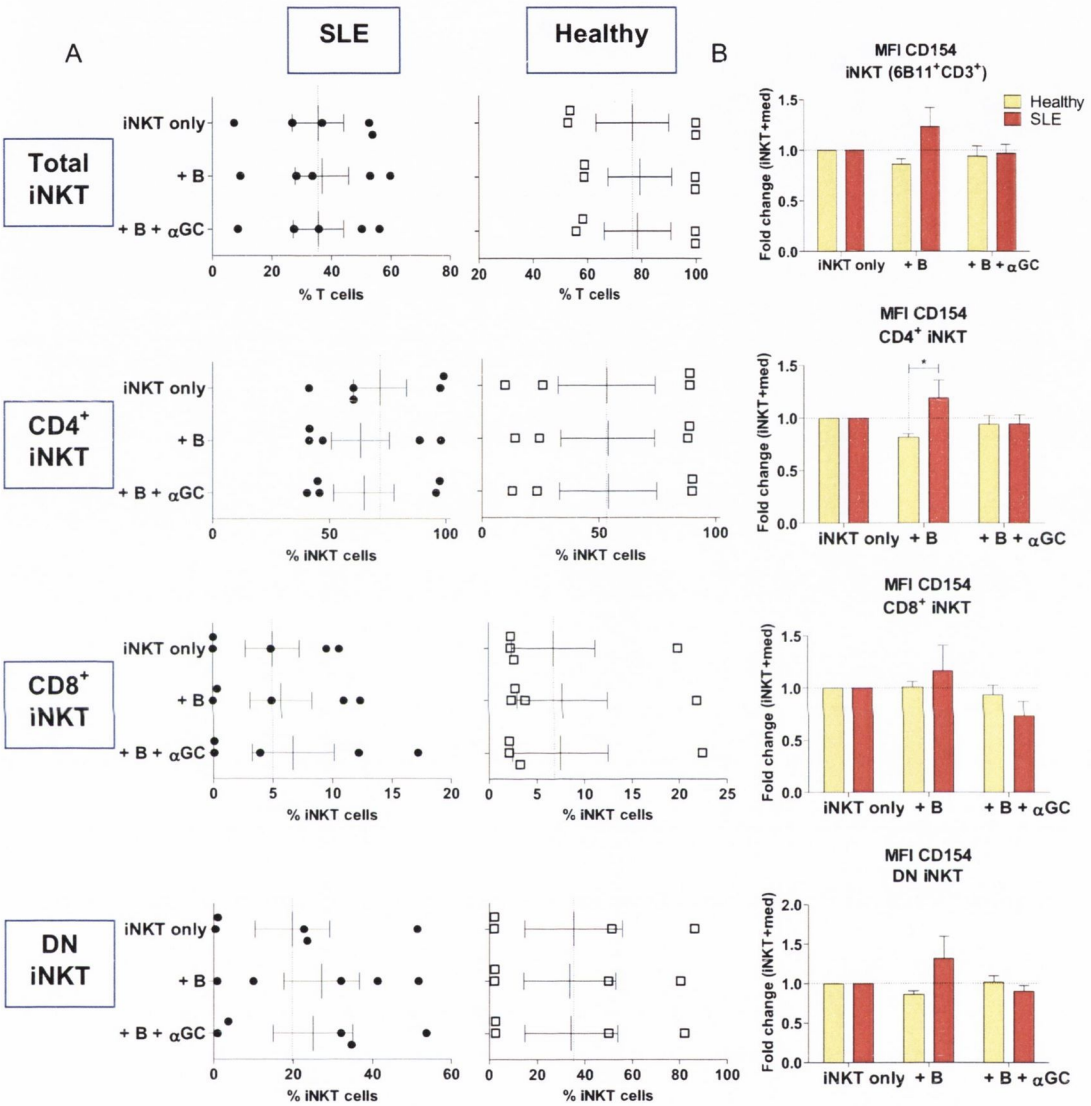

Figure 6.3.7. CD4$^+$ iNKT cells from SLE patients, but not healthy controls, upregulate CD154 expression when co-cultured with healthy B cells in the absence of α-GC. Healthy B cells were co-cultured with healthy iNKT cells (n=4) or SLE iNKT cells (n=5) in the absence or presence of α-GC for 3 days. Flow cytometric analysis was performed on total, CD4$^+$, CD8$^+$ and DN iNKT cells. (A) Scatter plots showing changes in frequencies of total (top row), CD4$^+$ (second row), CD8$^+$ (third row) and DN (bottom row) iNKT cells between SLE and healthy co-cultures. Vertical lines with error bars represent mean ± SEM. (B) Bar graphs depicting changes in MFI of CD154 expression on the corresponding iNKT cell subsets. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test to compare between treatments (B ± α-GC), *$p<0.05$. Two-way ANOVA with post-hoc Bonferroni test was used to compare changes in MFI between SLE and healthy co-cultures, *$p<0.05$; upregulation of CD154 expression was significantly higher on CD4$^+$ iNKT cells in SLE co-cultures (without α-GC) than healthy co-cultures.

6.3.3.2 iNKT cells from SLE patients are more potent than healthy iNKT cells in expanding naïve B cells but are unable to expand $CD27^-IgD^-$ memory B cells in response to α-GC.

iNKT cells expanded from SLE patients and healthy donors were co-cultured for 3 days with equal numbers of sorted B cells from healthy donors. Changes in the frequencies of naïve B cells, memory B cells and Breg cells were determined by flow cytometry.

iNKT cells from SLE patients were more potent than iNKT cells from healthy donors in expanding IgM^+ naïve B cells and decreasing frequencies of IgM^- naïve B cells, but appeared similar in their ability to expand total naïve B cells. (**Figure 6.3.8**A) These results suggest that iNKT cells from SLE patients expand IgM^+ naïve B cells more potently than iNKT cells from healthy donors.

Changes in the proportions of total, IgM^+ and IgM^- unswitched memory B cells were similar between SLE co-cultures and healthy co-cultures, suggesting that iNKT cells from SLE patients had similar effects on unswitched memory B cell frequencies as iNKT cells from healthy donors. (**Figure 6.3.8**B)

B cell co-culture with iNKT cells from SLE patients or with iNKT cells from healthy donors did not result in changes in proportions of total switched memory B cells. The increased expression of IgM on switched memory B cells was significant only when B cells were co-cultured with iNKT cells from SLE patients, however, trends of changes in IgM^+ and IgG^+ switched memory B cells were similar between SLE and healthy co-cultures. (**Figure 6.3.8**C) These data suggest that iNKT cells from SLE patients have similar effects on switched memory B cell frequencies as iNKT cells from healthy donors.

In the SLE co-cultures, frequencies of total $CD27^-IgD^-$ memory B cells were not increased in the presence of α-GC unlike that observed in healthy co-cultures, suggesting that the iNKT cells from SLE patients are less responsive to α-GC. Similarly, iNKT cells from SLE patients induced increases in proportions of IgM^+ and IgG^+ $CD27^-$ IgD^- memory B cells which remained upregulated to the same extent both in the presence or absence of α-GC. (**Figure 6.3.8**D) These data suggest that iNKT cells from SLE patients do not respond normally to α-GC with regards to their effects on $CD27^-IgD^-$ memory B cells.

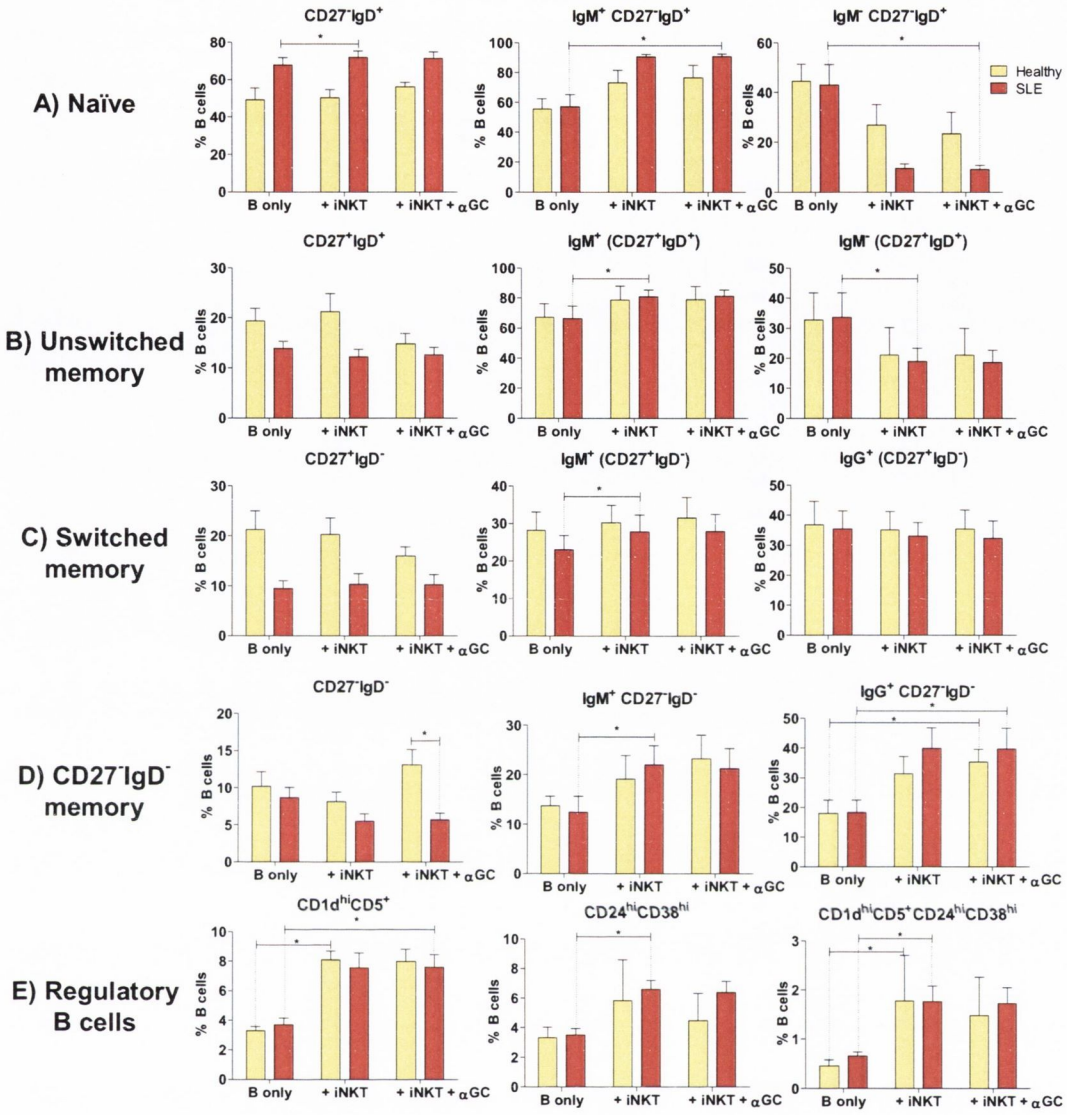

Figure 6.3.8. iNKT cells from SLE patients are more potent than healthy iNKT cells in expanding naïve B cells but are unable to expand CD27$^-$IgD$^-$ memory B cells in response to α-GC. Healthy B cells were co-cultured with iNKT cells from healthy donors (n=4) or iNKT cells from SLE patients (n=5) in the absence or presence of α-GC for 3 days. Flow cytometric analysis was performed on various B cell subsets. Bar charts showing changes in frequencies of (A) total, IgM$^+$ and IgM$^-$ naïve B cells, (B) total, IgM$^+$ and IgM$^-$ unswitched memory B cells, (C) total, IgM$^+$ and IgG$^+$ switched memory, (D) total, IgM$^+$ and IgG$^+$ CD27-IgD- memory and (E) CD1d^{hi}CD5$^+$, CD24^{hi}CD38^{hi} and CD1d^{hi}CD5$^+$CD24^{hi}CD38^{hi} regulatory B cells between SLE and healthy co-cultures. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test to compare between treatments (iNKT ± α-GC), *p<0.05, **p<0.01. Two-way ANOVA with post-hoc Bonferroni test was performed to compare changes in frequencies between SLE and healthy co-cultures, *p<0.05.

iNKT cells from SLE patients and healthy donors appeared to be equally efficient at inducing upregulation in frequencies of $CD1d^{hi}CD5^{+}$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$ B cells (denoted as Breg cells). (Figure 6.3.8E)

To summarise, iNKT cells from SLE patients were able to elicit expansion of naïve (total and IgM^{+}) and IgM^{+} and IgG^{+} $CD27^{-}IgD^{-}$ memory B cells in the absence of α-GC to the same extent as iNKT cells from healthy donors with the aid of α-GC. iNKT cells from SLE patients were also unable to induce expansion of total $CD27^{-}IgD^{-}$ memory B cells in response to α-GC, suggesting an innate defect in ability to respond to α-GC. However, iNKT cells from SLE patients were no different from iNKT cells from healthy donors in their ability to expand unswitched memory B cells, switched memory B cells and Breg cells. **Table 6.3.2** summarises the changes in frequencies of the B cell subsets in the SLE and healthy co-cultures.

6.3.3.3 iNKT cells from SLE patients maximally upregulate CD40 expression on memory and naïve B cells without need for α-GC.

Changes in CD40 expression on the various B cell subsets in both the SLE and healthy iNKT-B cell co-cultures were also analysed by flow cytometry.

Upregulation of CD40 expression on all three naïve B cell subsets was much higher in the SLE co-cultures than in healthy co-cultures. In particular, SLE iNKT cells in the absence of α-GC upregulated CD40 expression on total naïve B cells to a higher extent than healthy iNKT cells ($p<0.05$). Similar to previous observations, α-GC was not required for the potent upregulating effect of iNKT cells from SLE patients. (**Figure 6.3.9**)

The iNKT cells from SLE patients appeared to be equally efficient at upregulating CD40 on unswitched memory B cells regardless of the presence of α-GC. Upregulation of CD40 on unswitched B cells by iNKT cells from SLE patients was similar to iNKT cells from healthy donors. (**Figure 6.3.9**B)

Table 6.3.2. Summary of changes in frequencies of B cell subsets in co-cultures of B cells from healthy donors with iNKT cells from SLE patients and healthy donors.

Frequencies	Effects of iNKT cells (relative to unstimulated B cells)			
	Healthy		SLE	
B cell subsets	No α-GC	With α-GC	No α-GC	With α-GC
Naïve				
Total	-	↑	↑*	↑
IgM^+	↑	↑	↑	↑*
IgM^-	↓	↓	↓	↓*
Unswitched memory				
Total	-	↓	-	-
IgM^+	↑	↑	↑*	↑
IgM^-	↓	↓	↓*	↓
Switched memory				
Total	-	↓	-	-
IgM^+	↑	↑	↑*	↑
IgG^+	-	-	-	-
$CD27^-IgD^-$ memory				
Total	↓	↑*	↓	↓*
IgM^+	↑	↑↑	↑↑*	↑↑
IgG^+	↑	↑↑*	↑↑	↑↑*
Breg				
$CD1d^{hi}CD5^+$	↑↑*	↑↑	↑↑	↑↑*
$CD24^{hi}CD38^{hi}$	↑	-	↑*	↑
$CD1d^{hi}CD5^+$	↑↑*	↑↑	↑↑*	↑↑
$CD24^{hi}CD38^{hi}$				

Asterisk (*) denotes change observed is statistically significant, $p<0.05$. "↑" denotes upregulation, "↓" denotes downregulation, "-" denotes no change relative to B cells cultured in medium alone.

No observable differences were seen between iNKT cells from SLE patients and healthy controls in upregulating CD40 expression on total switched memory B cells. Unlike iNKT cells from healthy controls, iNKT cells from SLE patients did not require α-GC to maximally upregulate CD40 expression on IgM^+ and IgG^+ switched memory B cells. (**Figure 6.3.9**C)

iNKT cells from SLE patients induced slightly greater upregulation of CD40 expression on IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells in comparison to iNKT cells from healthy controls. As observed previously, potent upregulation of CD40 expression by iNKT cells from SLE patients did not require α-GC. These data suggest that SLE iNKT cells activate $CD27^-IgD^-$ memory B cells more potently than healthy iNKT cells. (**Figure 6.3.9**D)

iNKT cells from SLE patients and healthy individuals were comparable in their ability to enhance CD40 expression on $CD1d^{hi}CD5^+$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ B cells (denoted as Breg cells), both in the absence and presence of α-GC. (**Figure 6.3.9**E)

In summary, iNKT cells from SLE patients are able to potently upregulate CD40 expression on naïve and memory memory B cells without the need for α-GC. However, there were no differences between iNKT cells from SLE patients and healthy controls in upregulating CD40 expression on Breg cells.

6.3.3.4 iNKT cells from SLE patients are more potent than iNKT cells from healthy controls at upregulating CD80 expression on naïve B cells.

Changes in CD80 expression on the various B cell subsets in both the SLE and healthy iNKT-B cell co-cultures were also analysed by flow cytometry.

Upregulation of CD80 expression on total and IgM^+ naïve B cell subsets but not IgM^- naïve B cells, was higher in the SLE co-cultures than in healthy co-cultures. The iNKT cells from SLE patients did not require α-GC for maximal upregulation of CD80 expression on the naïve B cells. (**Figure 6.3.10**A)

iNKT cells from SLE patients and healthy controls were similar in their effect on CD80 expression on the memory B cells: they resulted in minimal upregulation of CD80 expression on unswitched and switched memory B cells (**Figure 6.3.10**B and C) and similar upregulation of CD80 expression on $CD27^-IgD^-$ memory B cells. (**Figure 6.3.10**D)

iNKT cells from SLE patients and healthy individuals did not affect CD80 expression by $CD24^{hi}CD38^{hi}$ B cells, caused slight upregulation of CD80 expression on $CD1d^{hi}CD5^+$ B cells and significant upregulation of $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ B cells ($p<0.05$). These

results suggest that iNKT cells from both sources were comparable in their ability to enhance CD80 expression on these B cell subsets, both in the absence and presence of α-GC. (**Figure 6.3.10**E)

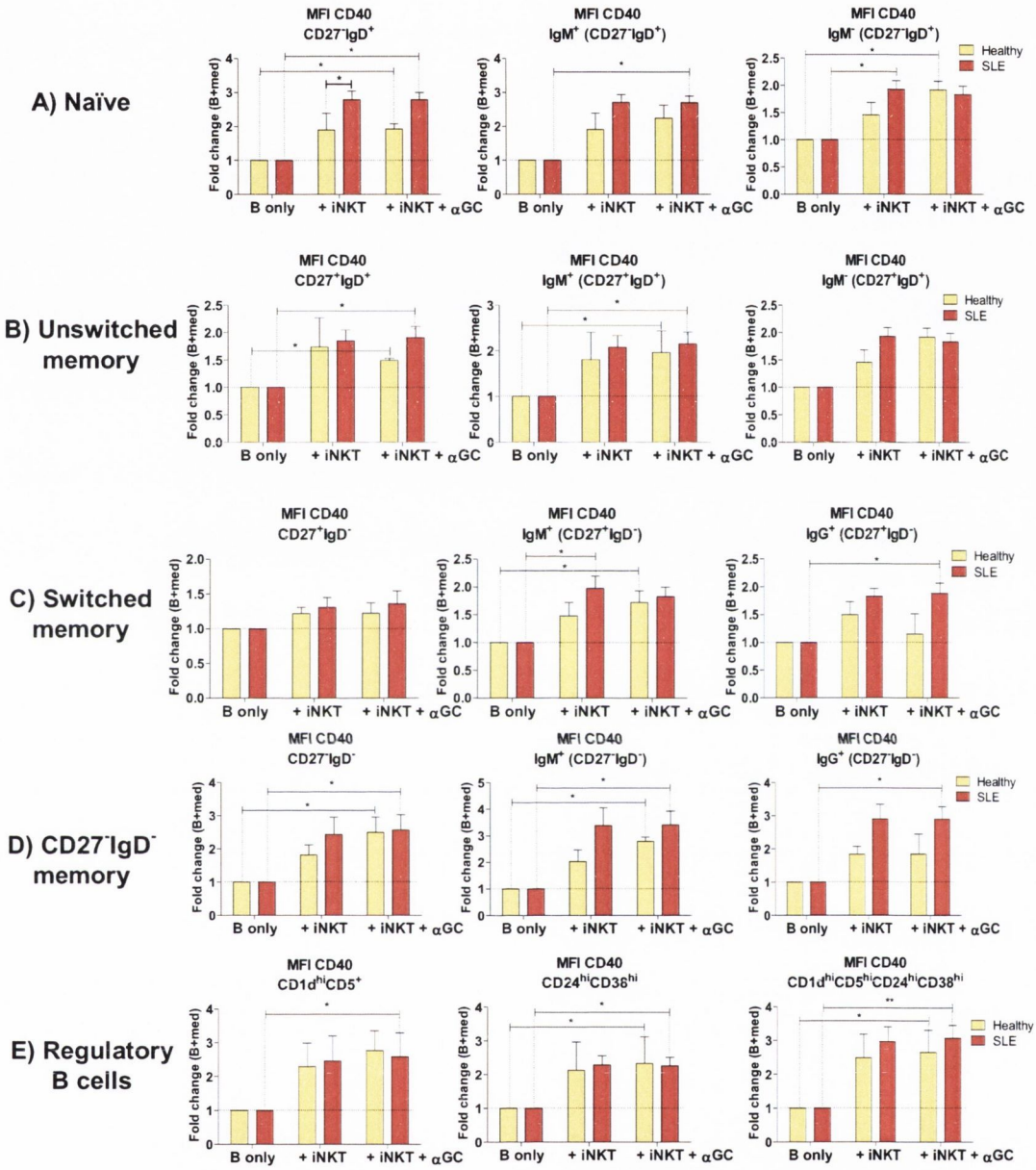

Figure 6.3.9. SLE iNKT cells maximally upregulate CD40 expression on naïve and memory B cells without need for α-GC. Healthy B cells were co-cultured with iNKT cells from healthy donors (n=4) or iNKT cells from SLE patients (n=5) in the absence or presence of α-GC for 3 days. Flow cytometric analysis was performed on the various B cell subsets. Bar graphs depict changes in MFI of CD40 expression on: (A) Total, IgM^+ and IgM^- naïve B cells, (B) Total, IgM^+ and IgM^- unswitched memory B cells, (C) Total, IgM^+ and IgG^+ switched memory B cells, (D) total, IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells and (E) $CD1d^{hi}CD5^+$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ regulatory B cells. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test to compare between treatments (iNKT ± α-GC), *p<0.05, **p<0.01. Two-way ANOVA with post-hoc Bonferroni test was used to compare changes in MFI between SLE and healthy co-cultures; *p<0.05.

In summary the data suggest that iNKT cells from SLE patients and healthy controls similarly induced CD80 expression by the memory B cell and Breg cell subsets. iNKT cells from SLE patients were slightly more potent in enhancing CD80 expression on naïve B cells than iNKT cells from healthy individuals and α-GC was not required to mediate this effect.

Table 6.3.3 summarises the changes in expression of CD40 and CD80 on the B cell subsets in response to iNKT cells from SLE patients and healthy controls.

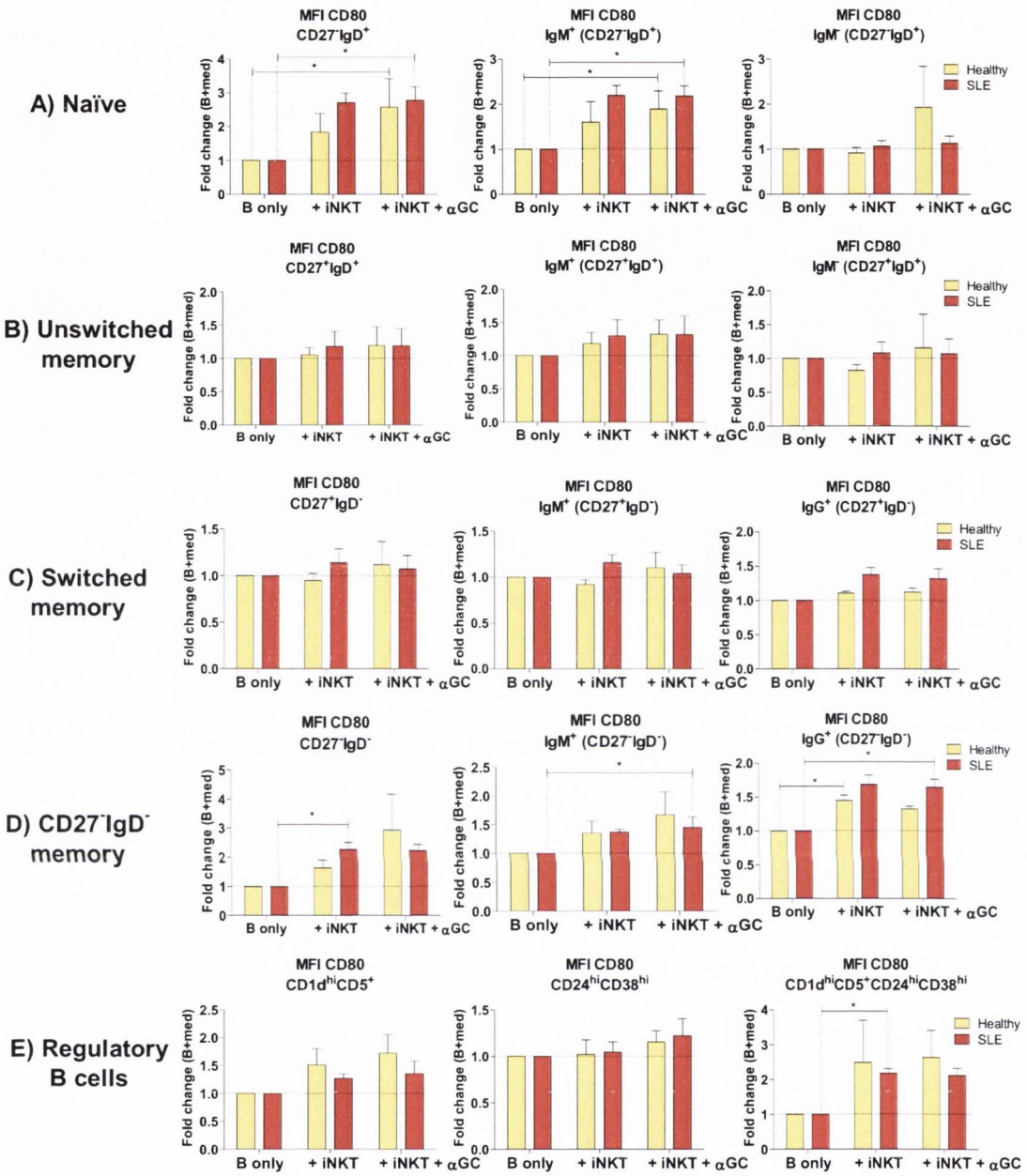

Figure 6.3.10. iNKT cells from SLE patients are more potent than iNKT cells from healthy controls at upregulating CD80 expression on naïve B cells. Healthy B cells were co-cultured with healthy iNKT cells (n=4) or SLE iNKT cells (n=5) in the absence or presence of α-GC for 3 days. Flow cytometric analysis was performed on memory B cells. Bar graphs depict changes in MFI of CD80 expression on: (A) Total, IgM^+ and IgM^- naïve B cells, (B) Total, IgM^+ and IgM^- unswitched memory B cells, (C) Total, IgM^+ and IgG^+ switched memory B cells, (D) total, IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells and (E) $CD1d^{hi}CD5^+$, $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ regulatory B cells. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test to compare between treatments (iNKT ± α-GC), *$p<0.05$. Two-way ANOVA with post-hoc Bonferroni test was used to compare changes in MFI between SLE and healthy co-cultures; no statistical difference was observed between them.

Table 6.3.3. Summary of changes in CD40 and CD80 expression on B cell subsets in co-cultures of B cells from healthy donors with iNKT cells from SLE patients and healthy donors.

CD40/CD80	Effects of iNKT cells (relative to unstimulated B cells)			
	Healthy		SLE	
B cell subsets	No α-GC	With α-GC	No α-GC	With α-GC
Naïve	**CD40/CD80**	**CD40/CD80**	**CD40/CD80**	**CD40/CD80**
Total	↑ / ↑	↑ * / ↑↑ *	↑↑ * / ↑↑	↑↑ * / ↑↑ *
IgM^+	↑ / ↑	↑ / ↑ *	↑↑ / ↑↑	↑↑ * / ↑↑ *
IgM^-	↑ / -	↑↑ * / ↑	↑↑ * / -	↑↑ / -
Unswitched memory				
Total	↑↑ / -	↑ * / -	↑↑ / -	↑↑ * / -
IgM^+	↑ / -	↑↑ * / ↑	↑↑ / ↑	↑↑ * / ↑
IgM^-	↑ / -	↑↑ / -	↑↑ / -	↑↑ / -
Switched memory				
Total	↑ / -	↑ / -	↑ / -	↑ / -
IgM^+	↑ / -	↑↑ * / -	↑↑ * / ↑	↑↑ / -
IgG^+	↑ / -	- / -	↑↑ / ↑	↑↑ * / ↑
$CD27^-IgD^-$ memory				
Total	↑ / ↑	↑↑ * / ↑↑	↑↑ / ↑↑ *	↑↑ * / ↑↑
IgM^+	↑ / ↑	↑ * / ↑	↑↑ / ↑	↑↑ * / ↑ *
IgG^+	↑ / ↑ *	↑ / ↑	↑↑ / ↑↑	↑↑ * / ↑↑ *
Breg				
$CD1d^{hi}CD5^+$	↑↑ / ↑	↑↑ / ↑↑	↑↑ / ↑	↑↑ * / ↑
$CD24^{hi}CD38^{hi}$	↑↑ / -	↑↑ * / ↑	↑↑ / -	↑↑ * / ↑
$CD1d^{hi}CD5^+$ $CD24^{hi}CD38^{hi}$	↑↑ / ↑↑	↑↑ * / ↑↑	↑↑ / ↑↑ *	↑↑ ** / ↑↑

Asterisk (*) denotes change observed is statistically significant, *p<0.05, **p<0.01. "↑" denotes upregulation, "↓" denotes downregulation, "-" denotes no change relative to B cells cultured in medium alone.

6.3.3.5 α-GC is not required for cytokine production in SLE iNKT-B cell co-cultures.

Supernatants from 3-day co-cultures of B cells sorted from healthy donors with iNKT cells from healthy controls (n=3) (denoted as "healthy co-cultures") or iNKT cells from SLE patients (n=5) (denoted as "SLE co-cultures") in the absence or presence of α-GC were analysed for cytokine production using multiplex cytometric bead arrays. Cytokines assayed for were IFN-γ, IL-2, IL-4, IL-5, TNF-α, IL-13 and IL-10.

The expanded SLE iNKT lines produced more cytokines (IFN-γ, IL-4, IL-5, TNF-α and IL-13) in the absence of further stimulation in comparison to expanded healthy iNKT lines. However, IL-2 production was comparable between SLE and healthy iNKT lines and did not vary even when the iNKT cells were co-cultured with B cells and with α-GC.

Enhanced production of IFN-γ, IL-4, IL-5, TNF-α and IL-13 was observed in co-cultures of B cells with iNKT cells from healthy subjects only in the presence of α-GC. In contrast, iNKT cells from SLE patients induced greater levels of these cytokines, in particular TNF-α ($p<0.05$), when co-cultured with B cells even in the absence of α-GC. The presence of α-GC in the SLE co-cultures did not induce further increases in cytokine production. IL-10 production was detected only in the SLE iNKT-B cell co-cultures; in the absence of α-GC enhanced IL-10 production was observed whereas in the presence of α-GC the enhanced IL-10 production was diminished. (**Figure 6.3.11**)
These data suggest that the iNKT cell lines from SLE patients have a more activated phenotype than those generated from healthy subjects.

6.3.3.6 SLE iNKT cells induce less immunoglobulin production from B cells.

Supernatants from 10-day co-cultures of B cells sorted from healthy donors with iNKT cells from healthy controls (n=3) (denoted as "healthy co-cultures") or iNKT cells from SLE patients (n=5) (denoted as "SLE co-cultures") in the absence or presence of α-GC were analysed for immunoglobulin production using multiplex cytometric bead arrays. Immunoglobulins assayed for were IgG_1, IgG_2, IgM and IgA.

iNKT cells from SLE patients induced less IgG_1, IgM ($p<0.05$) and IgA production from B cells in comparison to iNKT cells from healthy controls. Conversely, iNKT cells from SLE patients induced greater IgG_2 production from B cells than iNKT cells from healthy controls did, suggesting that iNKT cells from SLE patients may possibly be biased toward inducing a Th1-associated antibody response. In healthy co-cultures, addition of α-GC downregulated iNKT-induced antibody production. However, this was not observed for iNKT-induced IgG_1, IgM and IgA production in the SLE co-cultures, suggesting that the iNKT cells from SLE patients were not responsive to the feedback of α-GC on inhibiting antibody production from the B cells. (Figure 6.3.12)

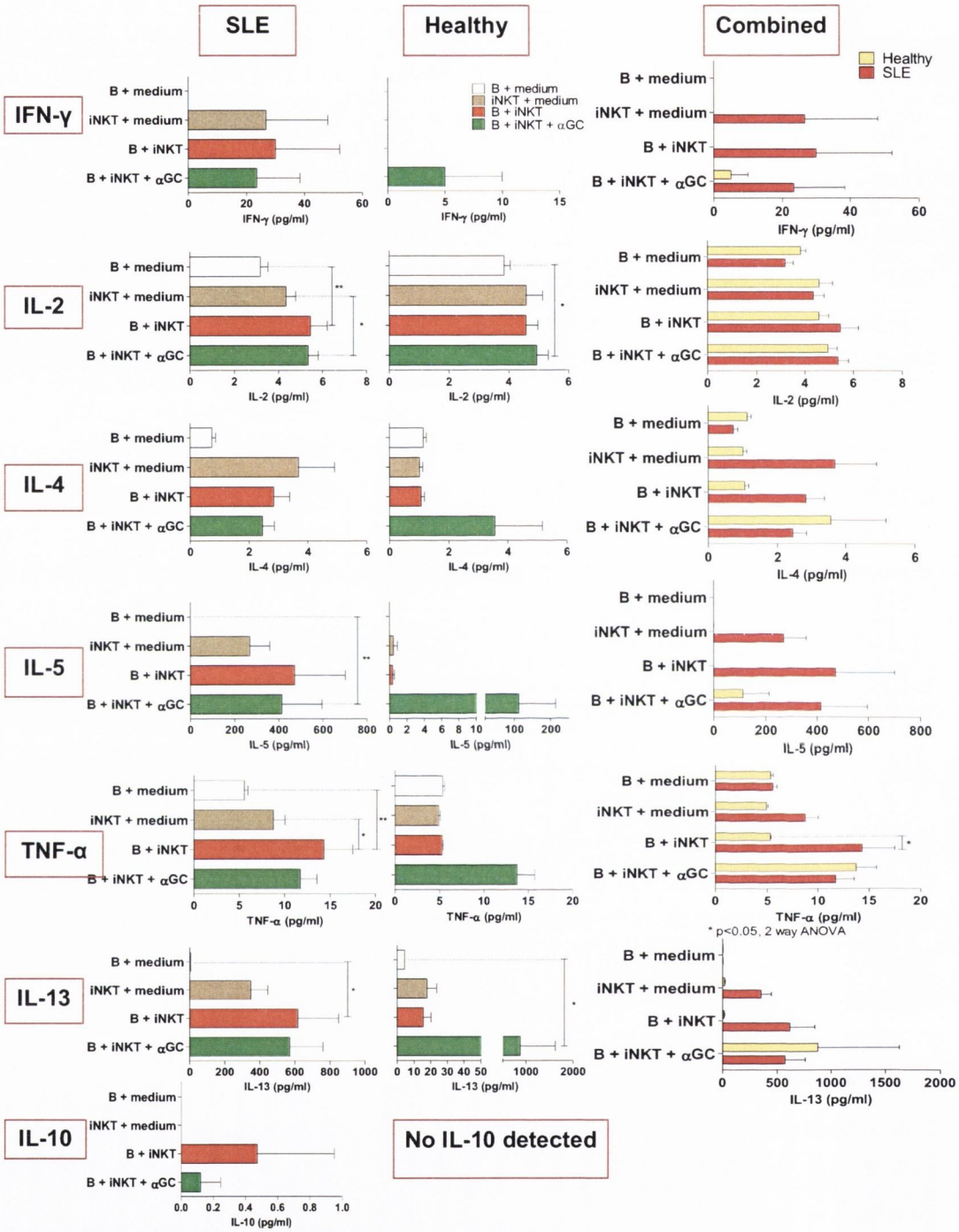

Figure 6.3.11. α-GC is not required for cytokine production in SLE iNKT-B cell co-cultures. Supernatants from 3-day co-cultures of healthy B cells with healthy iNKT cells (n=3) or SLE iNKT cells (n=5) in the absence or presence of α-GC were analysed by multiplex cytometric bead arrays for IFN-γ, IL-2, IL-4, IL-5, TNF-α, IL-13 and IL-10 production. Statistical analysis was carried out for SLE (left column) and healthy (middle column) co-cultures using Friedman's test with post-hoc Dunn's multiple comparison test, *p<0.05, **p<0.01. Statistical analysis was carried out for combined (right column) graphs using two-way ANOVA with post-hoc Bonferroni test, *p<0.05.

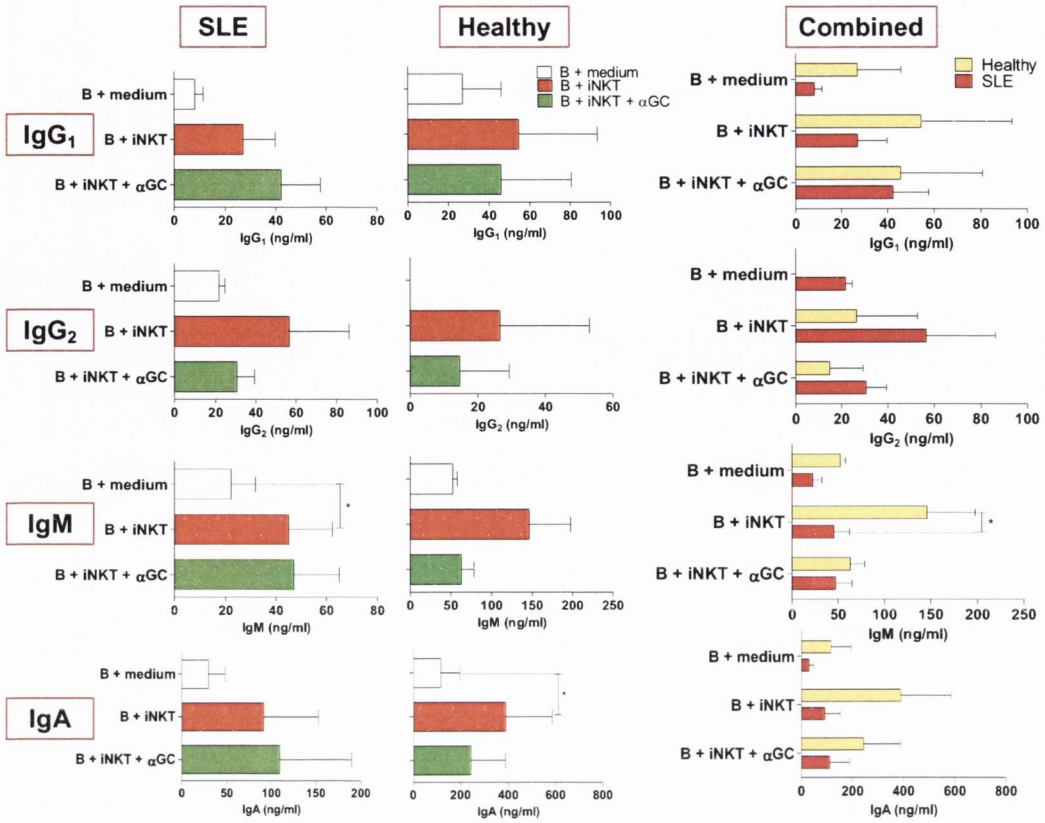

Figure 6.3.12. SLE iNKT cells induce less immunoglobulin production from B cells. Supernatants from 3-day co-cultures of healthy B cells with healthy iNKT cells (n=3) or SLE iNKT cells (n=5) in the absence or presence of α-GC were analysed by multiplex cytometric bead arrays for IgG1, IgG2, IgM and IgA production. Statistical analysis was carried out for SLE (left column) and healthy (middle column) co-cultures using Friedman's test with post-hoc Dunn's multiple comparison test, *p<0.05. Statistical analysis was carried out for combined (right column) graphs using two-way ANOVA with post-hoc Bonferroni test, *p<0.05.

6.4 Discussion

6.4.1 $CD4^+$ iNKT cell frequencies and CD154 expression are enhanced in SLE patients.

In our phenotyping of freshly-isolated PBMCs from SLE patients, we observed that the frequencies of total, $CD8^+$ and DN iNKT cells in SLE patients were not significantly different from healthy controls, whereas frequencies of $CD4^+$ iNKT cells were higher in the SLE patients compared to healthy controls. Enumeration of iNKT cell frequencies by flow cytometry phenotyping has resulted in a great variation of observations between papers. Cho et al observed decreased percentages and absolute numbers of total iNKT cells in the peripheral blood of SLE patients, but no significant differences between healthy controls and SLE patients with regards to the percentages of $CD4^+$, $CD8^+$ and DN iNKT cell subsets (Cho et al., 2011). In contrast, Kojo et al observed decreased numbers of both $CD4^+$ and DN iNKT cell subsets (Kojo et al., 2001) and van der Vliet et al observed overall decreased numbers of circulating $V\alpha24^+V\beta11^+$ iNKT cells in SLE patients. There are several reasons which could account for such disparities: firstly, classification of iNKT cells was different between the studies – Cho denoted iNKT cells as $6B11^+CD3^+$, whereas Kojo and van der Vliet denoted iNKT cells as $V\alpha24^+V\beta11^+$. Both methods of classification are valid for defining iNKT cells although the use of Vα24/Vβ11 staining is considered less optimal because it can include non-iNKT cells (Exley et al., 2008). Secondly, SLE is a heterogenous disease with patients exhibiting a variety of clinical manifestations and we can logically expect that there would be huge diversity between the cohorts of patients in these studies in terms of sample size, medications, disease severity and genetics. Thirdly, there is massive variation in iNKT cell frequencies even within the healthy population (O'Reilly et al., 2011). However, the variability in iNKT cell frequency between individuals does not appear to affect their ability to expand in culture conditions used in this thesis and hence it is still feasible to target iNKT cells in immunotherapy.

Only a third of the patients in our study were on steroids and/or immunosuppressant drugs, which are known to result in skewing of lymphocyte populations, increasing the percentage of $CD19^+$ lymphocytes and decreasing the percentage of $CD56^+$ lymphocytes (Gluhovschi et al., 2007). In contrast, the majority of the patients in the Cho and Kojo study were on steroid treatment (91% and 80% respectively) (Cho et al., 2011; Kojo et al., 2001), which may explain why these studies observed decreased numbers

of iNKT cells whereas in our study we observed that there were no differences in the percentages of iNKT cells between SLE patients and healthy controls. Since decreased iNKT cell numbers have been correlated with severity of disease (as measured by the SLEDAI score) (Cho et al., 2011), another possible reason that we did not observe lowered iNKT cell frequencies is because the majority of the patients did not have active disease (raised ESR, lowered C3 and C4).

Instead, we observed enhanced frequencies of $CD4^+$ iNKT cells in the cohort of SLE patients, which also displayed enhanced expression of CD154 in comparison to $CD4^+$ iNKT cells from healthy individuals. Increases in proportions of $CD4^+$ iNKT cells and decreases in proportions of DN iNKT cells have been known to result in an increase in Th2 cytokine secretion from iNKT cells in the elderly, who are more susceptible to infectious, malignant and autoimmune diseases (Jing et al., 2007). This Th2 predisposition was also observed in iNKT lines expanded by α-GC stimulation from multiple sclerosis patients in remission, which were found to be predominantly produced by $CD4^+$ iNKT cells (Araki et al., 2003). As $CD4^+$ iNKT cells are the main producers of Th2 cytokines, the enhanced frequencies of $CD4^+$ iNKT cells may explain the bias in Th2 cytokine secretion observed in iNKT lines expanded from SLE patients observed in our own studies and others (Yu and Wang, 2011). Previous results (section 5.3.1) also showed that in comparison to $CD8^+$ and DN iNKT cells, $CD4^+$ iNKT cells are most efficient at activating B cells through upregulation of CD40 expression on the B cells. Both Th1 and Th2 responses are thought to contribute to SLE pathogenesis (Charles et al., 2010; Chen et al., 2000; Tucci et al., 2008), with speculation that IL-12p70 contributes to disease activity, IL-10 causes autoantibody production and TNF-α possibly being protective (Gómez et al., 2004). In this respect, $CD4^+$ iNKT cells may play a dual role in the etiology of SLE by directly secreting Th2 cytokines and activating B cells which are the primary effectors in the pathogenesis of SLE.

Higher levels of CD154 expression have been observed on both T cells and B cells in SLE patients (Devi et al., 1998; Manea et al., 2009), than in healthy controls or patients with rheumatoid arthritis, suggesting that this T cell defect is specific to SLE. Given that the CD40-CD154 interaction is vital for development of memory B cell function, affinity maturation, antibody production, isotype switching and germinal centre formation (Clark, Foy, and Noelle, 1996; Leadbetter et al., 2008), the elevation of CD154 expression on the $CD4^+$ iNKT cells suggests that the characteristic autoantibody production and pathogenic B cell effector functions observed in SLE could be due to excessive CD40-CD154 signalling induced by the $CD4^+$ iNKT cells. It is possible that enhanced

frequencies and CD154 expression on $CD4^+$ iNKT skew the immune response towards a favourable Th2 environment for B cell function and antibody production, thus contributing to the mechanism of SLE pathogenesis.

6.4.2 Aberrations of B cell subsets in SLE.

6.4.2.1 SLE patients are deficient in Breg cells and memory B cells.

In our studies we observed that B cell populations in SLE patients were skewed, which was similar to observations reported in other studies (Odendahl et al., 2000; Rodríguez-Bayona et al., 2010). Defects in regulatory capacities of B cells have been described in studies in SLE patients (Pers et al., 2011) and in the NZB/W lupus murine model (Watanabe et al., 2010). $CD24^{hi}CD38^{hi}$ B cells (which are the only published subset of Breg cells in humans) derived from SLE patients were found to be defective in their ability to suppress Th1 cytokine (TNF-α and IFN-γ) production from $CD4^+$ T cells in comparison to $CD24^{hi}CD38^{hi}$ B cells derived from healthy individuals (Blair et al., 2010). This defect in Breg cell function appears to be specific to SLE as the authors report that it was not observed in other arthritic diseases such as Sjögren's syndrome or osteoarthritis. However, Blair et al observed an increase in the $CD24^{hi}CD38^{hi}$ B cell population in SLE patients, which contrasts with our results, where we observed a decrease in the $CD24^{hi}CD38^{hi}$ B cell population in SLE patients as compared to healthy controls. We also observed decreases in $CD1d^{hi}CD5^+$ and $CD1d^{hi}CD5^+CD24^{hi}CD38^{hi}$ B cells, which correlates with observed functional deficiencies of Breg cells in lupus (Blair et al., 2010; Watanabe et al., 2010).

Frequencies of total unswitched, IgM^+ unswitched, total switched memory and IgM^+ $CD27^-IgD^-$ memory B cells were also decreased in SLE patients. Reduced frequencies of switched memory B cells and $IgM^+CD27^+IgD^+$ (unswitched memory) have been previously reported, and the latter subset of B cells exhibit increased migratory potential and activation phenotype (upregulated expression of CD86 and CD80), allowing for greater chance of interaction with T cells (Rodríguez-Bayona et al., 2010). The decreased frequencies of memory B cells could be due to their re-activation and differentiation into antibody-secreting plasma cells, as CD27/CD70 signalling between B cells and T cells in the presence of IL-10 have been known to induce differentiation of $CD27^+$ memory B cells toward plasma cells (Agematsu et al., 1998). It could also be due to accumulation at inflamed sites, similar to the situation observed in patients with Sjögren's syndrome whereby there was accumulation of memory B cells in the inflamed

salivary glands and decreased peripheral blood memory B cells (Hansen et al., 2002). Hence these memory B cell subsets may be involved in disease pathogenesis and further investigation of their roles in SLE may be useful for targeted immunotherapy.

6.4.2.2 Naïve B cells are expanded in SLE patients.

In our cohort of SLE patients, we observed enhanced frequencies of naïve B cells in comparison to healthy individuals. Autoreactivity is thought to be enhanced in mature naïve B cells in SLE patients, which consists of an enriched population of cells that spontaneously produce auto-reactive antibodies (Yurasov et al., 2005). This subset of mature naïve B cells persistently produces auto-reactive antibodies even when the patient is in clinical remission, although their numbers are reduced compared to patients with active disease (Yurasov et al., 2006). It is thus possible that the expanded population of naïve B cells observed in our cohort of patients correlates with autoantibody production, as majority of them (11 out of 15) expressed antibodies against dsDNA.

6.4.2.3 Expression of CD40 and CD80 on B cell subsets in SLE patients.

The CD40-CD154 and CD28-CD86/CD80 co-stimulatory pathways are upregulated in active SLE patients and have been linked to disease pathogenesis (Toubi and Shoenfeld, 2004; Yellin and Thienel, 2000). CD40-CD154 signalling is vital for reciprocal activation of B and T cells, resulting in increased expression of CD86/CD80 which enhances B cell antigen presentation in combination with CD28 co-stimulation from T cells (Han et al., 1995; Yang and Wilson, 1996). In particular, prolonged expression of CD154 on T cells from SLE patients induced greater CD80 expression on B cells than T cells from healthy subjects when co-cultured together (Koshy, Berger, and Crow, 1996). This supports our observations that CD40 and CD80 expression are upregulated on switched memory and $CD27^{-}IgD^{-}$ memory B cells (with the exception of the IgM^{+} subsets of these cells where only CD40 expression was upregulated). Also, a subset of $CD95^{+}$ $CD27^{-}IgD^{-}$ memory B cells have been demonstrated to display an activated phenotype in SLE patients with active disease (Jacobi et al., 2008). Interestingly although CD40 expression was upregulated on unswitched memory and naïve B cells, CD80 expression was not. Similarly, CD40 expression was not upregulated on $CD1d^{hi}CD5^{+}$ and $CD24^{hi}CD38^{hi}$ B cells, but CD80 expression was upregulated on them. It is not known whether this discordance in upregulation of CD40 and CD80 expression

observed for these cells is physiologically significant. However, this observation could be due to these different subsets of B cells being at different stages of activation, since CD40 signalling precedes CD80 upregulation. Perhaps it would be more informative to monitor CD40 and CD80 expression on the B cell subsets of the SLE cohort over a fixed time period to better understand the temporal dynamics of CD40 and CD80 expression on them. Administration of rituximab, which depletes B cells, to SLE patients with severe disease resulted in downregulation of CD40 and CD80 expression on the reconstituted B cells and an associated decline in disease activity (Tokunaga et al., 2005), suggesting a link between CD40 and CD80 expression on B cells and clinical outcome in SLE.

6.4.3 iNKT cells from SLE patients appear to be dysfunctional.

The iNKT cells from SLE patients were notably more difficult to expand in culture than iNKT cells from healthy individuals, requiring enrichment and re-stimulation after the first four weeks of culture. iNKT cells from some SLE donors were refractory to mitogen-stimulated expansion (own observations) and in comparison to healthy controls exhibited a decreased ability to proliferate when PBMC from SLE patients were stimulated by α-GC and IL-2 (Cho et al., 2011).

iNKT cells from SLE patients upregulated CD154 expression to a greater extent than iNKT cells from healthy individuals when co-cultured with B cells in the absence of α-GC, and was particularly pronounced for $CD4^+$ iNKT cells ($p<0.05$). This suggests that the iNKT cells are hyper-responsive to B cell contact, resulting in an upregulation of CD40-CD154 signalling. However, when the iNKT cells from SLE patients were co-cultured with B cells in the presence of α-GC, CD154 expression decreased back to similar levels as observed in the iNKT-B cell co-cultures with healthy controls, suggesting that the iNKT cells from SLE patients still respond to downregulation of its own function by B cell presentation of α-GC.

iNKT cells from SLE patients did not seem significantly different than iNKT cells from healthy controls in their ability to expand and activate Breg cells. Notably, α-GC was not required for potent expansion of naïve B cells by iNKT cells from SLE patients. In contrast to iNKT cells from healthy controls, the iNKT cells from SLE patients were unable to increase frequencies of total $CD27^-IgD^-$ memory B cells in the presence of α-GC but did not require α-GC to enhance frequencies of IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells, suggesting that the iNKT cells from SLE patients do not respond normally to α-GC with respect to their effect on $CD27^-IgD^-$ memory B cells.

This lack of responsiveness to α-GC was also observed in the ability of iNKT cells from SLE patients to maximally upregulate CD40 expression on the memory B cells and upregulate CD40 and CD80 expression on the naïve B cells in the absence of α-GC. This was further corroborated by the observations that cytokine production in the SLE iNKT-B cell co-cultures was independent of α-GC and cytokine production was greater in the SLE co-cultures than in the co-cultures with healthy controls. Surprisingly, the iNKT cells from SLE patients induced less IgM, IgA and IgG_1 production, suggesting defects in iNKT cell-mediated regulation of normal B cell antibody production. It may be possible that the iNKT cells in SLE are defective in inducing normal antibody production but display a propensity towards inducing autoantibody production instead. Natural antibodies, which are produced by B cells in the absence of exogenous antigenic exposure, recognise non-self epitopes and also possess weak affinity for self-ligands such as oxidized phospholipids and apoptotic antigens (Binder et al., 2008; Fleming, 2006). These natural antibodies have anti-inflammatory properties in healthy subjects and mediate apoptotic clearance to maintain homeostasis (Schwartz-Albiez et al., 2009). Based on our observations, it is likely that iNKT cells are defective in inducing protective natural antibodies from B cells in SLE, thus allowing the propagation of pathogenic autoantibodies by the accumulation of uncleared autoantigens. The iNKT cells from SLE patients also induced greater production of IgG_2, which are known to be produced in Th1 responses (Silfverdal et al., 2002; Wheeler, Marshall, and Ulrich, 2001), suggesting that the iNKT cells skew the B cells towards pathogenic Th1 antibody production in SLE. IgG_2 subclass antibodies have been linked with lupus nephritis and renal relapse in SLE (Bijl et al., 2002). Work by Fang et al showed that IgG_2 is the dominant antibody subclass in anti-C1q autoantibodies produced by SLE patients with nephritis, the latter of which shows a strong positive correlation with disease activity (Fang et al., 2009).

Our work demonstrates that the iNKT cells from SLE patients are able to potently expand and activate B cells in the absence of α-GC, thus suggesting that they are hyper-activated in comparison to iNKT cells from healthy controls. However, iNKT cells from SLE patients are still responsive to B cell-mediated regulation by α-GC as evidenced by the downregulation of CD154 when α-GC was present in the co-cultures of healthy B cells with iNKT cells from SLE patients. Further work is thus needed to look at the signalling mechanisms that are activated or defective when these iNKT cells interact with B cells to provide greater insight into iNKT cell function in SLE.

The conclusions from this chapter are:

1. $CD4^+$ iNKT cells are enhanced in frequencies and activation (based on CD154 expression) in SLE patients, suggesting that the iNKT cells are primed to cause enhanced B cell activation.

2. B cell subsets are disproportionate in SLE patients, whereby naïve B cells are increased in frequencies while memory B cells and Breg cells are decreased in frequencies, suggesting that differentiation of naïve to memory B cells are inhibited in SLE. Naïve and memory B cells exhibited increased CD40 expression. In contrast, CD80 expression was upregulated on Breg cells and the IgG^+ subsets of switched and $CD27^-IgD^-$ memory B cells but decreased on unswitched memory B cells and naïve B cells. On the whole, all of the B cell subsets appear to be activated but the discordance in upregulation of CD40 and CD80 expression may suggest that they are at different stages of activation. The observations from the phenotyping studies suggest that enhanced frequencies of $CD4^+$ iNKT cells may result in decreased maturation of B cells into memory and Breg cells.

3. iNKT cells expanded from SLE patients exhibit enhanced ability to activate B cells from healthy controls and induce cytokine production without need for α-GC, suggesting that they are aberrant in function. However, these iNKT cells induce less antibody production from B cells from healthy controls, suggesting that B cells themselves contribute to the high titers of autoantibodies seen in SLE.

7 Discussion

7.1 Reciprocal regulation between iNKT cells and B cells, and the role of α-GC.

The interesting interplay between iNKT cells and B cells has shown that the two cell types reciprocally regulate each other's function. Using the setup of co-culturing expanded iNKT cells with sorted B cells in vitro, we observed that the iNKT cells induced co-stimulatory and activation markers on B cells in a contact-independent manner. There was little change in cytokine production observed in the iNKT-B cell co-cultures (with polyclonal iNKT cells), but TNF-α, IL-4 and IL-13 was significantly enhanced by α-GC. iNKT cells also induced antibody production by B cells in a contact-dependent manner, which was inhibited by α-GC. Although interaction between iNKT cells and B cells results in B cell activation through upregulation of co-stimulatory markers and enhanced antibody production, the antigen-presenting function of B cells to conventional T cells may have been inhibited. We also observed that the interaction between iNKT cells and B cells was dependent on CD1d, IL-13 and the CD40-CD154 co-stimulatory pathway.

The induction of B cell activation and antibody production but possible inhibition of B cell antigen presentation by iNKT cells, may be a regulatory mechanism for maintenance of tolerance in the immune system by preventing the activated B cells from triggering inflammatory T cell responses. Cognate B-T cell interaction and antigen presentation by B cells to T cells is crucial for clonal expansion and memory generation in both B cells and T cells (Linton, Harbertson, and Bradley, 2000; Shimoda and Koni, 2007). The antigen presentation function of B cells is crucial to development of autoimmunity. Non-obese diabetic (NOD) mice that are genetically deficient in B cells fail to develop T cell autoreactivity to administered self peptides and do not develop diabetes or insulitis, suggesting that antigen presentation by B cells contributes to pathogenesis of diabetes in the NOD mice (Falcone et al., 1998). In patients with multiple myeloma, a B cell neoplasia characterised by clonal expansion of malignant myeloma plasma cells from the bone marrow, the plasma cells exhibit ability to present soluble antigens such as purified protein derivative (PPD) and tetanus toxoid to autologous T cells in a MHC-restricted manner, leading to T cell proliferation and IFN-γ secretion (Yi et al., 1997). Deficiencies in MHC II-mediated B cell antigen presentation and hence ability to activate alloreactive $CD4^+$ T cells has also been associated with prolonged cardiac allograft

survival in mice (Noorchashm et al., 2006), thus supporting our theory that iNKT-mediated inhibition of B cell antigen presentation induces tolerance.

If iNKT cells do indeed induce B cell tolerance, how do we explain the enhanced antibody production as a consequence of interaction with iNKT cells? A possible theory is that in healthy individuals, iNKT cells induce natural antibody production (described further in the following paragraph) but inhibit autoreactive antibody production. A study by Yang et al has shown that activation of iNKT cells by α-GC in a murine model that spontaneously produces autoantibodies resulted in inhibition of IgG autoantibody production with concurrent upregulation of activation markers such as CD69 on the B cells. However, normal polyclonal IgG responses in response to LPS challenge remained intact (Yang et al., 2011). This inhibition of autoantibody production required direct contact and was CD1d-dependent, which agrees well with our observations that iNKT-induced antibody production was contact- and CD1d-dependent. In our studies in SLE patients, we observed that iNKT cells from these patients were not able to induce as much antibody production from healthy B cells as iNKT cells from healthy controls, suggesting that perhaps the ability of iNKT cells to induce natural antibody production whilst inhibiting autoantibody production is defective in SLE. This also suggests that a dichotomy exists in B cell antibody production whereby the mechanisms of autoantibody production are distinct from the mechanisms of natural antibody production.

Based on our results and those of others' we speculate that in healthy individuals, iNKT cells inhibit autoantibody production but positively regulate natural antibody production. Natural antibodies are thought to be produced by B cells in the absence of antigenic stimuli (Schwartz-Albiez et al., 2009). Primarily produced by the B1 B cell subset, natural antibodies are able to recognise self, altered self and foreign antigens comprising phospholipids, carbohydrate sequences, single-stranded DNA, peptides and surface glycoproteins (Griffin, Holodick, and Rothstein, 2011; Schwartz-Albiez et al., 2009). They are vital for first line defense against invading pathogens (Ochsenbein and Zinkernagel, 2000; Rapaka et al., 2010) but also have a role in maintaining tissue homeostasis via clearance of intracellular molecules, necrotic and apoptotic cells (Hu and Späth, 2003). Their important function in waste removal has been speculated to be beneficial for preventing autoimmune and inflammatory reactions, especially since dysfunctional clearance of apoptotic cells is thought to be a contributing factor to the etiology of SLE (Gaipl et al., 2006; Rosen and Casciola-Rosen, 2001; Shao and Cohen, 2011). These natural antibodies also have the potential to bind to inhibitory FcγRIIB receptors found on lymphocytes (B cells and plasma cells), myeloid cells (monocytes,

macrophages and dendritic cells) and granulocytes (neutrophils, basophils and mast cells) for the purpose of maintaining homeostasis by downregulating inflammatory responses (Smith and Clatworthy, 2010).

Although natural antibodies are generally recognized to be protective, there is increasing speculation that they may be propelled towards autoreactivity due to their weak affinity for self and increased availability of autoantigens caused by defective clearance of apoptotic cells (Fleming, 2006; Jordö et al., 2011). In the case of SLE, this transition from protective to pathogenic antibody responses is linked to genetic mutations in molecules such as C1q and the Mer receptor, leading to physiological dysregulation of apoptotic cell clearance (Botto et al., 1998; Scott et al., 2001). The protective role of iNKT cells has been demonstrated in this aspect. When apoptotic cells are injected into mice, iNKT cells rapidly alter their cytokine profile to decrease pro-inflammatory cytokine secretion, and also reduce autoreactive B cell activation and autoantibody (anti-dsDNA) production (Wermeling et al., 2010). The iNKT ligand α-GC may also play a role in regulation of B cell antibody production by iNKT cells, as we observed that α-GC downregulated iNKT-enhanced antibody production. This secondary mechanism of regulation by α-GC highlights a possible role of B cell presentation of α-GC to iNKT cells being a mechanism for eliminating excessive immune responses induced by potent stimuli (during infection) and maintaining homeostasis. Apart from directly inhibiting B cell function, the iNKT-B cell interaction can also induce tolerance by supporting the development of regulatory T cells in vivo (Sonoda and Stein-Streilein, 2002; Wei et al., 2005). Reconstitution of B cell knockout mice with marginal zone B cells resulted in generation of antigen-specific regulatory T cells that induced anterior chamber associated immune deviation (ACAID) (peripheral tolerance in the eye). However, when CD1d expression was ablated on the marginal zone B cells used for reconstitution, both peripheral tolerance and associated regulatory T cell development was abrogated (Sonoda and Stein-Streilein, 2002).

The phosphoMAPK arrays provided a fascinating insight into the dynamics of signalling processes taking place inside the B cells and iNKT cells as a result of their interaction and of the addition of modulators such as α-GC and the PI3Kδ inhibitor, IC87114. MAPK phosphorylation in the B cells was decreased when B cells were co-cultured with iNKT cells in the absence of α-GC, while MAPK phosphorylation was enhanced in the iNKT cells, in particular phosphorylation of Akt2 and CREB. However, in the presence of α-GC, MAPK phosphorylation was decreased in iNKT cells but restored in B cells. Inhibition of PI3Kδ in the iNKT-B cell co-cultures by IC87114 then abrogated MAPK

phosphorylation in B cells and restored levels of MAPK phosphorylation in iNKT cells back to levels similar to when it was co-cultured with B cells in the absence of α-GC.

The widespread downregulation of signalling (via decreases in intracellular kinase phosphorylation) in B cells when in contact with iNKT cells but without the ligand α-GC showed that the iNKT cells had an overall inhibitory effect on B cell function, and corresponds well with the observed inhibition of B cell antigen-presentation mediated by iNKT cells in our experiments. Contact with B cells resulted in activation of iNKT cell signalling instead, with marked increases in Akt2 and CREB phosphorylation. Akt2 is well-characterised for its role in glucose metabolism and diabetes (Yang et al., 2004), being localized in the mitochondria (Santi and Lee, 2010). However virtually nothing is known about the role of Akt2 in iNKT cell immunoregulation and further study is required to elucidate its role in regulation of B cells by iNKT cells. When α-GC was present in the interaction, B cells mediated a reciprocal decrease in iNKT cell signalling whilst B cell signalling was partially restored. Although α-GC appeared to restore partial B cell signalling, it also paradoxically downregulated antibody production, showing that we still do not fully understand the intricate mechanisms of how iNKT cells and B cells reciprocally regulate each other. Inhibition of PI3Kδ by IC87114 then relieved the inhibitory effect mediated by B cells and α-GC and restored MAPK phosphorylation in iNKT cells whilst totally abolishing MAPK phosphorylation in the B cells, demonstrating that PI3Kδ is probably more crucial to B cell signalling than iNKT cell signalling.

iNKT cells had differential effects on various subsets of B cells. They were shown to be able to expand known ($CD24^{hi}CD38^{hi}$) and possible ($CD1d^{hi}CD5^{+}$ and $CD1d^{hi}CD5^{+}CD24^{hi}CD38^{hi}$) Breg cells, upregulate their CD40 and CD86 expression and increase their IL-10 production, suggesting that iNKT cells could be used therapeutically to stimulate Breg expansion and activation in autoimmune diseases with excessive Th1 or Th17 responses such as multiple sclerosis (Miyamoto, Miyake, and Yamamura, 2001) and type 1 diabetes (Li, Braun, and Wei, 2011). In our experiments we observed that iNKT cells also expanded unswitched memory B cells and induced isotype switching to IgG^{+} switched memory B cells, suggesting that they have an adjuvant effect on B cell memory generation. This was observed also by Galli et al (Galli et al., 2007), who found that co-administration of the iNKT cell ligand α-GC with protein antigen into mice enhanced their antigen-specific antibody recall response, a hallmark of B cell memory.

Frequencies of naïve B cells were observed to be enhanced at 3 days and then decreased at 10 days following iNKT help, suggesting that the iNKT cells induced an

initial expansion and activation of the naïve B cells, followed by differentiation into memory B cells. We also observed that the $CD27^{-}IgD^{-}$ memory B cells, which are reportedly expanded in SLE (Sanz et al., 2008) and suspected to be pathogenic (Wei et al., 2007), were also decreased in frequency when co-cultured with iNKT cells, highlighting yet another homeostatic mechanism of iNKT cells in their immunoregulation of B cells. The fact that iNKT cells exert such disparate effects on the frequencies of different subsets of B cells provides food for thought and alludes to the need to understand the roles and functions of these different B cell subsets in the immune system more intimately. For instance, IL-10 generation by naïve B cells is thought to function primarily in maintaining homeostasis by preventing inflammatory responses that lead to autoimmune disease, whereas IL-10 generation by memory B cells may function primarily in resolution of active disease exacerbation during active inflammatory responses (Fillatreau, Gray, and Anderton, 2008; Rieger and Bar-Or, 2008).

7.2 Harnessing $CD4^{+}$ iNKT cells in therapy.

Our results show that $CD4^{+}$ iNKT cells are most potent at activating B cells through upregulation of CD40 expression but did not seem superior to $CD8^{+}$ and DN iNKT cells at inducing antibody production by the B cells. $CD4^{+}$ iNKT cells also induced expansion and activation of IL-10-producing B cells and a population of $CD1d^{hi}CD5^{+}$ B cells which could be Breg cells as they contain a population of IL-10-producers, and co-culture with iNKT cells increases the proportions of $CD1d^{hi}CD5^{+}$ IL-10 producers. IL-10-producing B cells (or Breg cells) have been shown to be successful in downregulating inflammation in autoimmune disease murine models (Fillatreau et al., 2002), and show great promise in therapy for autoimmune diseases. Since $CD4^{+}$ iNKT cells increase frequencies of IL-10-producing B cells, it may be beneficial to target the $CD4^{+}$ iNKT subset for autoimmune disease therapy.

$CD4^{+}$ iNKT cells are thought to contribute to immunological tolerance. iNKT cell activation was shown to inhibit development of type 1 diabetes in non-obese diabetic mice and this was primarily mediated by $CD4^{+}$ iNKT cells (Chen et al., 2006). They are able to suppress the generation of anti-tumour cytotoxic T lymphocyte responses to peptide-pulsed dendritic cells by secreting massive amounts of Th2 cytokines (Osada et al., 2005). Work by Roelofs-Haarhuis et al also showed that in response to nickel challenge, murine $CD4^{+}$ iNKT cells are able to generate nickel-specific regulatory T cells by mediating the tolerization of naïve T cells by tolerogenic antigen-presenting cells

(Roelofs-Haarhuis, Wu, and Gleichmann, 2004). We have observed similar results with total iNKT cells, which inhibited antigen presentation by B cells, suggesting that iNKT cells can also tolerise B cells and could potentially be used to induce tolerogenicity of pathogenic B cells in antibody-mediated autoimmune disorders (Wang and Zhu, 2004). However, further work will need to be carried out to determine the type of antibody-mediated autoimmune disorder in which $CD4^+$ iNKT cells can be targeted to induce tolerogenicity in B cells, because $CD4^+$ iNKT cells were observed to be pathogenic in a murine model of allergic asthma, exhibiting cytotoxicity towards a protective subset of regulatory T cells (Nguyen, Vanichsarn, and Nadeau, 2008).

7.3 iNKT cells and B cells in SLE.

7.3.1 iNKT cells and Breg cells – is inducing IL-10 production the solution?

In our work with SLE patients, we observed enhanced frequencies of $CD4^+$ iNKT cells, which were also enhanced in their CD154 expression in comparison to $CD4^+$ iNKT cells from healthy controls, suggesting that the $CD4^+$ iNKT subset may be hyper-activated and primed to provide B cell help in SLE patients. Distribution of B cell subsets were changed in SLE patients - increased frequencies of naïve B cells and decreased frequencies of memory B cells and Breg cells were observed. In comparison to healthy controls, CD40 expression was upregulated on naïve B cells and memory B cells but not Breg cells, whereas CD80 expression was upregulated on Breg cells and the IgG^+ subsets of switched and $CD27^-IgD^-$ memory B cells but decreased on unswitched memory B cells and unchanged on naïve B cells. The upregulation of CD40 and CD80 expression suggests that the B cell subsets are activated in comparison to healthy controls, as signalling through CD40 and CD80/CD86 is known to induce B cell effector functions such as antibody production, proliferation and antigen presentation (Brine and Klaus, 1993, Yang and Wilson, 1996, Leadbetter et al., 2008). However, the B cell subsets are perhaps at different activation states as suggested by the discordance in upregulation of CD40 and CD80 expression. iNKT cells expanded from SLE patients exhibited enhanced ability to activate B cells from healthy controls and stimulate cytokine production without the need for α-GC, suggesting that they might be hyper-activated. They also induced less antibody production from the B cells, suggesting that they may be defective in inducing natural antibody responses from B cells. Activation of iNKT cells in healthy iNKT-B cell co-cultures with α-GC (in chapter 4) has previously been shown to result in decreased antibody production, lending support to the

observation that the iNKT cells from healthy patients may be hyper-activated. On overall our work suggests that the iNKT cells are aberrant in function in SLE.

SLE patients produce abnormally large amounts of IL-10 which correlate with higher disease activity (Hagiwara et al., 1996) and production of autoreactive antibodies e.g. anti-dsDNA (Llorente et al., 1995). In SLE, there appears to be an intrinsic defect in the ability of IL-10 to induce suppression of IL-6 and TNF-α synthesis, which are elevated and pro-inflammatory (Mongan, Ramdahin, and Warrington, 1997; Steinvinkel et al., 2005). This could explain the increased levels of IL-10 and IL-6 found in this condition, and may also be responsible for the characteristic polyclonal B-cell activation as these cytokines exert mitogenic effects on B cells and induce their differentiation (Mongan, Ramdahin, and Warrington, 1997; Swinnen and Fisher, 1993; Urashima et al., 1996). This suggested defect in B cell function could mean that inducing Breg cells in treatment for SLE would backfire as (i) Breg cells may be dysfunctional in SLE patients, and (ii) increased IL-10 secretion from Breg cells would exacerbate autoreactive antibody production. There is thus a need to characterise fully the role of Breg cells in human SLE and their cytokine production profile.

Intracellular flow cytometry for assaying cytokine production from apparent Breg subsets ($CD1d^{hi}CD5^{+}$ and $CD24^{hi}CD38^{hi}$) showed that these cells are extremely heterogenous in the combinations of cytokines that these B cells produce. In both Breg subsets, we observed subsets of single cytokine producers (IL-10^{+}, IL-13^{+} or IFN-γ^{+}), dual cytokine producers (IL-10^{+}IL-13^{+}, IL-13^{+}IFN-γ^{+} and IL-10^{+}IFN-γ^{+}) and triple cytokine producers (IL-10^{+}IL-13^{+}IFN-γ^{+}). Based on the premise that IFN-γ is a Th1 cytokine (Bradley, Dalton, and Croft, 1996), IL-10 is a Treg cytokine (O'Garra et al., 2004) and IL-13 is a Th2 cytokine (McKenzie et al., 1998), it appears that these B cell subsets can exhibit a wide variety of cytokine production phenotypes, such as Th1/Treg, Th1/Th2 and Th2/Treg. Current knowledge is still lacking about the physiological significance of B cells that are able to produce apparently conflicting combinations of cytokines, such as Th1/Treg (IL-10^{+}IFN-γ^{+}) cytokines. This also highlights the need for more detailed classification of Breg cells with respect to their cytokine production profile, as the current consensus for classification of Breg cells is IL-10 production (Matsushita and Tedder, 2011). However, we have shown that they are able to produce other cytokines in combination with IL-10. Further studies are also needed to observe the behaviour of these different subsets in both healthy individuals and SLE patients for conclusive classification of Breg cells.

The role of IL-10 in lupus murine models is also controversial. IL-10-deficient MRL-Fas^{lpr} mice develop more severe glomerulonephritis, enhanced pathogenic Th1 responses and autoantibody production than wild-type controls with normal IL-10 levels (Yin et al., 2002), suggesting that IL-10 has a protective role in lupus. However, administration of anti-IL-10 mAbs in NZB/W F_1 mice delayed proteinuria, glomerulonephritis and autoantibody production, suggesting that IL-10 has a pathogenic role in lupus (Ishida et al., 1994). In humans, administration of anti-IL-10 mAbs to 6 SLE patients in a clinical study alleviated disease symptoms and decreased disease activity (Llorente et al., 2000), lending further support to the idea that IL-10 is pathogenic in SLE. Interestingly the paradoxical role of IL-10 in the two different murine models parallels the paradoxical role of iNKT cells in the respective models – iNKT cells are thought to be protective in MRL mice (Yang et al., 2003) but pathogenic in NZB/W F_1 mice (Forestier et al., 2005). Could iNKT cells be the source of IL-10 production in SLE and/or induce IL-10 production by B cells? After all, our results have shown that proportions of IL-10-producing $CD4^+$ iNKT cells increase during co-culture with B cells and α-GC, and $CD4^+$ iNKT cells are also able to increase the proportions of IL-10-producing B cells in both the presence and absence of α-GC. However it is likely that iNKT cells are inducers of IL-10 production rather than the producers themselves as both intracellular flow cytometry and cytometric bead arrays to assay for intracellular and extracellular cytokine production respectively have shown that the iNKT cells are not significant producers of IL-10 in both healthy individuals and SLE patients under the experimental conditions used. This merits further investigation to identify the source of IL-10 production in SLE.

7.3.2 Can iNKT cells be targeted in SLE therapy?

Rituximab treatment in rheumatoid arthritis patients overcomes deficiencies in circulating iNKT cells and restores their numbers back to normal (Parietti et al., 2010), suggesting that B cells also exert an inhibitory effect on iNKT cells in the context of autoimmune disease. The subsequent restoration of iNKT cell numbers was associated with clinical remission of rheumatoid arthritis. As such, many scientists have speculated on the potential of targeting iNKT cells in SLE, which have also been reported to be reduced in the peripheral blood of patients (Gabriel, Morley, and Rogers, 2009). In healthy individuals, we observed that iNKT cells achieved enhanced activation of B cells with the aid of α-GC. However, as observed in the SLE patients, iNKT cells did not

require the aid of α-GC to exert its effects on B cells and perhaps are more activated in phenotype.

The immunoregulatory nature of iNKT cells has led to a glut of studies investigating how they can be targeted in therapy for various diseases. The fact that iNKT cells recognise glycolipids instead of protein antigens means that they can be specifically targeted with a glycolipid agonist without activating other cells such as conventional T cells. Several studies have since screened panels of modified glycolipids (usually analogs of α-GC) in murine models for their ability to bias the immune environment towards a Th1 response needed for anti-tumour activity or towards a Th2 response needed for autoimmune diseases like multiple sclerosis (Chang et al., 2007; Li et al., 2010; Miyamoto, Miyake, and Yamamura, 2001). In a murine model for experimental autoimmune encephalitis (EAE) (the murine equivalent of multiple sclerosis in human), the synthetic analog OCH, which consists of a truncated acyl chain and truncated sphingoid base, can induce predominant IL-4 production and hence Th2 bias by iNKT cells, leading to suppression of EAE (Miyamoto, Miyake, and Yamamura, 2001). In contrast, synthetic analogs containing aromatic rings in their acyl or sphingosine tails selectively induced Th1 responses by iNKT cells and are able to confer protection against murine breast and lung cancers (Chang et al., 2007).

Doubts exist as to whether iNKT cells can be targeted in therapy for SLE due to their ability to enhance antibody production (Mars et al., 2004), considering that autoantibody production is a hallmark of SLE and thought to contribute to disease pathogenesis (Koffler, 1974; Yung and Chan, 2008). However, our work has shown that iNKT cells from SLE patients actually induce less antibody production from healthy B cells than iNKT cells from healthy controls do, suggesting that mechanisms of normal antibody production may be discrete from autoantibody production by B cells, and perhaps iNKT cells induce greater autoantibody production and less natural antibody production in SLE patients. The protective role of iNKT cells in controlling autoantibody production was also suggested by Green et al, who observed that high levels of autoreactive IgG antibodies in SLE patients was linked to deficiencies in iNKT cell numbers (Green et al., 2007). Despite this, there is mounting evidence that iNKT cells are dysfunctional in SLE (Cho et al., 2011; Kojo et al., 2001) and our results have shown that iNKT cells are hyporesponsive to α-GC in terms of its modulatory effect on B cells. In light of this knowledge, the question of whether iNKT cells can be targeted in immunotherapy for SLE must be re-evaluated because if an innate defect exists in the iNKT cells then triggering their activation or expansion would not resolve SLE. In fact, it could result in

more severe disease if iNKT cells are pathogenic rather than protective, as has been observed in several studies with the NZB/W F_1 murine model (Morshed et al., 2002; Zeng et al., 2003).

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9 Appendix

This section consists of results that have been omitted from the main text because of lack of statistical significance or low impact on the study subject.

9.1 iNKT interaction with allogeneic or autologous B cells results in little change in cytokine production.

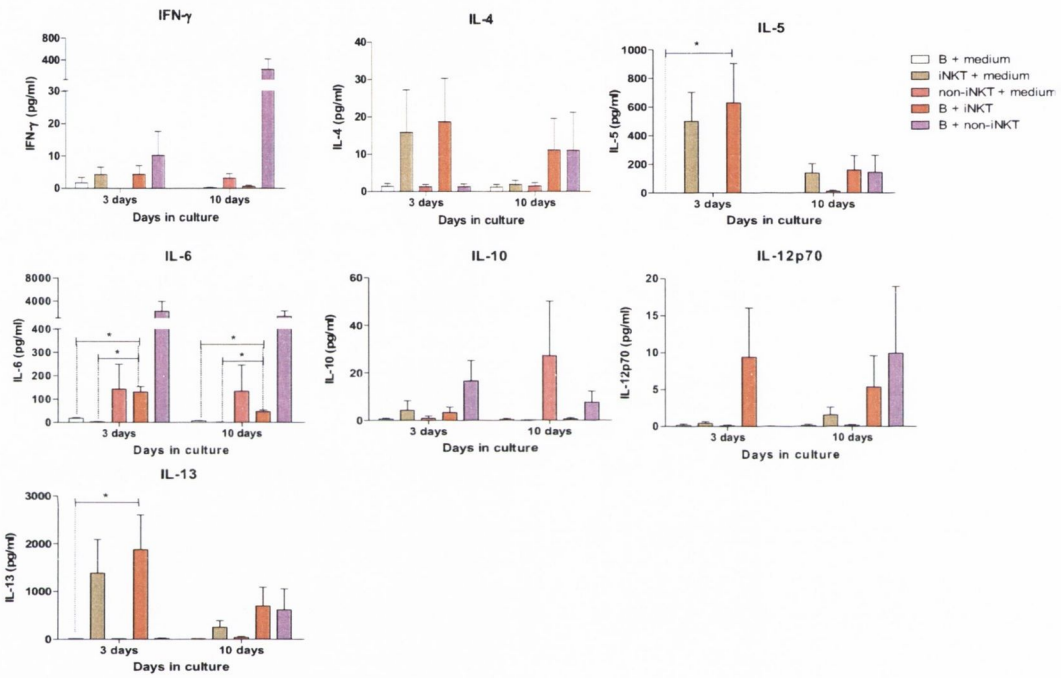

Figure 9.1.1. Multiplex cytometric bead arrays showed little change in cytokine production when iNKT cells were co-cultured with allogeneic B cells. Concentrations of cytokines produced when B cells, iNKT or non-iNKT cells were incubated with medium only (negative controls), and when B cells were incubated with allogeneic iNKT or non-iNKT. Bars represent the mean ± SEM, data was obtained from 4 independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test. *$p<0.05$.

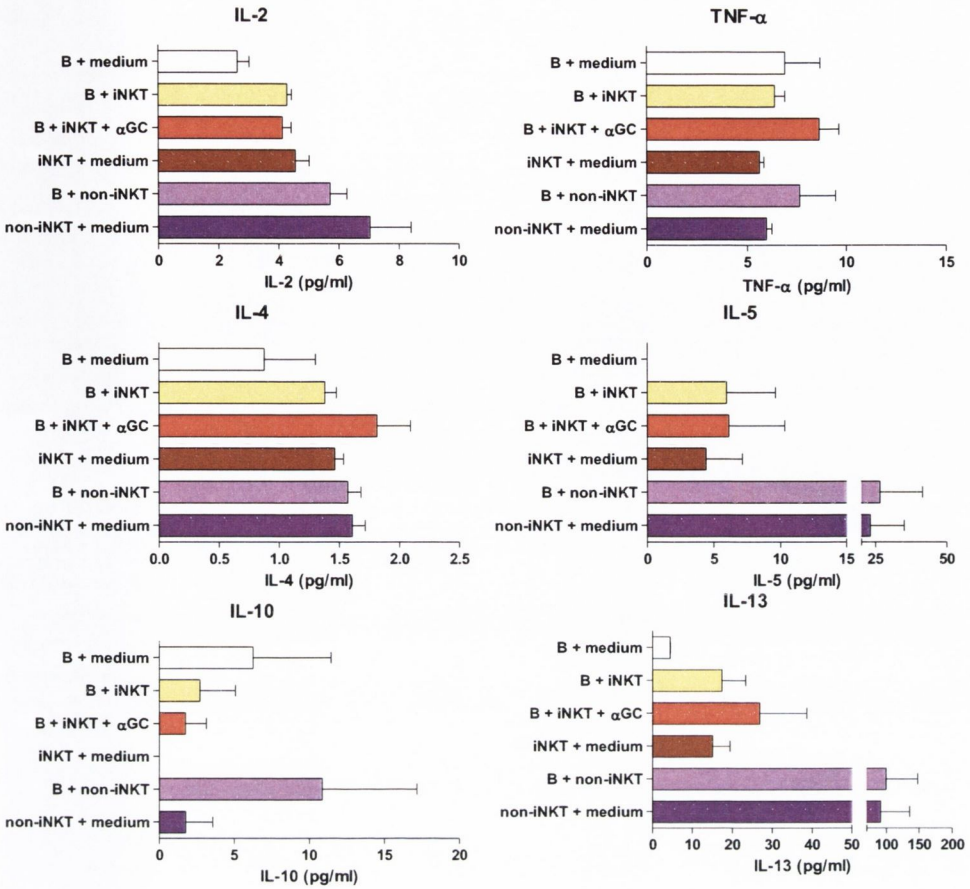

Figure 9.1.2. There was little change in cytokine production observed in autologous iNKT-B cell co-cultures. To assay for cytokine production, multiplex CBA of IFN-γ, IL-2, TNF-α, IL-4, IL-5, IL-10 and IL-13 was performed on supernatants from 3 day co-cultures of iNKT cells with autologous B cells. Bars represent mean ± SEM. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

9.2 iNKT cells are stimulated by B cells independent of contact.

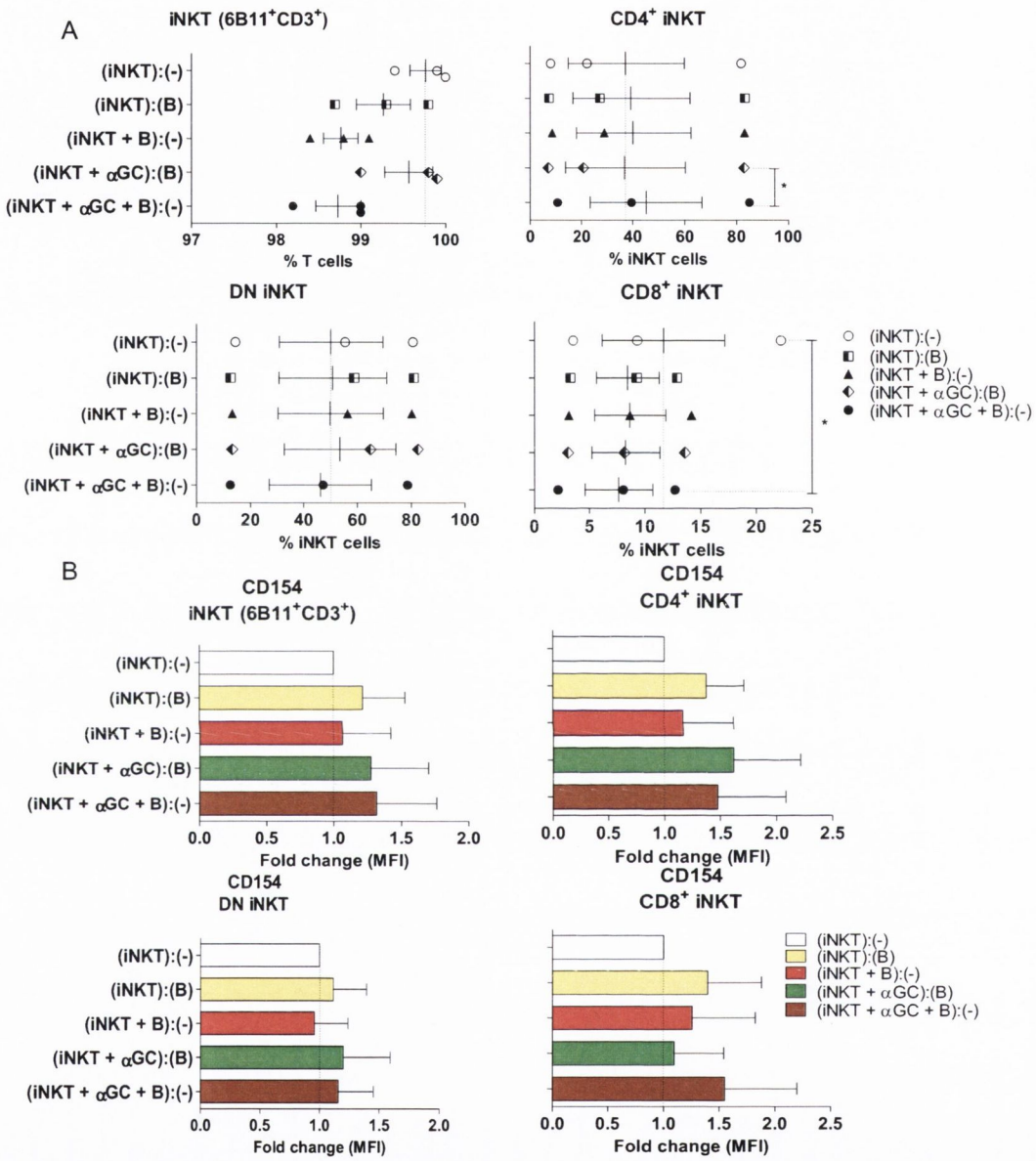

Figure 9.2.1. iNKT cells are stimulated by B cells independent of contact. iNKT cells were co-cultured with B cells in the absence or presence of α-GC for 3 days in a transwell system containing a membrane in the middle, preventing cell-cell contact. The cells were also co-cultured together in contact to compare between the two. In the figure legends, brackets represent each side of the membrane, with the colon symbol (:) representing the membrane. (A) Changes in proportions of total, $CD4^+$, $CD8^+$ and DN iNKT cells were assayed by flow cytometry. (B) Corresponding fold changes in MFI of CD154 expression for the respective iNKT subsets. Bars represent mean ± SEM, data was obtained from three independent experiments. Friedman's test with post-hoc Dunn's multiple comparison test was used to compare differences in means between the different treatments, *$p<0.05$.

9.3 iNKT cell-enhanced frequencies and activation of unswitched memory B cells requires cell-cell contact.

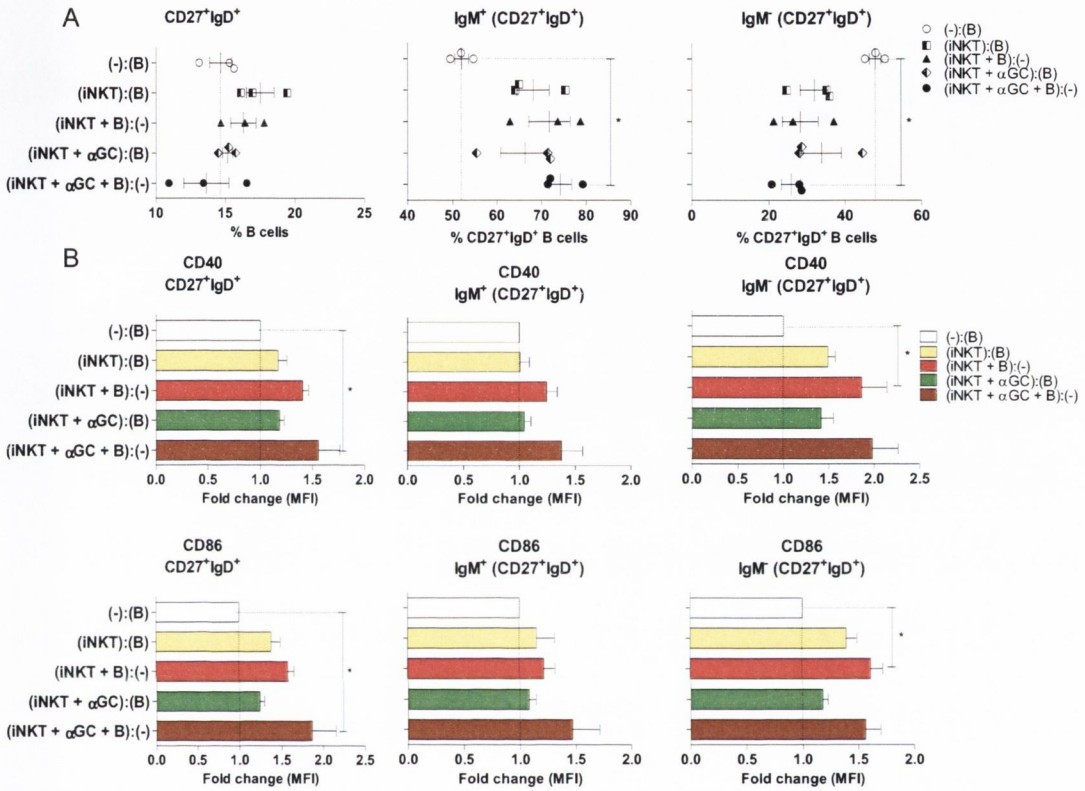

Figure 9.3.1. iNKT cell-enhanced frequencies and activation of unswitched memory B cells requires cell-cell contact. iNKT cells were co-cultured with B cells in the absence or presence of α-GC for 3 days in a transwell system containing a membrane in the middle, preventing cell-cell contact. The cells were also co-cultured together in contact to compare between the two. In the figure legends, brackets represent each side of the membrane, with the colon symbol (:) representing the membrane. (A) Changes in proportions of total, IgM^+, and IgM^- unswitched memory B cells were assayed by flow cytometry. (B) Corresponding fold changes in MFI of CD40 and CD86 expression for the respective B cell subsets. Bars represent mean ± SEM, data was obtained from three independent experiments. Friedman's test with post-hoc Dunn's multiple comparison test was used to compare differences in means between the different treatments, *$p<0.05$.

9.4 Cell-cell contact is necessary for activation of total and IgM$^+$ but not IgG$^+$ switched memory B cells.

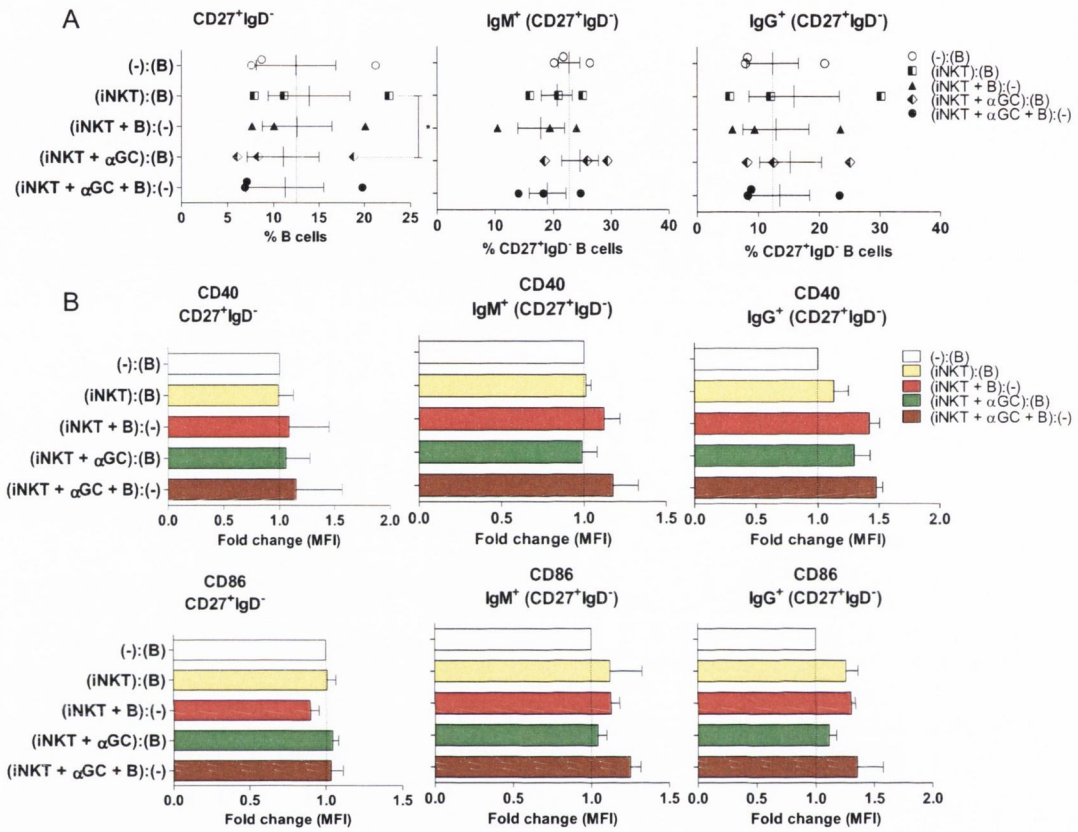

Figure 9.4.1. Cell-cell contact is necessary for activation of total and IgM$^+$ but not IgG$^+$ switched memory B cells. iNKT cells were co-cultured with B cells in the absence or presence of α-GC for 3 days in a transwell system containing a membrane in the middle, preventing cell-cell contact. The cells were also co-cultured together in contact to compare between the two. In the figure legends, brackets represent each side of the membrane, with the colon symbol (:) representing the membrane. (A) Changes in proportions of total, IgM$^+$, and IgG$^+$ switched memory B cells were assayed by flow cytometry. (B) Corresponding fold changes in MFI of CD40 and CD86 expression for the respective B cell subsets. Bars represent mean ± SEM, data was obtained from three independent experiments. Friedman's test with post-hoc Dunn's multiple comparison test was used to compare differences in means between the different treatments, *$p<0.05$.

9.5 Cell-cell contact was required for iNKT-enhanced CD40 and CD86 expression on naïve B cells.

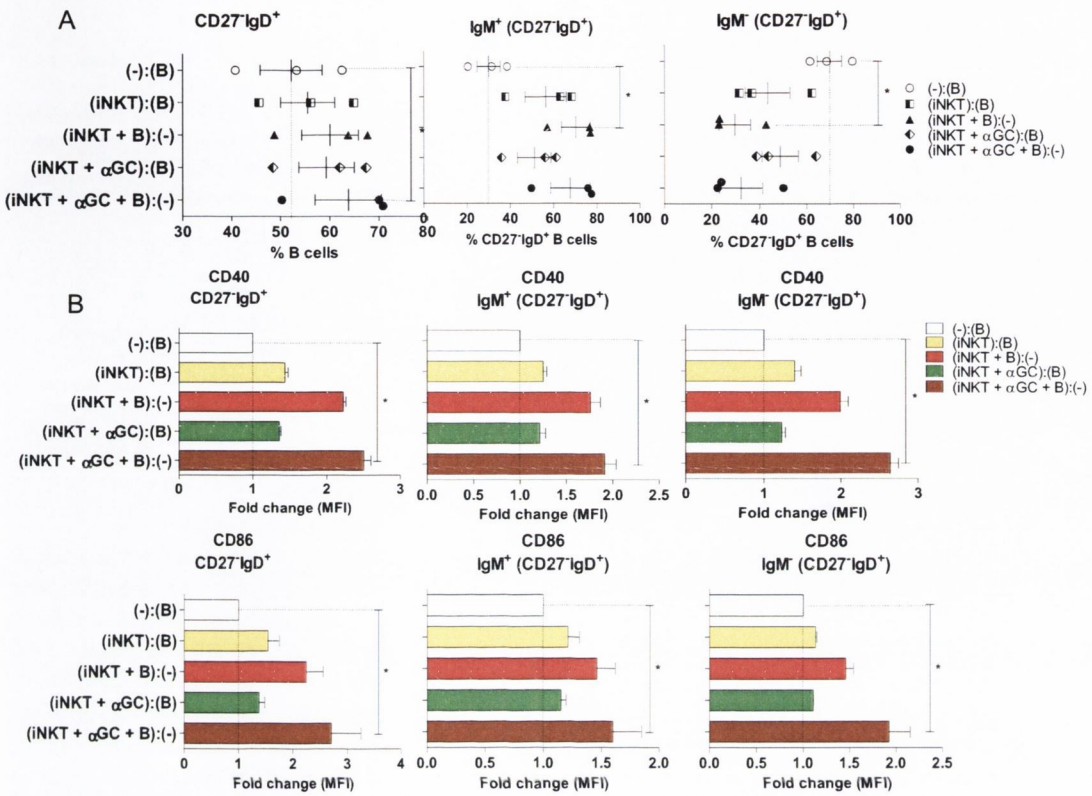

Figure 9.5.1. Cell-cell contact was required for iNKT-enhanced CD40 and CD86 expression on naïve B cells. iNKT cells were co-cultured with B cells in the absence or presence of α-GC for 3 days in a transwell system containing a membrane in the middle, preventing cell-cell contact. The cells were also co-cultured together in contact to compare between the two. In the figure legends, brackets represent each side of the membrane, with the colon symbol (:) representing the membrane. (A) Changes in proportions of total, IgM^+, and IgM^- naïve B cells were assayed by flow cytometry. (B) Corresponding fold changes in MFI of CD40 and CD86 expression for the respective B cell subsets. Bars represent mean ± SEM, data was obtained from three independent experiments. Friedman's test with post-hoc Dunn's multiple comparison test was used to compare differences in means between the different treatments, *$p<0.05$.

9.6 Cell-cell contact is required for iNKT-enhanced CD40 and CD86 expression on CD27$^-$IgD$^-$ memory B cells.

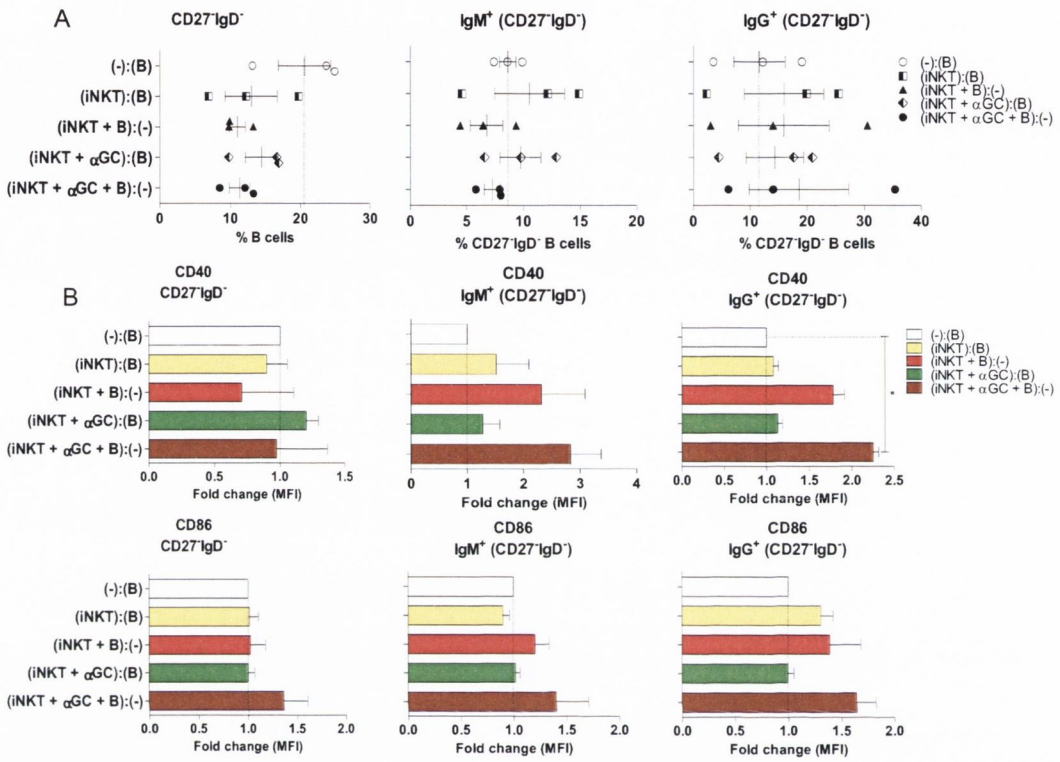

Figure 9.6.1. Cell-cell contact is required for iNKT-enhanced CD40 and CD86 expression on CD27$^-$IgD$^-$ memory B cells. iNKT cells were co-cultured with B cells in the absence or presence of α-GC for 3 days in a transwell system containing a membrane in the middle, preventing cell-cell contact. The cells were also co-cultured together in contact to compare between the two. In the figure legends, brackets represent each side of the membrane, with the colon symbol (:) representing the membrane. (A) Changes in proportions of total, IgM$^+$, and IgG$^+$ CD27$^-$IgD$^-$ memory B cells were assayed by flow cytometry. (B) Corresponding fold changes in MFI of CD40 and CD86 expression for the respective B cell subsets. Bars represent mean ± SEM, data was obtained from three independent experiments. Friedman's test with post-hoc Dunn's multiple comparison test was used to compare differences in means between the different treatments, *p<0.05.

9.7 Anti-CD1d mAb had no effect on cytokine production in iNKT-B cell co-cultures.

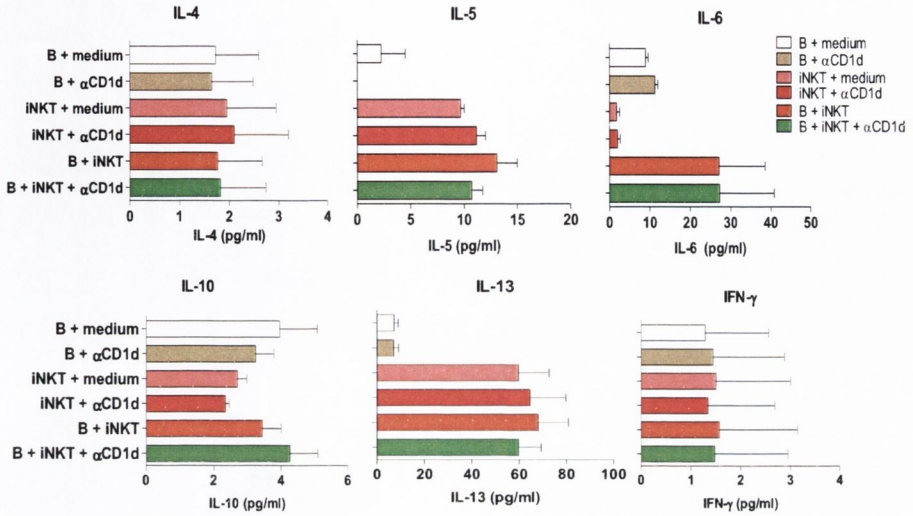

Figure 9.7.1. Anti-CD1d mAb had no effect on cytokine production in the iNKT-B cell co-cultures. Supernatants collected after three days from the iNKT-B cell co-cultures were analysed using multiplex cytometric bead arrays for IL-4, IL-5, IL-6, IL-10, IL-13 and IFN-γ. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test. Differences between B + iNKT (orange bars) and B + iNKT + αCD1d (green bars) were not statistically significant.

9.8 Anti-IL-4 antibodies did not influence cytokine production in the iNKT-B cell co-cultures.

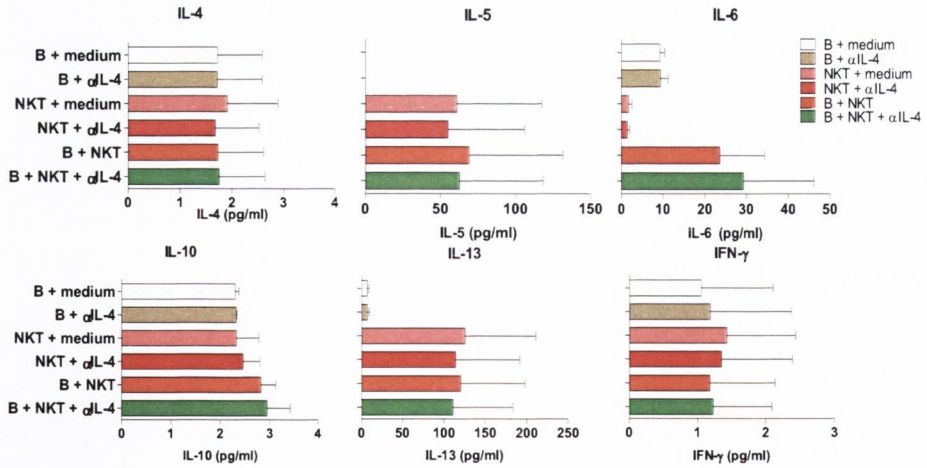

Figure 9.8.1. Anti-IL-4 mAb did not influence cytokine production in iNKT-B cell co-cultures. Supernatants collected after three days from the iNKT-B cell co-cultures were analysed using multiplex cytometric bead arrays for IL-4, IL-5, IL-6, IL-10, IL-13 and IFN-γ. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test. Differences between B + iNKT (orange bars) and B + iNKT + αCD1d (green bars) were not statistically significant.

9.9 Blocking IL-13 downregulated IL-6 production in iNKT help to B cells.

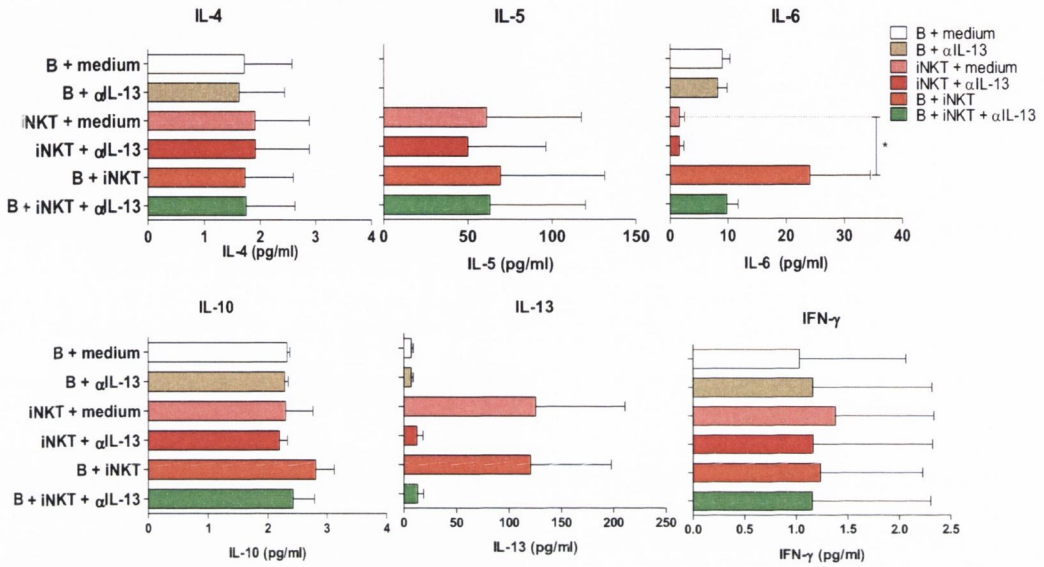

Figure 9.9.1. Blocking IL-13 downregulated IL-6 production in the iNKT-B cell co-cultures. Supernatants collected after three days from the iNKT-B cell co-cultures were analysed using multiplex cytometric bead arrays for IL-4, IL-5, IL-6, IL-10, IL-13 and IFN-γ. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, *p<0.05.

9.10 Blocking CD154 inhibits iNKT-induced upregulation of CD40 and CD86 on unswitched memory ($CD27^+IgD^+$) B cells.

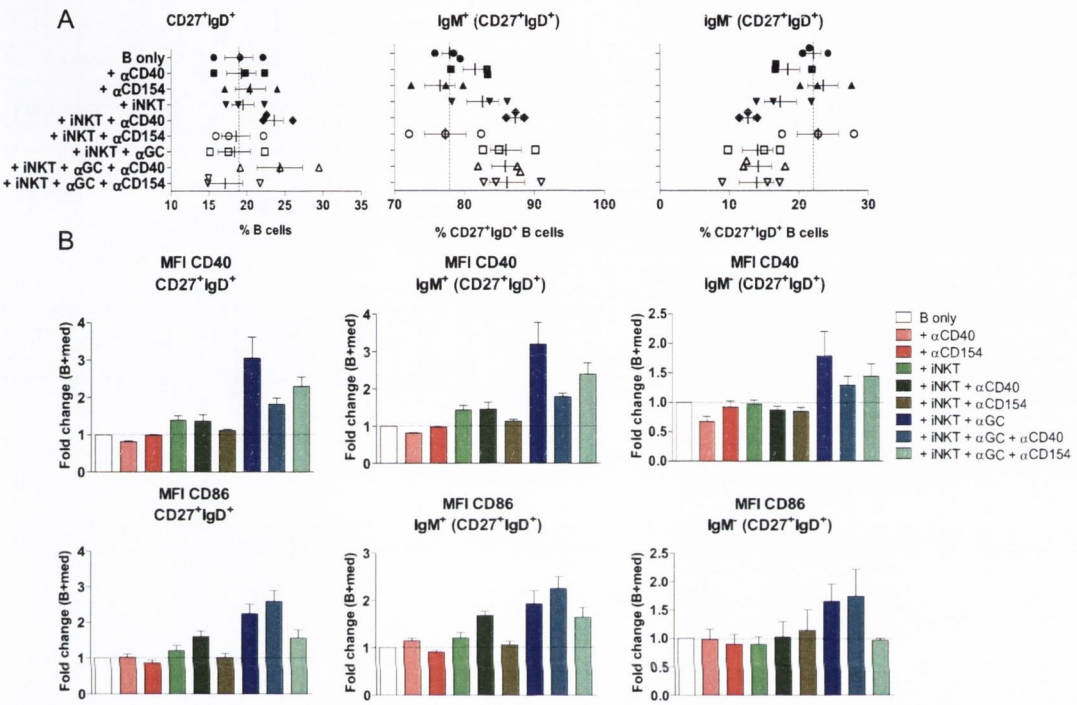

Figure 9.10.1. Blocking CD154 inhibits iNKT-induced CD40 and CD86 upregulation on unswitched memory ($CD27^+IgD^+$) B cells. Total, IgM^+ and IgM^- unswitched memory B cells were analysed by flow cytometry after three days of B cell co-culture with iNKT cells and blocking antibodies. Data shown was obtained from three independent experiments. (A) Graphs showing changes in proportions of total (left), IgM^+ (middle) and IgM^- (right) unswitched memory B cells with different treatments. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on total (left), IgM^+ (middle) and IgM^- (right) unswitched memory B cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

9.11 Blocking CD154 inhibits iNKT-induced CD40 and CD86 upregulation on IgM$^+$ and IgG$^+$ switched memory B cells.

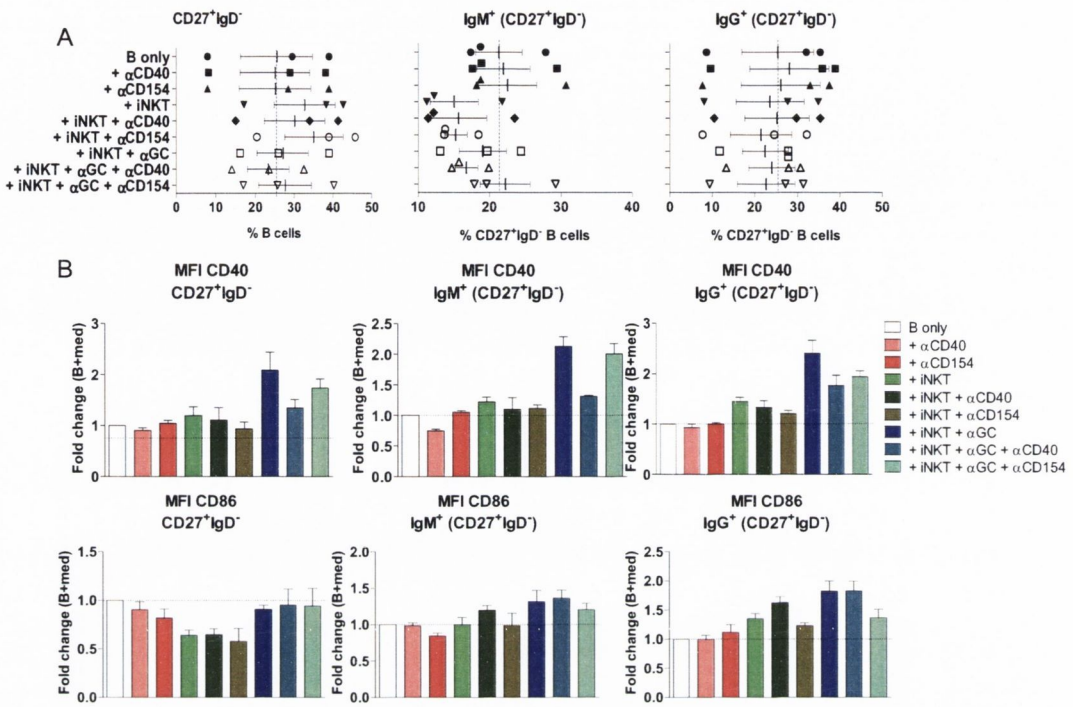

Figure 9.11.1. Blocking CD154 inhibits iNKT-induced CD40 and CD86 upregulation on IgM$^+$ and IgG$^+$ switched memory B cells. Total, IgM$^+$ and IgG$^+$ switched memory B cells were analysed by flow cytometry after three days of B cell co-culture with iNKT cells and blocking antibodies. Data shown was obtained from three independent experiments. (A) Graphs showing changes in proportions of total (left), IgM$^+$ (middle) and IgG$^+$ (right) switched memory B cells with different treatments. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on total (left), IgM$^+$ (middle) and IgG$^+$ (right) switched memory B cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

9.12 Blocking CD154 inhibits IgM expression and activation in naïve B cells.

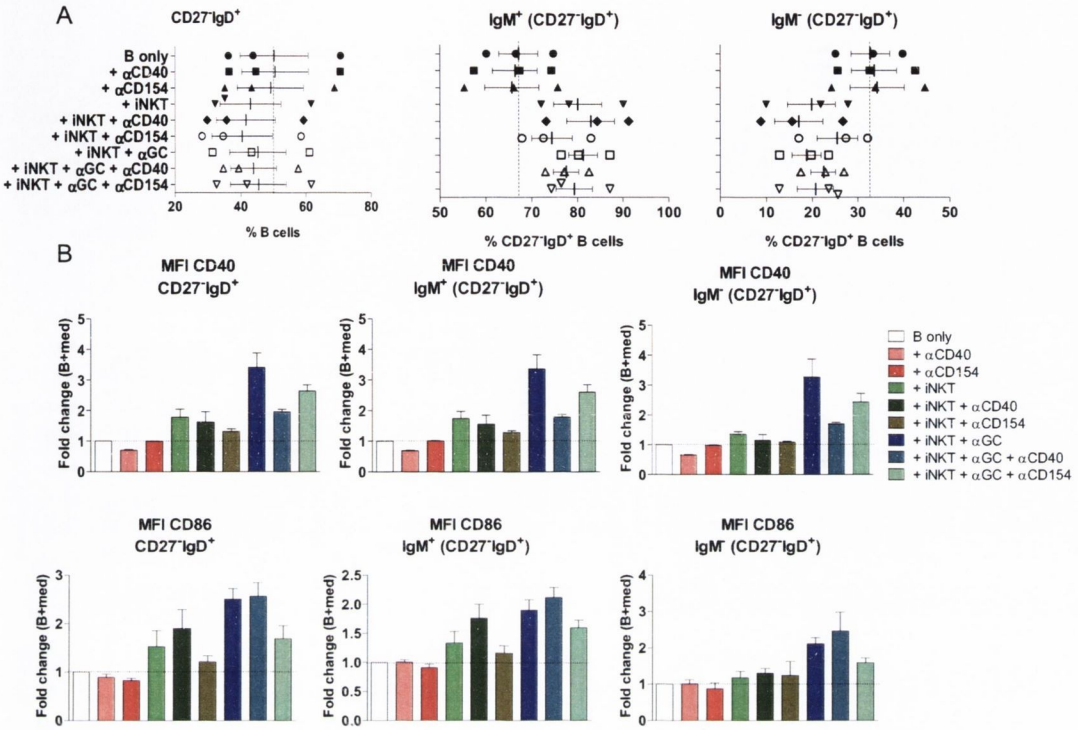

Figure 9.12.1. Blocking CD154 inhibits IgM expression and activation in naïve B cells. Total, IgM^+ and IgM^- naïve B cells were analysed by flow cytometry after three days of B cell co-culture with iNKT cells and blocking antibodies. Data shown was obtained from three independent experiments. (A) Graphs showing changes in proportions of total (left), IgM^+ (middle) and IgM^- (right) naïve B cells with different treatments. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on total (left), IgM^+ (middle) and IgM^- (right) naïve B cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

9.13 Blocking CD154 inhibits activation of $CD27^-IgD^-$ memory B cells.

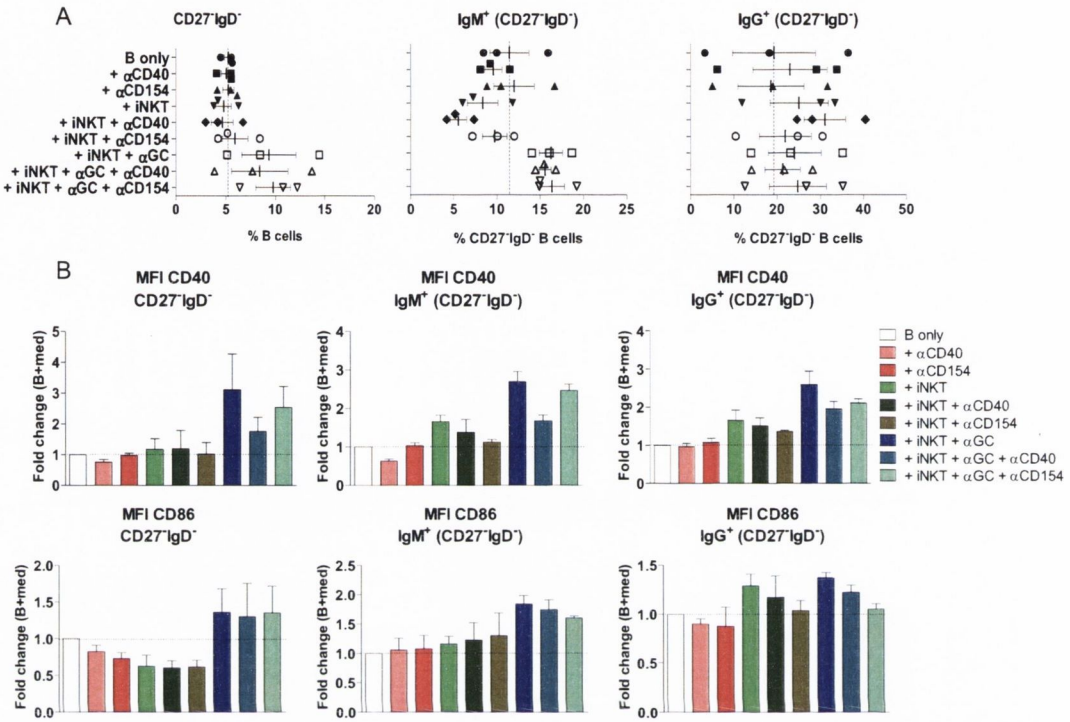

Figure 9.13.1. Blocking CD154 inhibits the activation of $CD27^-IgD^-$ memory B cells. Total, IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells were analysed by flow cytometry after three days of B cell co-culture with iNKT cells and blocking antibodies. Data shown was obtained from three independent experiments. (A) Graphs showing changes in proportions of total (left), IgM^+ (middle) and IgG^+ (right) $CD27^-IgD^-$ memory B cells with different treatments. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on total (left), IgM^+ (middle) and IgG^+ (right) $CD27^-IgD^-$ memory B cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using Friedman's test with post-hoc Dunn's multiple comparison test, results were not statistically significant.

9.14 Upregulation of CD40 and CD86 expression on unswitched memory B cells is greatest with CD4$^+$ iNKT cells and α-GC.

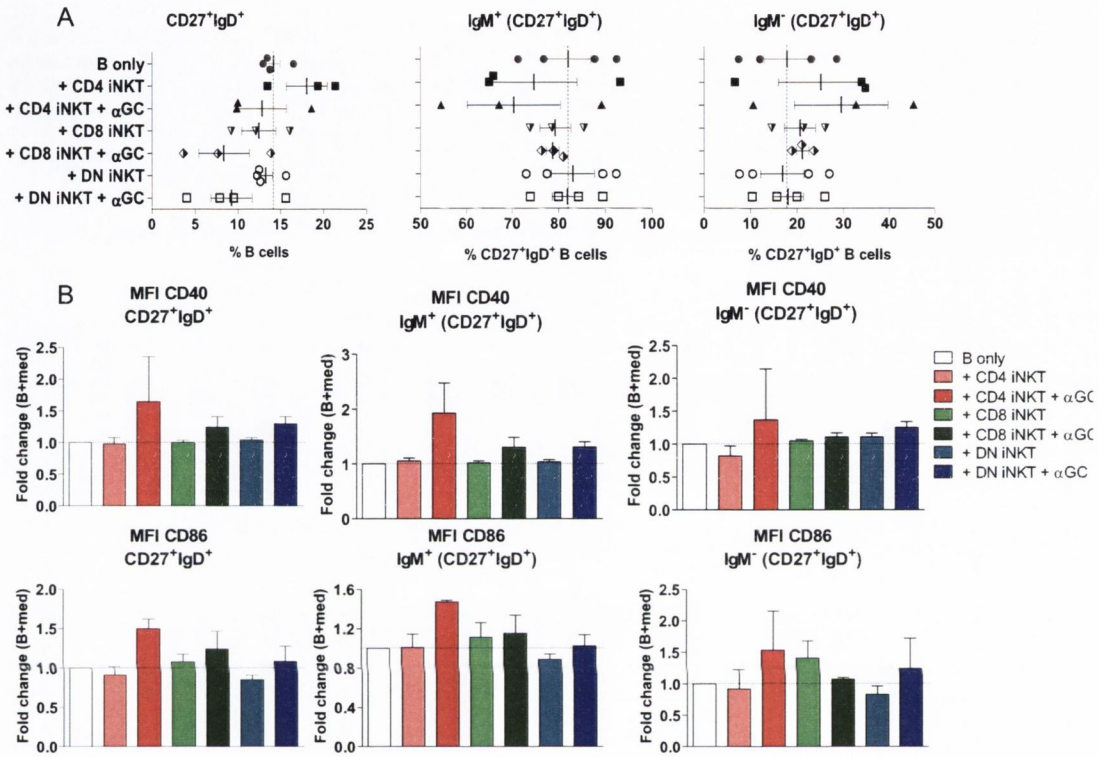

Figure 9.14.1. Upregulation of CD40 and CD86 expression on unswitched memory B cells is greatest with CD4$^+$ iNKT cells and α-GC. Total, IgM$^+$ and IgM$^-$ unswitched memory B cells were analysed by flow cytometry after three days of B cell co-culture with CD4$^+$, CD8$^+$ or DN iNKT subsets. (A) Graphs showing changes in proportions of total (left), IgM$^+$ (middle) and IgM$^-$ (right) unswitched memory B cells when co-cultured with different iNKT subsets and α-GC. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on total (left), IgM$^+$ (middle) and IgM$^-$ (right) unswitched memory B cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using the Kruskal-Wallis with post-hoc Dunn's multiple comparison test, results were not statistically significant.

9.15 $CD4^+$, $CD8^+$ and DN iNKT cells have similar effect on frequencies and activation of switched memory B cells.

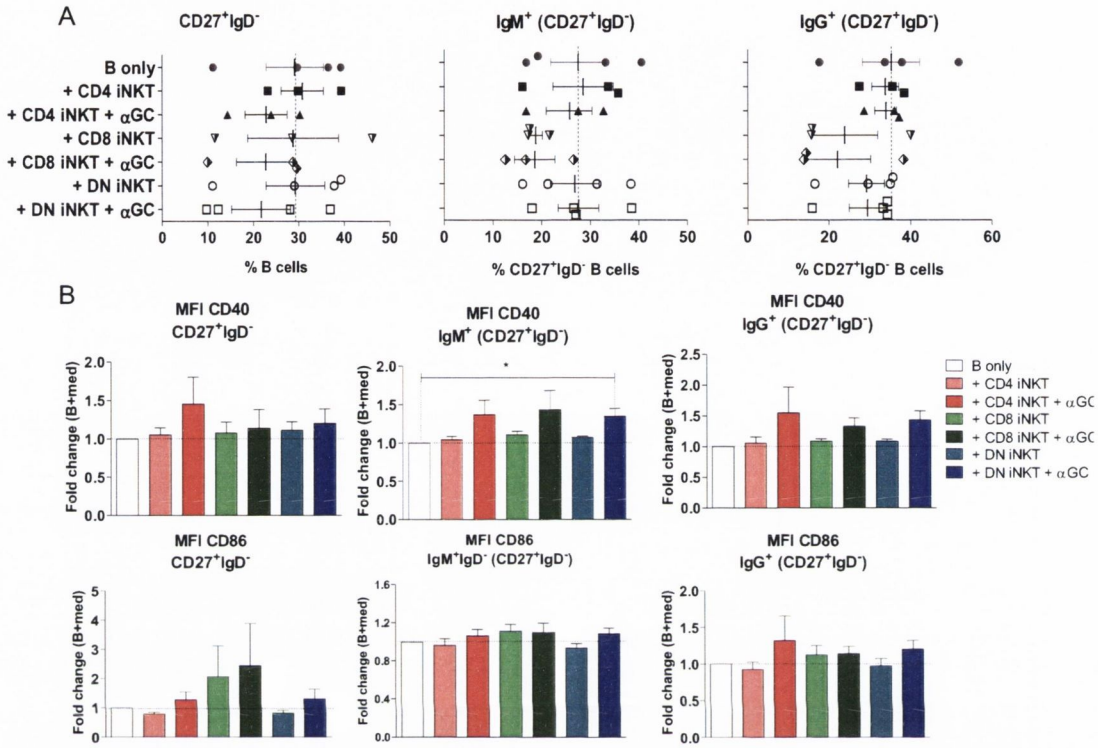

Figure 9.15.1. $CD4^+$, $CD8^+$ and DN iNKT cells have similar effect on frequencies and activation of switched memory B cells. Total, IgM^+ and IgG^+ switched memory B cells were analysed by flow cytometry after three days of B cell co-culture with $CD4^+$, $CD8^+$ or DN iNKT subsets. (A) Graphs showing changes in proportions of total (left), IgM^+ (middle) and IgG^+ (right) switched memory B cells when co-cultured with different iNKT subsets and α-GC. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on total (left), IgM^+ (middle) and IgG^+ (right) switched memory B cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using the Kruskal-Wallis with post-hoc Dunn's multiple comparison test, *$p<0.05$.

9.16 CD4$^+$ iNKT cells were most efficient at enhancing CD40, CD86 and IgM expression on naïve B cells.

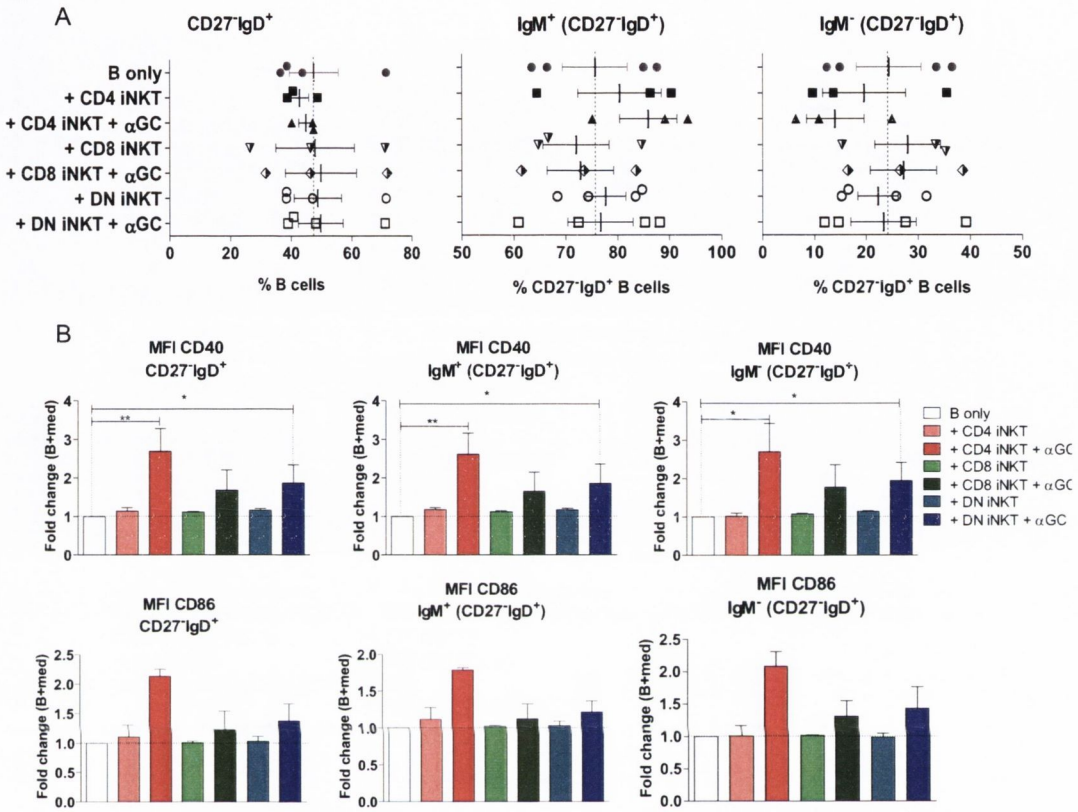

Figure 9.16.1. CD4$^+$ iNKT cells were most efficient at enhancing CD40, CD86 and IgM expression on naïve B cells. Total, IgM$^+$ and IgM$^-$ naïve B cells were analysed by flow cytometry after three days of B cell co-culture with CD4$^+$, CD8$^+$ or DN iNKT subsets. (A) Graphs showing changes in proportions of total (left), IgM$^+$ (middle) and IgM$^-$ (right) naïve B cells when co-cultured with different iNKT subsets and α-GC. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on total (left), IgM$^+$ (middle) and IgM$^-$ (right) naïve B cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using the Kruskal-Wallis with post-hoc Dunn's multiple comparison test, *p<0.05, **p<0.01.

9.17 $CD27^-IgD^-$ memory B cells are increased in IgM and IgG expression by $CD4^+$ iNKT cells.

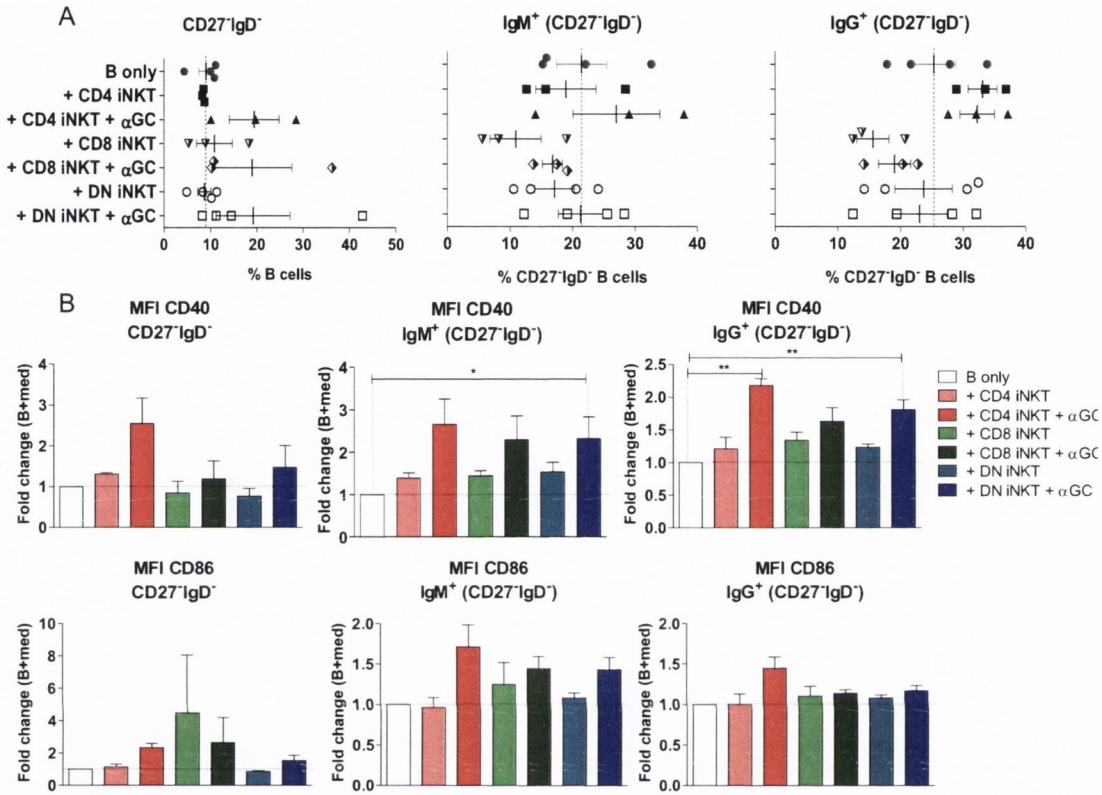

Figure 9.17.1. $CD27^-IgD^-$ memory B cells are increased in IgM and IgG expression by $CD4^+$ iNKT cells. Total, IgM^+ and IgG^+ $CD27^-IgD^-$ memory B cells were analysed by flow cytometry after three days of B cell co-culture with $CD4^+$, $CD8^+$ or $CD27^-IgD^-$ iNKT subsets. (A) Graphs showing changes in proportions of total (left), IgM^+ (middle) and IgG^+ (right) $CD27^-IgD^-$ memory B cells when co-cultured with different iNKT subsets and α-GC. Vertical lines in each row represent mean ± SEM from three independent experiments. (B) Graphs showing corresponding changes in MFI of CD40 and CD86 expression on total (left), IgM^+ (middle) and IgG^+ (right) $CD27^-IgD^-$ memory B cells. Changes are expressed as fold change relative to B only. Bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using the Kruskal-Wallis with post-hoc Dunn's multiple comparison test, *$p<0.05$, **$p<0.01$.

9.18 Adding IC87114 to iNKT-B cell co-cultures increases the frequencies of memory B cells but decreases their IgM and IgG expression.

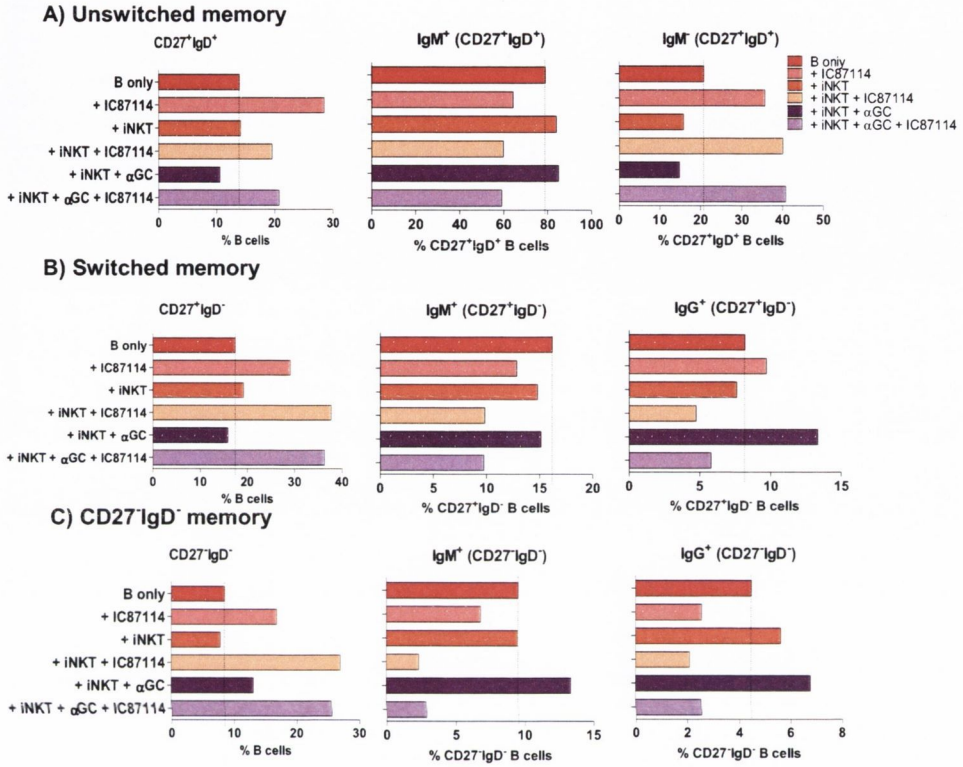

Figure 9.18.1. Adding IC87114 to the co-cultures increases frequencies of memory B cells but decreases their IgM and IgG expression. B cells were co-cultured with iNKT cells for 3 days in the absence or presence of α-GC, with or without IC87114. As negative controls, B cells were cultured in medium only or with IC87114. Flow cytometric analysis of changes in proportion of (A) unswitched, (B) switched and (C) $CD27^-IgD^-$ memory B cells was performed. N=1.

9.19 Naïve B cells are decreased in frequency and IgM expression by IC87114.

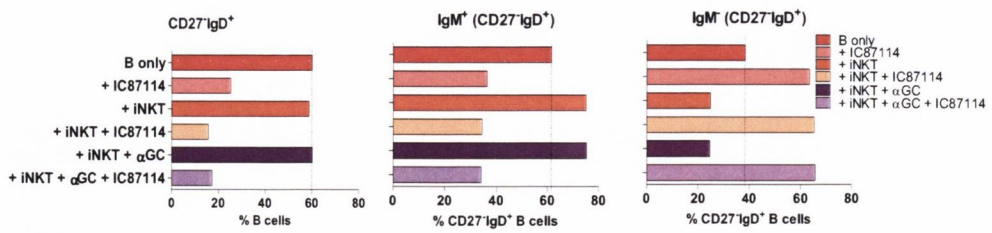

Figure 9.19.1. Naïve B cells are decreased in frequency and IgM expression by IC87114. B cells were co-cultured with iNKT cells for 3 days in the absence or presence of α-GC, with or without IC87114. As negative controls, B cells were cultured in medium only or with IC87114. Flow cytometric analysis of changes in proportion of total, IgM^+ and IgM^- naïve B cells was performed. N=1.