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TRINITY COLLEGE DUBLIN
COLÁISTE NA TRÍONÓIDE

THE
UNIVERSITY
OF DUBLIN

Defining functional interactions between human B cells and invariant natural killer T cells from healthy donors and patients with chronic lymphocytic leukaemia

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of Doctor of Philosophy (PhD) – February 2015



IRISH RESEARCH COUNCIL
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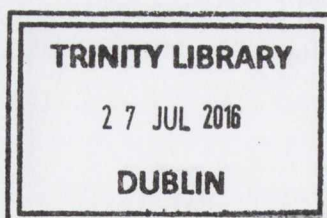
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Ghnewa Y G, McElligott T AM, Vandenberghe E, O'Reilly V P, Doherty D G. Retinoic acid receptor agonists upregulate CD1d on chronic lymphocytic leukaemia cells and improve alpha galactosylceramide mediated cytotoxicity of CD8⁺ Invariant natural killer T cells. Manuscript in preparation.

Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
APL	Acute promyelocytic leukaemia
ATRA	All <i>trans</i> retinoic acid
B-CLPDs	B-cell chronic lymphoproliferative disorders
BCR	B cell receptor
BFA	Brefeldin A
Breg	Regulatory B
CD	Cluster of differentiation
cDMEM	complete DMEM.
CLL	Chronic lymphocytic leukaemia
cRPMI	Complete RPMI.
CSR	Class-switch recombination
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DCS	Dead cell stain
DMEM	Dulbecco's Modified Eagle Medium.
DN	Double negative
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticulum
FA	Fatty acid
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FCS	Foetal calf serum
FMO	Fluorescence minus one
FSC	Forward scatter
GC	Germinal center
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSL-1	Glycosphingolipid-1
hCD1d	human CD1d

HCV	Hepatitis C virus
HMBPP	(E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate
IBTS	Irish Blood Transfusion Service
iDC	Immature dendritic cell
iGB3	Isoglobotrihexosylceramide
ILC	Innate lymphoid cell
iNKT	Invariant natural killer T
iNKT _{FH}	Invariant naturel killer T follicular helper
IPP	Isopentenyl pyrophosphate
IRF	Interferon-regulatory factors
KIR	Killer cell immunoglobulin-like receptor
LC	Langerhans cell
LPS	Lipopolysacharide
mAb	monoclonal antibody
MAIT	Mucosal-associated invariant T
MCS	Magnetic cell sorting
mDC	Myeloid dendritic cell
MEM	Minimum essential medium
MHC	Major histocompatibility
moDC	Monocyte-derived dendritic cell
MZB	Marginal zone B
NF-κB	Nuclear factor-κB
NK	Natural killer
NKT	Natural killer T
NLR	NOD-like receptor
PAMP	Pathogen-associated molecular patterns
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Plasma cell
pDC	Plasmacytoid dendritic cell
PHA	Phytohaemagglutinin
PIM	Phosphotidylinositol mannoside
PMA	Phorbol 12-myristate 13-acetate
PRR	Pathogen recognition receptor
PTLDs	Post-transplantation lymphproliferative disorders

RAR α	Retinoic acid receptor alpha
RLR	Retinoic acid-inducible gene-I-like receptor
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SHM	Somatic hypermutation
SLAM	Signaling lymphocytic activation molecule
SLE	Systemic lupus erythematosus
SSC	Side scatter
TCM	T cell medium.
TCR	T cell receptor
TD	Thymus dependent
T _{FH}	T follicular helper
TI	Thymus independent
TLR	Toll-like receptor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory
WHO	World Health Organisation
α -GalCer	α -Galactosylceramide
β -GlcCer	β -D-Glucopyranosylceramide
β 2M	β 2-microglobulin

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Abstract.

Invariant natural killer T (iNKT) cells are a minor population of innate T cells that express semi-invariant T cell receptors that recognise glycolipid antigens (Ag) presented by CD1d. Upon activation, iNKT cells kill target cells, release cytokines that activate and regulate cellular and antibody-mediated immune responses, and promote maturation of dendritic cells into antigen presenting cells. Studies in mice and humans have shown that iNKT cells mediate immunity against tumours, and clinical trials targeting iNKT cells for the treatment of cancer are ongoing.

Human B cells express CD1d and have recently been shown to stimulate iNKT cells, and iNKT cells can reciprocally provide help for antibody (Ab) production by B cells. However, iNKT cells include functionally distinct subsets based on expression of CD4 and CD8. In the present study, we investigated whether CD4⁺, CD8⁺, and DN iNKT cell subsets can differentially promote B cell differentiation, antibody production, as well as cytokine production *in vitro*. We optimised methods for the generation of CD4⁺, CD8⁺, and DN iNKT cell lines, and investigated the roles B cells have in activating iNKT cells through the presentation of various glycolipid antigens. In view of the well-documented roles that iNKT cells play in anti-tumour immunity and in cognate interactions with B cells, we investigated the role and treatment potential of iNKT cells in chronic lymphocytic leukaemia (CLL), a human B cell malignant neoplastic disease.

A robust method for the generation of functional CD4⁺, CD8⁺, and DN iNKT cell lines from as little as 3 ml of fresh human blood was achieved. Upon co-culture with either autologous or allogeneic B cells, all subsets of iNKT cells were capable of IFN- γ and IL-4 production as well as cytotoxic degranulation. However, glycolipid antigen presentation by B cells required higher concentration of glycolipid to elicit a functional response by iNKT cells when compared to CD1d-transfected HeLa cells or dendritic cells.

Independently of the exogenous antigen α -GalCer, CD4⁺, CD8⁺, and DN iNKT cells were capable of inducing B cell activation through the upregulation of CD69 and IL-21R, the production of IgG, IgA, and IgM, and the induction of phenotypic changes to B cells. These data indicate that B cells can present glycolipid antigens to iNKT cells leading to their activation and that iNKT cells can reciprocally modulate B cell differentiation and function.

Phenotypic analysis of circulating lymphocytes from patients with CLL revealed depletions of iNKT cells as well as reduced CD1d expression by B cells. However, iNKT cells were capable of killing CLL cells and we found that retinoic acid receptor agonists could induce upregulated expression CD1d on CLL cells resulting in enhanced cytotoxic degranulation by CD8⁺ iNKT cells. Hence, targeting the antitumour activities of iNKT cells, directly by adoptive cellular immunotherapy, and indirectly by administration of retinoic acid receptor agonists for the modulation of CD1d expression by CLL B cells, may provide a new treatment option for improving the prognosis of patients with CLL.

Chapter 1 Introduction.

1.1 Innate and adaptive immunity.

The human immune system holds the important role of protecting the host from a large number of different types of internal and external threats. A fully functional immune system requires the active interplay of a variety of specialised immune organs, immune cells (i.e. leukocytes), and biomolecules that anatomically span almost all parts of the human body. Homeostasis relies on communication between the various immune cells and other cells in the body. Immune cells can be found circulating in the blood and lymph vessels, arranged in organised structures in tissues strategically located to detect foreign threats (e.g. Peyer's patches), or found within lymphoid organs whose function can be either dedicated to the development and maturation of these cells (e.g. thymus), or their functional maturation to allow them to respond to foreign threats (e.g. lymph nodes) (Kindt et al. 2007a).

Immune responses are generally categorised as innate or adaptive based on specificity, speed of response, and immunological memory. Innate immune responses are first line responses, whereby foreign organisms or damaged self are efficiently and effectively removed. Although they are rapid, they are non-specific, responding in a similar fashion to different groups of pathogens, based on the recognition of common pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Adaptive immune responses are second-line responses that rely on members of the innate immune system for their recruitment and activation. Adaptive immune responses can take up to 7 days to take effect, but they are highly specific and give rise to protective antibodies and long term memory cells that result in a more heightened response on subsequent antigenic encounters. The specificity of adaptive immune cells relies on highly specific cell surface receptors on B cells and T cells that recognise specific epitopes on foreign antigens (Ag) (Kindt et al. 2007a).

Although the division between innate and adaptive immunity is historical and very artificial, it can help in our understanding and study of the immune system. When looking at both innate and adaptive immunity, it is important to know that immune

responses are fluid and therefore so are the functional divisions between innate and adaptive immune responses.

1.1.1 Cells of the immune system.

An effective immune response requires the functional co-ordination between a variety of different leukocytes. Where some threats can be dealt with by the innate leukocytes, others require subsequent recruitment of adaptive lymphocytes.

Leukocytes are broadly divided based on their ontogeny either into lymphoid (i.e. lymphocytes) or myeloid cells. Myeloid cells include neutrophils, eosinophils, and basophils, all of which are granulocytes. Granulocytes are important first-line innate cells, responding within hours to bacterial, viral, and parasitic threats. Generally speaking these cells clear pathogens through phagocytosis or degranulation and contain anti-microbial compounds within their granules, which can neutralise various pathogens. The myeloid lineage also includes agranular cells such as monocytes, macrophages, and myeloid dendritic cells (DC), all of which are phagocytes. A major role of these cells is to transactivate cells of the adaptive immune system through Ag-presentation and as such monocytes, macrophages, and DC are antigen presenting cells (APC).

Lymphoid cells include T cells, B cells, natural killer cells (NK), and plasmacytoid DC (pDC). T and B cells are primary members of the adaptive immune system, responding later than their innate counterparts. Their responses require the engagement of Ag-specific T cell and B cell receptors (TCR and BCR respectively) on their cell surface with Ag presented to them by APC in the context of specialised protein complexes such as major histocompatibility complex (MHC) molecules. TCRs and BCRs are clonally specific and are generated during cellular development through somatic recombination of TCR and BCR gene segments. This results in a vast repertoire potentially consisting of over 10^{11} clones of different antigenic specificities (Takeda et al. 2003).

NK cells are innate lymphocytes that are considered part of a wider group of non-T cell non-B cell innate lymphoid cells (ILC). In humans, NK cells are phenotypically identified as $CD56^+ CD3^-$ cells, the majority of which also express the C-type lectin

CD161. They are well known for their killer or cytotoxic activities, but also have important roles in innate and adaptive immune regulation. NK cells are equipped with a variety of “sensing” receptors that bind to complementary surface ligands/receptors on host cells. NK cells can become activated upon engagement of receptors such as NKG2D with stress-induced ligands on target cells (e.g. virus infected or cancer cell), NKG2C with HLA-E, or Fc receptors with IgG bound to target cells. NK cells can also become activated in the absence of negative signals provided by the engagement of inhibitory receptors such as killer cell immunoglobulin-like receptors (KIR) with MHC class I on target cells, or NKG2A with HLA-E. Hence, in the presence of pathological down regulation of MHC class I, NK cells become activated. NK cells can also become activated by cytokines produced by other immune cells such as IFN- γ , IFN- α , IL-2, IL-12, IL-15, and IL-18. Upon activation, NK cells can produce a number of cytokines including IFN- γ and IL-12, and can kill target cells through the release of cytolytic molecules such as granzyme B and perforin, or through TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis of target cells (Vivier et al. 2008).

1.1.2 Pathogen recognition by the innate immune system.

Innate immune cells rely on pattern recognition receptors (PRR) for the recognition of foreign organisms or danger signals from host cells. PRRs comprise a number of soluble and membrane bound receptors that can recognise conserved motifs on microorganisms called pathogen-associated molecular patterns (PAMP), or on damaged cells called damage-associated molecular patterns (DAMPs). There are four main types of PRRs: TLR, C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors (NLRs). The innate immune system relies on PRRs to recognise and respond to threats, but PRRs can also be found on cells of adaptive immune system such as B cells and T cells. The same PAMPs can be found on a variety of organisms, hence immune recognition through PRR is non-specific, but due to their ubiquitous nature, responses to PAMPs are rapid and effective. The best-studied types of PRRs are toll-like receptors (TLR). TLR are trans-membrane proteins that are found on the cell surface or within the cell, with 10 subsets identified in humans (TLR1-10). Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, and TLR6, which recognise either

components of gram-positive bacteria, gram-negative bacteria, yeasts, or fungi. Intracellular TLRs include TLR3, TLR7, TLR8, and TLR9, which recognise RNA or DNA components of viruses or bacteria (Barr et al. 2007; Takeuchi & Akira 2010). Upon recognition of the TLR with its ligand on a target cell, TLRs dimerise causing the initiation of downstream intracellular signaling through a series of kinases and adaptor proteins, which ultimately lead to the activation of transcription factors with major pro-inflammatory consequences. Such transcription factors include nuclear factor- κ B (NF- κ B), and the interferon-regulatory factors (IRFs), which ultimately lead to the production of pro-inflammatory cytokines. Another major consequence of TLR engagement for cells such as neutrophils, monocytes, macrophages, and DC is to phagocytose and degrade target cells (O'Neill et al. 2013).

Phagocytosis is initiated by the engagement of some PRRs, Fc receptors, complement receptors, or apoptotic corpse receptors with their ligands on target cells. Phagocytic receptors along with other transmembrane proteins co-ordinate cytoskeletal rearrangement to allow for pseudopods to form, encapsulating the target in a membrane bound phagosome. The phagosome then travels through the phagocytic cell, where it's contents can be targeted for degradation. Professional APC such as DC can also incorporate Ag presentation at this stage to present various antigenic epitopes that may be recognised by specific TCRs and BCRs on T and B cells respectively (Freeman 2014).

1.1.3 Pathogen recognition by the adaptive immune system.

T and B cells recognise and respond to specific antigenic epitopes detected by their highly specific TCR and BCR respectively. TCRs can only recognise Ag if it is presented to them by APCs such as DCs. APCs take up Ags by a variety of methods, including phagocytosis, pinocytosis, and receptor mediated endocytosis. Protein Ags are degraded within intracellular vesicles into smaller peptides and incorporated into major histocompatibility (MHC) complexes. MHCs are diverse protein structures that differ between individuals, and are categorised into two major classes: MHC class I and MHC class II. Generally peptides originated outside the APC are incorporated and presented on the surface of APC in the

context of an MHC class II, while peptides sampled from within the APC (i.e. intracellular Ag) are generally presented in the context of MHC class I (Fig. 1.1). Presentation of intracellular Ags allows the immune system to detect changes in normal intracellular protein expression, which may be caused by spontaneous aberrations (e.g. tumours) or intracellular pathogens. The recognition of Ag-MHC complexes by T cells differs between T cell subsets, where CD8⁺ T cells recognise peptide Ag presented on MHC class I, and CD4⁺ T cells recognise Ag presented on MHC class II. Functional consequences of TCR interaction with peptide bound MHC depends on the engagement of other receptors on the T cell surface, including receptors for cytokines, co-stimulatory, and co-inhibitory molecules. Signals through such receptors are provided by professional APC such as DC. Cytokines are required for appropriate T cell differentiation, while co-stimulation is required for T cell activation and expansion (Coquet et al. 2015).

Similar to T cells, B cells express Ag specific BCRs, but unlike T cells they do not require MHC for Ag recognition. BCRs are membrane bound Abs that bind native Ag, triggering Ag internalisation via receptor mediated endocytosis and subsequent presentation of peptide fragments on MHC to Ag specific T cells. Cognate interaction with Ag specific T cells known as follicular helper (T_{FH}) cells is required for subsequent B cell activation and differentiation into Ab producing plasma cells (PC) and memory B cells (Kindt et al. 2007b).

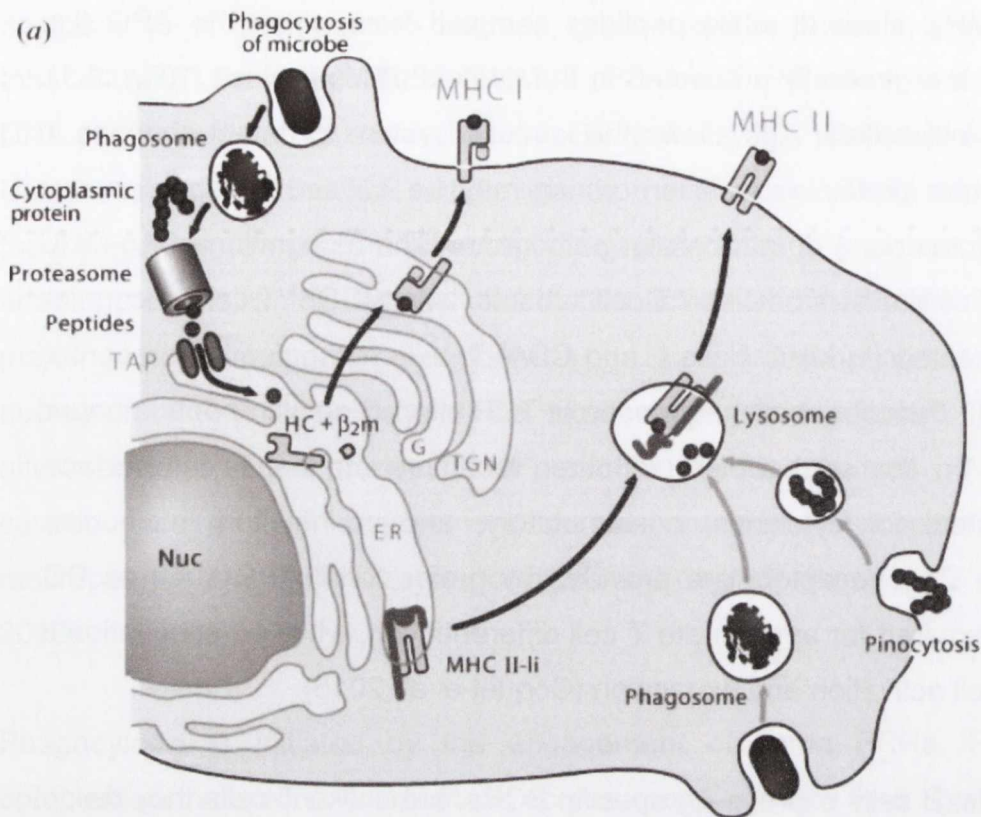


Fig. 1.1. Intracellular trafficking of MHC class I and II. Extracellular pathogens are phagocytosed and transported into the cytoplasmic compartment in phagosomes. Phagosomes fuse with lysosomes, where proteins are degraded into peptide Ags. MHC class II is assembled in the endoplasmic reticulum (ER), bound to an invariant chain (Ii) that prevents binding of intracellular Ag, and is transported via endosomes to the lysosome where it can bind processed Ag, and is then transported to the plasma membrane for Ag presentation. MHC class I is coupled to β_2 -microglobulin (β_2 M) in the ER and subsequently loaded with peptide Ags that have been produced through protein degradation by cytoplasmic proteases. Ags loaded on MHC class I are mainly self-Ags but can also include foreign Ags, which are then cross-presented on MHC class I. This illustration is taken from (Brigl & Brenner 2004).

1.1.4 Antigen presenting cells.

A number of cell types including DCs, macrophages, monocytes, B cells, neutrophils, T cells, epithelial and endothelial cells have been shown to be able to present Ag to T cells. However, DCs are considered professional APC due to their efficiency and effectiveness at antigen presentation and ability to modulate subsequent T cell responses. DCs are consequently used as tools in human clinical trials as part of cell-based or cellular immunotherapy regimens, aimed at manipulating Ag specific responses *in vivo*, targeting a variety of cancers. While there are many ways of targeting the immune system as part of treatment regimen (i.e. immunotherapy), cellular immunotherapy involves administering immune cells

that have been manipulated ex-vivo to patients in the form of a vaccine or infusions. DC-based therapies involve taking a sample of blood, expanding the DCs in culture, treating the DCs to make them more immunogenic, then re-administering the DCs back to the patient (Motohashi et al. 2011).

DCs are a heterogeneous population of professional APC whose primary roles include antigen presentation, and the activation of Ag-naive T cells. A major aspect of DC function is their ability to migrate from tissues where they are activated, through the circulation to local lymph nodes, where they act as potent T and B cell activators. DCs are broadly divided based on their lineage or ontogeny into myeloid (mDC), pDC, CD14⁺ DC, and Langerhans cells (LC). mDC express various myeloid cell markers, and are further divided into CD1c⁺ and CD141⁺ mDC. pDC lack myeloid lineage markers and share common lymphoid features, they express markers such as CD123, and are the most abundant peripheral blood DC subset. CD14⁺ DC are thought to originate from monocytes, and are found mainly in tonsils and LN. LCs are tissue-resident cells found in the skin, and are capable of self-renewal. During inflammation, a subset of DC called inflammatory DC emerges at the site of inflammation. Suspected to be monocyte in origin, both CD14⁺ DC and inflammatory DC can be termed monocyte-derived DC (moDC) (Collin et al. 2013; Smits et al. 2015).

DCs present Ag in the context of MHC class II to naive CD4⁺ T cells. Upon activation, CD4⁺ T cells can proliferate and mature into a number of types of helper T cells, which orchestrate adaptive immune responses through the secretion of cytokines and through contact-dependent interaction with other cells. DCs can also present Ag of intracellular origin such as self Ag, viral particles, and tumour Ag in the context of MHC class I to CD8⁺ T cells or cytotoxic T lymphocytes (CTL). A process called cross presentation allows DCs to present extracellular Ag on MHC class I. This allows for the induction of CD8⁺ T cell responses against viruses or tumours that arise outside of the DC itself (Segura & Amigorena 2014). DCs release a variety of cytokines that influence T cell differentiation including IL-1 β , IL-4, IL-6, IL-12, IL-18, IFN- γ , and TGF- β . DCs also provide co-stimulation required for naive T cell activation and expansion including CD80, CD86, CD40, signaling lymphocytic activation molecule (SLAM) family members among others. Inhibition of T cell activation is also achieved through

engagement of co-inhibitory receptors on the T cell, such as programmed cell death 1 (PD1) receptor and cytotoxic T lymphocyte antigen 4 (CTLA4) to name a couple, by their ligands on DCs. While DC can be stimulated through TLR engagement, they require T cell help to become fully mature and activated. On the other hand T cells require a mature and activated DC to provide all the necessary signals for full T cell activation and function (Lieping & Flies 2013; Coquet et al. 2015).

DCs are present in one of two maturation states: immature DC (iDC) and mature DC. Immature DC are those that are functionally equipped to sample the microenvironment through processes such as phagocytosis. They have low MHC expression and do not induce T cell activation. Mature DCs are those that are functionally geared towards antigen presentation and T cell activation. They undergo changes in adhesion and chemokine receptor expression resulting in their migration to lymph nodes. Mature DC express high levels of MHC and co-stimulatory molecules (e.g. CD40, CD80, and CD86), and produce an array of pro-inflammatory cytokines including IL-1, IL-6, TNF- α , IL-12 and IL-23 (Collin et al. 2013).

Myeloid DC can be generated from peripheral blood monocytes *in vitro* and these moDC have been found to functionally and phenotypically resemble inflammatory DCs. CD14⁺ monocytes are typically cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 for 5-6 days, where they differentiate into iDC. iDC can then be matured using a number of cytokines or ligands that bind to PRRs, collectively known as adjuvants. The use of DC for the basis of cancer immunotherapy has been exploited since the mid 1990s. Autologous moDC generated *in vitro* can be pulsed with the desired target Ag, and infused back into patients. These primed DCs theoretically can educate the appropriate cells (e.g. CD8⁺ CTL) to effectively target the tumour in question (Smits et al. 2015).

1.2 B lymphocytes.

B cells along with T cells make up the cellular pillars of the adaptive immune system and are exclusively responsible for Ab production, protecting the host against pathogens that evade innate immune defenses. Similar to T cells, the specificity of B cell responses are dictated by a large repertoire of Ag-specific B cell clones generated through BCR gene rearrangement during B cell development in the bone marrow. Along with Ab production B cells are capable of Ag presentation to Ag-specific T cells called T_{FH} , a cognate interaction that reciprocally provides help for B cell activation and function. Within secondary lymphoid tissue B cells are found in organised lymphoid follicles and are thus termed follicular B cells. The specific interaction between the BCR of a naive B cell and an Ag generally attached to a DC or macrophage can lead to Ag internalisation, processing, and peptide presentation on MHC class II to $CD4^+$ T_{FH} cells. T_{FH} cells provide necessary signals for the B cell to proliferate and differentiate into Ab secreting PC and memory cells. The general understanding is that activated B cells that enter a lymphoid follicle germinal center reaction will give rise to memory cells and long lived PC with enhanced Ag specificity due to somatic hypermutation (SHM). In contrast, B cells activated outside of a lymphoid follicle (i.e. extra-follicular B cells) will not give rise to memory cells and generated PC are short lived. However, evidence from murine studies suggests the existence of germinal center independent memory generation. Extra-follicular B cell responses include those involving marginal zone B (MZB) cells in the spleen (LeBien & Tedder 2008; Vinuesa & Chang 2013).

1.2.1 Antigen recognition by B cells.

All mature B cells express the surface co-stimulatory CD19 protein, and an Ag-specific BCR. The BCR is a membrane bound IgD that recognises specific protein or non-protein Ag in native form. Successful Ag recognition leads to internalisation of Ag via receptor-mediated endocytosis, and Ag presentation in the context of MHC class II to $CD4^+$ helper T cells. The type of Ag will determine the type of helper cell activated by the B cell, which in turn can dictate the type of response elicited by that B cell.

Ags recognised by B cells are generally categorised into thymus dependent (TD) protein and thymus independent (TI) non-protein Ags. B cell responses to TD Ag ultimately require cognate help by Ag-specific T_{FH} cells, while TI responses do not require T cell help and are categorised into TI-1, TI-2, and TI-3. TI-1 responses involve the engagement of the BCR with Ag as well as TLR with an appropriate ligand (e.g. LPS). TI-2 involves a multivalent BCR engagement with a sequence of repeating epitopes from encapsulated bacteria or viral capsids. A more recently described TI-3 response involve innate myeloid helper cells, including neutrophils, monocytes, mast cells, or basophils, providing co-stimulation through BAFF, APRIL and CD40L (Vinuesa & Chang 2013). Engagement of the BCR with Ag triggers the formation of the immunological synapse and subsequent Ag internalisation, processing, and presentation (Yuseff et al. 2013).

1.2.2 Antigen presentation by B cells.

B cells are considered a member of the professional APC group, which include DC, macrophages, and B cells. However, unlike other professional APC, the primary function of B cells lies in the provision of humoral immunity via the production of high affinity Abs. While there is no doubt on the ability of B cells to present Ag to T cells and its requirement for TD responses, the necessity of this cognate interaction for T cell function has been debated (Chen & Jensen 2008).

Ag presentation by B cells is short lived when compared to DC, whose Ag presentation functions are long lived. However, both APC have been shown to co-operate and potentially require the other for optimal APC function and immune modulation. It is well documented that B cells can efficiently capture Ag that is bound to DC cell surface through the formation of immunological synapse. It is also suggested that B cells can reciprocally transfer BCR-specific Ag to DC. BCR-specific Ag transfer to DC provides a greater Ag load than that obtained through phagocytosis of particulate Ag, and also increases the Ag specificity of DC-mediated immune responses (Raman et al. 2010).

Once BCR-Ag complex are internalised by B cells, peptide fragments are then presented to conventional CD4⁺ T_{FH} cells. This cognate interaction allows for Ag-specific T_{FH} cells to provide the necessary cognate co-stimulation (e.g. CD40L)

and cytokine signals (e.g. IL-21) for full activation of B cells. Although much less effective than DC, B cells can reciprocally modulate T cell function, causing activation or inducing tolerance in an Ag dependent manner (Chen & Jensen 2008).

1.2.3 Immune regulation by B cells.

B cells secrete a variety of high affinity Abs including IgG, IgM, IgA, IgE, and IgD subclasses, providing protection for various tissues of the body against bacteria and viruses. IgM Abs are expressed by all naive B cells; they are broadly reactive, and are produced and secreted rapidly during primary immune responses, prior to SHM and class-switch recombination (CSR). IgG Abs are the most abundant serum Ab of all subclasses. They are highly specific and their main functions include complement activation, Ag neutralisation, and opsonisation. IgA Abs are the most abundant subclass found at mucosal surfaces and secretions, where they protect associated tissue from toxins, viruses, and bacteria via direct neutralisation. IgE is the least stable Ab but with very potent activity; its involved mainly in allergic reactions, hypersensitivity, and response to parasitic worms (Schroeder & Cavacini 2013).

Activated B cells can differentiate into short-lived extra-follicular PCs that provide low affinity IgM Ab protection early after activation. Activated B cells can also enter a lymphoid follicle, proliferate forming germinal centers, undergo SHM and CSR, providing appropriate classes of high affinity Ab that emerge later through the generation of long-lived PC and memory B cells (LeBien & Tedder 2008).

While antibody-mediated responses are well characterised (LeBien & Tedder 2008; Elgueta et al. 2010), B cells also elicit antibody independent B cell responses. These include the previously mentioned APC and co-stimulation function, as well as cytokine-mediated transactivation and immune regulation of other immune cell functions. B cells can produce and secrete a variety of cytokines including the stimulatory cytokines IFN- γ , TNF- α , IL-2, IL-4, IL-6, and IL-12, and regulatory cytokine IL-10. Antibody independent B cell responses have been implicated in tumour immunity, transplant rejection, wound healing,

regulating DC function, and balancing T helper cell responses. On the other hand, regulatory B (Breg) cells have been described that down-regulate immune responses in both IL-10 independent and IL-10 dependent manners. Breg cells can also function by trans-activating regulatory T cells (Treg), which in turn suppress immune function. Breg cells are a heterogeneous population of B cells with various phenotypes in both mouse and human. While there is no distinctive phenotypic marker(s) to distinguish Breg cells from other B cells, Breg cells are enriched within specific B cell populations (e.g. transitional B cells with a CD24^{hi}CD38^{hi} phenotype). Whether these cells are inducible, and capable of differentiating from different B cell subsets, or are a distinct subset with a unique regulatory function, is yet to be uncovered (Mauri & Menon 2015).

1.3 T lymphocytes: cells of the innate and adaptive immune system.

1.3.1 Conventional T cells.

Adaptive T cells, which have previously been introduced as either CD4⁺ or CD8⁺ T cells, are given the term conventional T cells. They express a TCR complex composed of the transmembrane signaling protein CD3, and an Ag-specific TCR composed of an $\alpha\beta$ chain. Each TCR is generated through gene rearrangement events during development in the thymus, and recognises Ag presented in the context of MHC by APC. Recognition of Ag by the TCR can result either in T cell activation or anergy (i.e. a state of non-responsiveness), depending on the signals it receives from the APC. Engagement of the TCR with Ag-MHC in the presence of other co-stimulatory signals brings about the activation of a naive T cell, clonal expansion and differentiation into effector and memory T cells that contribute to the clearance of the infection.

CD4⁺ and CD8⁺ T cells have different effector roles. CD8⁺ T cells become CTL and play a major role in clearing infections with intracellular pathogens, and tumour cells. Cytolysis is achieved by CTL through perforin and granzyme release or TRAIL and Fas/Fas ligand (FasL) induced apoptosis. CD4⁺ T cells can differentiate into various types of helper cells that can direct immune responses

through cognate interaction with other cells and cytokine production (Harty et al. 2000).

CD4⁺ T helper cells can be subdivided into distinct functional subsets based on the cytokines they produce upon activation. Principal T helper subsets include Th1, Th2, Th17, Treg and T_{FH} cells, while other less defined subsets include Th9, Th22, and Th1/17. The type of signals/cytokines a naive CD4⁺ T cell receives during Ag presentation dictates the commitment to a specific T helper lineage. Each T helper lineage is functionally controlled by a master transcription factor, which can contribute to their identification (Fig. 1.2). Briefly, Th1 cells are involved in promoting immune responses against intracellular bacteria and viruses, through IFN- γ and TNF- α mediated transactivation of macrophages, NK cells, and DC. Th2 cells promote immune responses against extracellular parasites through cytokine-mediated (IL-4, IL-5, and IL-13) trans-activation of B cells, eosinophils, and mast cells. Th17 cells promote immune responses against fungi and extracellular bacteria through IL-17, IL-21, IL-22, and IL-26-mediated neutrophil recruitment. Follicular helper T (T_{FH}) cells are anatomically associated with B cell-rich areas of the spleen and LN, and provide help for B cell activation and differentiation. T_{FH} cells express a master transcription factor that is antagonistic to Th1, Th2, and Th17 cell differentiation, making them a separate subset of Th cells. IL-21 is an important cytokine for T_{FH} function and differentiation. Alongside effector CD4⁺ T cells, there are also regulatory T cells (Treg), which promote immune tolerance and homeostasis mainly through the production of immuno-suppressive cytokines such as IL-10 and TGF- β . Modulating the function of Treg cells has been a major target for immune based therapies, as they are central regulators of the immune system (Crotty 2011; Geginat et al. 2013).

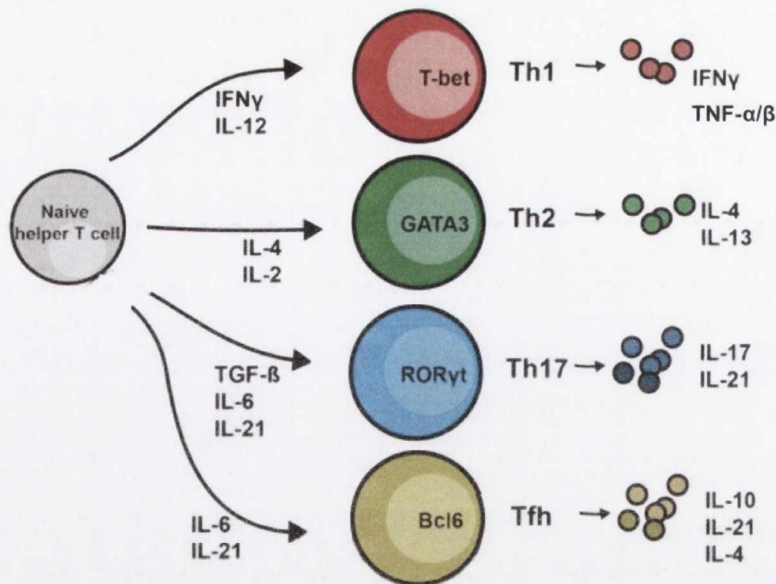


Fig. 1.2. CD4⁺ T helper cell subsets. Naive CD4⁺ T helper cells respond to specific cytokine stimulation by APCs and differentiate accordingly into one of four distinct effector T helper cell subsets with distinct functions determined by the cytokines they produce: Th1, Th2, Th17, or Tfh cells. Naive T cells receiving IFN- γ and/or IL-12 stimulation by APC can differentiate into Th1 cells, which are controlled by the master transcription factor T-bet, and produce IFN- γ and TNF- α/β . Naive T cells receiving IL-4 and IL-2 stimulation can differentiate into Th2 cells, which are controlled by GATA3, and produce IL-4 and IL-13. Naive T cells receiving TGF- β , IL-6, and IL-21 stimulation can differentiate into Th17 cells, which are controlled by ROR γ t, and IL-17 and IL-21. Naive T cells receiving IL-6 and IL-21 stimulation can differentiate into Tfh cells, which are controlled by Bcl6 and produce IL-10, IL-21, and IL-4. This illustration is a modification from (Geginat et al. 2013).

1.3.2 Innate T cells.

Innate T cells represent a heterogeneous group of CD3⁺ cells all of which express a form of TCR, and combine features of both T cells and natural killer cells. Innate T cells include $\gamma\delta$ T cells, mucosal-associated invariant T (MAIT) cells, and natural killer T (NKT) cells.

1.3.2.1 $\gamma\delta$ T cells.

$\gamma\delta$ T cells are first line responders during infection and inflammation, appearing days before adaptive immune cells. $\gamma\delta$ T cells are found in various organs in the body, and in the peripheral blood (PB) where mature $\gamma\delta$ T cells represent ~4% of total T cells. $\gamma\delta$ T cells express a TCR composed of one gamma (γ) chain and one delta (δ) chain, and based on δ chain usage, there are three main subsets in

humans: V δ 1, V δ 2, and V δ 3. Similar to the $\alpha\beta$ TCR, $\gamma\delta$ TCR are diverse with the potential to recognise a large repertoire of Ags. While $\alpha\beta$ T cells recognise Ag presented to them in the context of MHC, the mechanism by which $\gamma\delta$ T cells recognise Ag via their TCR is less well understood. Irrespective of the mechanism, $\gamma\delta$ T cells respond to a wide range of biomolecules, including various microbial and self-metabolites, suggesting their heterogeneity. $\gamma\delta$ T cells can expand *in vivo* during a variety of human infections to reach up to 60% of total PB T cells, most of which belong to the V δ 2 T cell subset V γ 9V δ 2 T cells. V γ 9V δ 2 T cells respond strongly to the microbial isoprenoid intermediate (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMBPP), and to a lesser extent to the human mevalonate pathway intermediate isopentenyl pyrophosphate (IPP). As well as their response to phosphate-containing Ag, V γ 9V δ 2 T cells can also respond to aminobisphosphonates (e.g. zoledronate), and alkylamines. Aminobisphosphonates indirectly stimulate V γ 9V δ 2 T cells by interfering with the mevalonate pathway, causing the accumulation of IPP, which directly stimulates the cells. V γ 9V δ 2 T cells were shown *in vitro* upon antigenic activation to express LN homing receptors (CCR7) and become professional APC. Treatment of cancer patients with *in vitro* expanded autologous V γ 9V δ 2 T cells or with zoledronate and IL-2 resulted in relatively better prognostic outcomes (Chien et al. 2014). V δ 1 T cells are the second most abundant $\gamma\delta$ T cell in humans, some which recognise the MHC class I like molecules CD1c and CD1d. They respond to CD1d⁺ cells presenting sulfatide or α -GalCer as well as the stress-inducible MICA/B on tumour or virally infected cells (Pellicci et al. 2014; Chien et al. 2014). V δ 3 T cell responses are less well understood, and ligands to enhance their responses have yet to be identified. However, V δ 3 T cell are expanded in patients with HIV and CMV infection, as well as patients with chronic lymphocytic leukaemia (CLL) (Mangan et al. 2013).

In general, $\gamma\delta$ T cells can produce an array of Th1, Th2, and Th17 cytokines by which they influence cells of both adaptive and innate immune responses. They are capable of direct cytotoxicity, and produce a number of antimicrobial peptides (Marischen et al. 2009). Alongside the array of immunostimulatory functions of $\gamma\delta$ T cells, some $\gamma\delta$ T cells have also been found to be immunosuppressive, abrogating DC and T cell function in patients with breast cancer (Chien et al. 2014).

1.3.2.2 MAIT cells.

MAIT cells are innate T cells that express a highly conserved TCR composed of a conserved α chain (V α 7.2-J α 33) that combines with a restricted number of β chains, as well as expressing CD161. MAIT cells are the most abundant innate T cell in humans, representing up to 10% of total T cells in the peripheral blood, and up to 40% of liver T cells, frequencies higher than those in mice. They recognise Ag presented in the context of a highly conserved protein called MR1. Riboflavin metabolites from some bacteria and fungi represent Ags that cause MAIT cell activation, in an MR1-dependent manner. Unlike other innate T cells, MAIT cells do not seem to respond to self-Ag. In general, MAIT cells are capable of cytotoxic killing, and can produce a range of inflammatory cytokines including IFN- γ , TNF- α , and IL-17, trans-activating a variety of innate and adaptive cells, and can express the multi-drug resistance transporter ABCB1. However, unlike other T cells, MAIT cells do not proliferate *in vitro* in response to IL-2, IL-7, or IL-15, making them challenging to study and restrict their use for cellular immunotherapy (Le Bourhis et al. 2013).

1.4 NKT cells.

1.4.1 Type 1 and type 2 NKT cells.

Natural killer T (NKT) cells are a heterogeneous group of $\alpha\beta$ and $\gamma\delta$ T cells, which are considered part of the innate lymphocyte population. While other $\alpha\beta$ T cells recognise protein Ag presented in the context of MHC molecules, NKT cells recognise lipid based Ag presented in the context of the MHC class I-like CD1d molecule. Within the large NKT cell family there are 2 main subsets: a) Type 1 NKT cells, with a well-characterised semi invariant TCR, are also termed invariant NKT (iNKT) cells. b) Type 2 NKT cells, are a heterogeneous group with a variable TCR repertoire but contains some TCR biases. Type 2 NKT include $\alpha\beta$ T cells, as well as some $\gamma\delta$ T cells. Although type 2 NKT cells are diverse; there is a concentration of V α 3 and V α 8 TCR chains coupled mainly with V β 8. Type 2 NKT cells are not as well understood as type 1, but some of the antigens that have been identified include sulfatide, lysophosphatidylcholine, cardiolipin, β -glucosylceramide (β -GlcCer) variants and other non-lipid small molecules. Phenotypically human type 2 NKT cells are either CD4⁺ or CD4⁻CD8⁻, while human

type 1 NKT cells also comprise CD8⁺ NKT cells. One of the main function of type 1 NKT cells is their cytotoxic capabilities, promoting anti-tumour immune responses, while type 2 NKT cells suppress tumour immunity, and each can cross-regulate the other's function (Godfrey et al. 2010; Terabe & Berzofsky 2015).

1.4.2 Invariant natural killer T (iNKT) cells: an introduction.

iNKT cells are type 1 NKT cells, and are the most studied and characterised NKT cell. In humans they express a common TCR α chain (V α 24-J α 18) that couples with the β chain (V β 11). Mouse iNKT cells express a V α 14-J α 18 and can couple with one of three β chains. Both human and mouse iNKT cells are capable of recognising the marine sponge glycolipid Ag α -galactosylceramide (α -GalCer) presented in the context of CD1d on the surface of APC. In humans, the proportion of iNKT cells varies greatly between individuals, but remains stable within the same individual. They represent 0.01–0.1% of human peripheral blood mononuclear cells (PBMC), 1% of liver lymphocytes, and 10% of omental lymphocytes. In contrast, in mice they are much more abundant representing up to 0.5% of PBMC, and up to 35% of liver lymphocytes (Berzins et al. 2011). Following development in the thymus, iNKT cells migrate to various tissues in the body, including the gut, lungs, spleen, lymph nodes, liver, bone and skin (Johnston 2015).

NKT cells were first named based on a population of $\alpha\beta$ T cell in mice, which uniquely expressed the V α 14 T cell receptor chain along with the NK receptor NK1.1, a structural homologue of CD161 in humans (Makino et al. 1995). Subsequently a homologous NKT cell was identified in humans, expressing the $\alpha\beta$ T cell receptor chains V α 24-V β 11 (Prussin & Foster 1997). Later it was determined that CD161 is not always expressed on NKT cells, hence rendering the name inaccurate. Type 1 NKT cells are more specifically given the name iNKT cells, to distinguish them from other non-invariant type 2 NKT cells.

Although CD1 recognition by T cells was first described in 1989 (Porcelli et al. 1989), it was only in 1995 that CD1d recognition by NK1.1 murine T cells was reported (Bendelac et al. 1995). Later, a glycolipid Ag isolated from marine

sponge was found to activate murine iNKT cells in a CD1d-dependent manner, this glycolipid was α -galactosylceramide or α -GalCer (Kawano et al. 1997). However, it was not until 2000 that a breakthrough was made in the specific phenotypic identification of both human and murine iNKT cells using α -GalCer-loaded CD1d tetramers (Benlagha et al. 2000). iNKT cells are therefore better defined as CD1d restricted T cells that recognize glycolipid Ag (e.g. α -GalCer) via a semi invariant α/β T cell receptor.

1.4.3 Antigen recognition by iNKT cells.

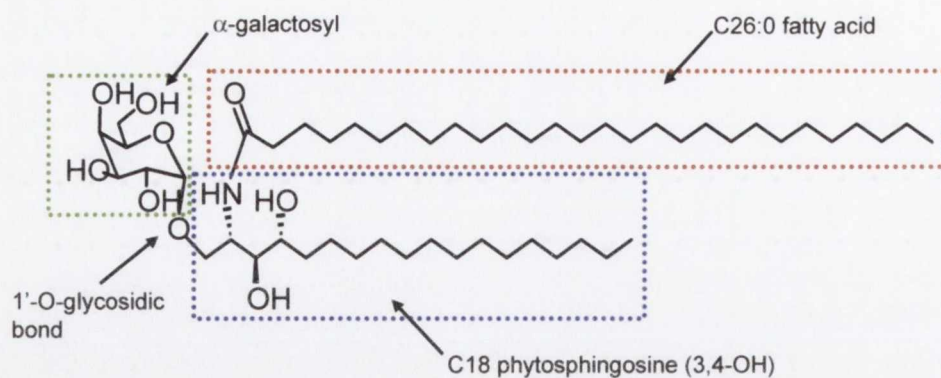
Similar to conventional T cells, iNKT cell development takes place in the thymus, where the iTCR undergoes VDJ gene rearrangement and maturation into CD4⁺, CD8⁺, and CD4⁻CD8⁻ (DN) iNKT cells in humans, or CD4⁺ and DN iNKT cells in mice, through CD1d-dependent interactions with cortical thymocytes (Bendelac 1995). α -GalCer (Fig. 1.3.A), a glycolipid originally isolated from a marine sponge and now synthetically produced as KRN7000, is recognised by all iNKT cells from both mice and humans. α -GalCer has been until recently the most potent iNKT cell Ag known, inducing cytokine production, anti-tumour cytotoxicity, and proliferation of iNKT cells *in vivo* and *in vitro* (Kobayashi et al. 1995). A number of endogenous glycolipids capable of activating iNKT cells have been identified, which may play a role in the development and function of iNKT cells *in vivo*. Among these lipid Ags are isoglobotrihexosylceramide (iGB3) and GD3. iGB3 is a lysosomal glycosphingolipid capable of activating both murine and human iNKT cells (Zhou et al. 2004), while GD3 is a ganglioside expressed on tumours of neuroectodermal origin, which has been shown to stimulate iNKT cells in mice (Wu et al. 2003). More recently, the glycolipid β -GlcCer was identified as a self-antigen that potently activates iNKT cells in both in mice and humans. β -GlcCer is upregulated in response to TLR agonists during infection, resulting in iNKT cells activation.

A number of microbial derived lipid-based Ags are capable of stimulating iNKT cells in humans and/or mice, inducing IFN- γ production or IFN- γ and IL-4 production. Foreign glycolipids generally isolated from bacteria are recognised by iNKT cells in either or both mice and humans, stimulating proliferation and

cytokine production. The first microbial glycolipid identified as an iNKT cell Ag was the mycobacterial phosphatidylinositol mannoside (PIM) isolated from *Mycobacterium bovis*, which stimulated IFN- γ production and cytotoxicity by a fraction of iNKT cells in both mice and humans (Fischer et al. 2004). A diacylglycerol isolated from *Borrelia burgdorferi* was able to stimulate both IFN- γ and IL-4 production by murine and human iNKT cells (Kinjo et al. 2006). α -glucuronosylceramides expressed by *Sphingomonas* and *Ehrlichia* genera were found to be potent stimulators of murine and human iNKT cells, and the synthetic form of the *Sphingomonas* glycosphingolipid GSL-1 potently stimulates iNKT cells in mice (Kinjo et al. 2008). Lipid Ags isolated from *Leishmania donovani* and *Entamoeba histolytica* were also capable of stimulating iNKT cells in mice (Venkataswamy & Porcelli 2012).

A number of α -GalCer analogues have been synthesized, some inducing a polarised cytokine response by iNKT cells. OCH is one such analogue, inducing an IL-4 polarised response by murine but not human iNKT cells (Miyamoto et al. 2001). The addition of an aromatic ring onto α -GalCer analogues (e.g. C13 glycolipid) (Fig. 1.3.B) have been shown to induce a potent Th1-biased response by iNKT cells from both humans and mice (Chang et al. 2007). A multitude of other α -GalCer-based synthetic glycolipids have been described, with the aim of inducing specific iNKT cell responses in both mouse and human (Venkataswamy & Porcelli 2012).

A.



B.

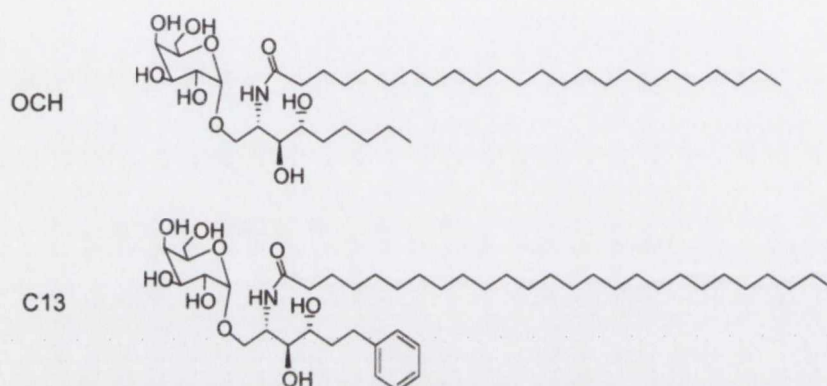


Fig. 1.3. Synthetic glycolipid iNKT cell antigens. A. The iNKT cell prototypic Ag α -GalCer can be modified at the hydrophilic head (green), fatty acid chain (red), or at the sphingosine (blue). B. The glycolipid OCH is produced by truncating the sphingosine part of α -GalCer, while the addition of an aromatic ring as well as a truncation gives rise to the glycolipid C13.

1.4.4 CD1d and glycolipid antigen presentation to iNKT cells.

CD1d is an MHC class I-like molecule composed of a heavy chain ($\alpha 1$ - $\alpha 3$) non-covalently bound to $\beta_2 M$ (Fig. 1.4.A). CD1d is highly conserved between species, and unlike MHC CD1d does not induce allo-reactivity. It is a member of the CD1 family of which in humans there are five isotype molecules divided into two broad groups: group I (CD1a, CD1b, CD1c, and CD1e), and group II (CD1d). All CD1 molecules except CD1e bind and present a variety of lipid Ags to T cell (Koch et al. 2005).

The structure of CD1d allows for the binding of a wide range of glycolipid Ags. Crystallisation of mouse CD1d molecules revealed two connected hydrophobic pockets (A' and F') in the CD1d binding groove, which are occupied by the fatty acid (FA) acyl chain and sphingosine respectively from the lipid Ag (Fig. 1.4.B). While precise hydrogen bonding along the center of the groove helps position the glycolipid into the hydrophobic pockets, the hydrophilic sugar head group of the Ag is exposed for TCR recognition (Zajonc et al. 2005). CD1d is expressed on many professional APC, including dendritic cells (DCs), monocytes, macrophages, and B cells (Exley et al. 2000). CD1d is also found on some activated conventional T cells, as well as epithelial cells, parenchymal cells, and vascular smooth muscle cells. Ag presented in the context of CD1d identified to date include self-lipids, microbial glycolipids, and synthetic glycolipids such as KRN700 or α -GalCer. Like MHC, human CD1d heavy chain assembles with $\beta_2 M$ in the ER, and is loaded with glycolipid Ag. Loaded CD1d molecules are then transported to the cell surface, but subsequently are re-internalised and join sorting endosomes and early endosomes, where they survey the glycolipid content and traffic to the surface. Some human CD1d molecules by physically associating with MHC class II can also enter late endosomes and lysosomal vesicles for antigenic sampling. Ags presented by CD1d on the cell surface are recognised by CD1d-restricted T cells (e.g. iNKT cells), eliciting both tolerogenic and inflammatory responses depending on the type of Ag (Brigl & Brenner 2004; Gumperz 2006).

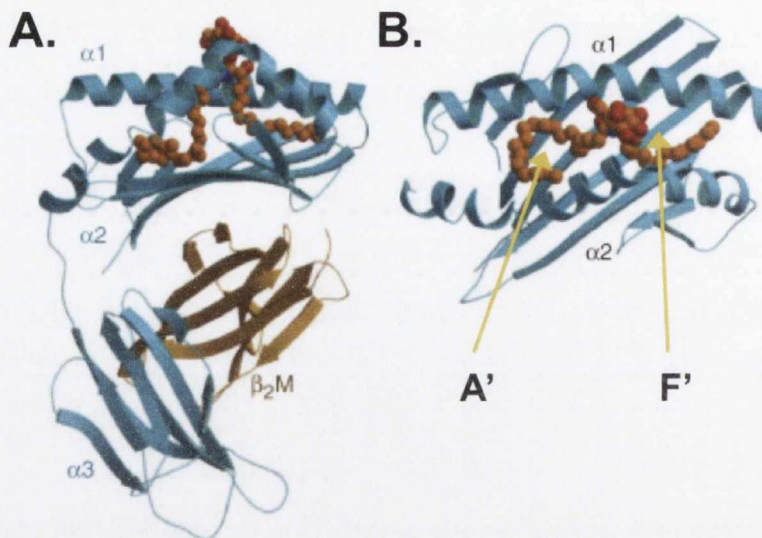


Fig. 1.4. Crystal structure of CD1d presenting α-GalCer. A. Side view of CD1d quaternary structure, showing α-helix and β-pleated sheets peptide configuration of α1, α2, and α3 of the heavy chain (blue), and β₂M (mustard). B. Top view of α1 and α2 part of the heavy chain, making up the lipid binding groove. Two connected hydrophobic pockets A' and F' are shown to house the FA acyl chain and sphingosine of α-GalCer respectively. α-GalCer is demonstrated with a red sugar head group and orange hydrophobic chains. This illustration is a modification from (Koch et al. 2005).

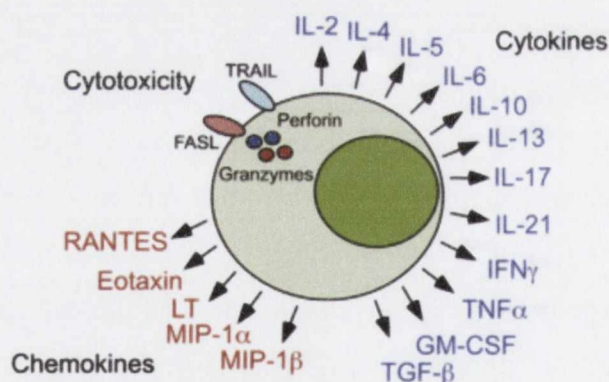


Fig. 1.5. iNKT cell effector functions. iNKT cells can directly kill target cells through the release of granzymes and perforin, or through the engagement of FasL and TRAIL with apoptosis-inducing receptors on target cells. The cytokines (blue) produced by iNKT cells include pro- and anti-inflammatory as well as regulatory cytokines, by which iNKT cells can influence the effector functions of other cells. Through the production of chemokines (red), iNKT cells can recruit other cells of the immune system. This illustration is taken from (Matsuda et al. 2008).

1.4.5 iNKT cell subsets, phenotype, and functions.

iNKT cells phenotypically and functionally encompass elements of both adaptive and innate immune cells. Human iNKT cells express the T cell co-receptors CD4 and CD8, and are subdivided accordingly into CD4⁺, CD8⁺, and double negative (DN) iNKT cells. However, murine iNKT cells do not express CD8. iNKT cells also express CD161, or NK1.1 in mice, which is an innate marker generally associated with NK cells. iNKT cells are known for their innate-like rapid effector responses. They are described as having natural memory, where they acquire a memory or Ag-experienced phenotype from the time of development in the thymus. They constitutively express activation and memory markers such as CD25, CD44, CD69, CD122 (Engel & Kronenberg 2012; Wu & Van Kaer 2013; Nair et al. 2015).

iNKT cell activation can occur by direct and indirect means. Direct activation involves the cognate interaction with CD1d-expressing APC and consequent recognition of a foreign lipid Ag. Indirect activation involves responding to other innate stimuli (e.g. cytokines) in conjunction with self ligand recognition presented by APC (Godfrey & Rossjohn 2011).

Upon TCR recognition of a glycolipid Ag, iNKT cells are capable of rapid cytokine release and cytotoxicity (Fig. 1.5). iNKT cells rapidly release cytokines including TNF- α , IFN- γ , IL-4, IL-10, IL-13 and GM-CSF, both pro-inflammatory and anti-inflammatory. Unlike conventional T cells, iNKT cells can produce both Th1 and Th2 type cytokines simultaneously. Shortly after their discovery in humans, iNKT cells were found to produce large amounts of IL-4 and IFN- γ ; both immunomodulatory cytokines produced by the same cells and still heavily studied as hallmarks of iNKT cell function (Exley et al. 1997; Yoshimoto et al. 1994; Prussin & Foster 1997; Matsuda et al. 2008; Berzins et al. 2011). The distribution of Th1 and Th2 cytokines between iNKT cell subsets varies. CD4⁺ iNKT cells tend to produce more IL-4 when compared to CD8⁺ and DN iNKT cells, and the opposite is true for IFN- γ production. CD8⁺ iNKT cells are more pro-inflammatory, eliciting more anti-tumour effector functions through direct cytotoxicity and transactivation of conventional CTLs and NK cells (Lin, Nieda, Rozenkov, et al. 2006; O'Reilly et al. 2011; Lin, Nieda, Hutton, et al. 2006).

A well-known function of iNKT cells, which has been demonstrated in both mice and humans, is their ability to induce DC maturation (Kitamura et al. 1999; Hogan et al. 2011). They achieve this through CD40L-dependent cognate interactions with DC, inducing their phenotypic maturation and IL-12 production, a cytokine required for iNKT cell activation by DCs. When iNKT cell subsets were further investigated, CD4⁺ iNKT cells were found to be responsible for DC maturation, while DN iNKT cells were more likely to induce DC death (Liu et al. 2008).

1.4.6 iNKT cells promote B cell-mediated immunity.

iNKT cells are constantly referred to as a bridge between innate and adaptive immunity. Due to the plethora of cytokines and chemokines produced by stimulated iNKT cells, the range of cellular targets influenced by iNKT cells are vast. Innate by nature, iNKT cells respond rapidly when activated, trans-activating various early innate responders such as NK cells, DC, macrophages, $\gamma\delta$ T cells, neutrophils, and eosinophils, as well as later adaptive counterparts (e.g. CD4⁺ and CD8⁺ T cells, B cells, and Treg cells (Matsuda et al. 2008; Berzins et al. 2011).

B cells express CD1d and are capable of presenting glycolipid Ag to iNKT cells, which in turn reciprocally provide help to B cells (Dellabona et al. 2014). iNKT cells have been shown extensively in mouse models to provide help for Ab production by B cells. A study carried out in humans showed iNKT cells to provide such help *in vitro* for IgG and IgM production by B cells in a CD1d-dependent and IL-4 and IL-13-dependent manner. iNKT cells were also able to induce the proliferation of both naive and memory B cells. Specifically, CD4⁺ iNKT cells were more efficient at inducing such B cell responses than their CD4⁻ counterparts (Galli et al. 2003). The use of α -GalCer as an adjuvant to enhance Ab responses in mice was found to be comparable to other adjuvants currently used in human vaccines (Galli et al. 2007).

Based on studies in mouse models, the provision of help by iNKT cell to B cells can occur in one of two ways: non-cognate iNKT cell help (TD-1) (Fig. 1.6.a) or cognate iNKT cell help (TD-2) (Fig. 1.6.b). As previously mentioned (section 1.2.1) TD responses by B cells are those requiring cognate interaction with CD4⁺

T_{FH} cells. Due to the ability of iNKT cells to also provide cognate help to B cells, TD responses have been subdivided into TD-1 (conventional T_{FH} cell-dependent) and TD-2 (iNKT_{FH}-dependent). While TD-1 B cell responses provide robust GC formation, SHM, affinity maturation, Ab class switching, long-lived PC, and B cell memory, murine studies have shown that TD-2 responses promote extra-follicular B cell foci, poor GC formation, low affinity maturation, Ab class switching, short-lived PC, and no B cell memory (King et al. 2011; Chang et al. 2011; Tonti et al. 2013).

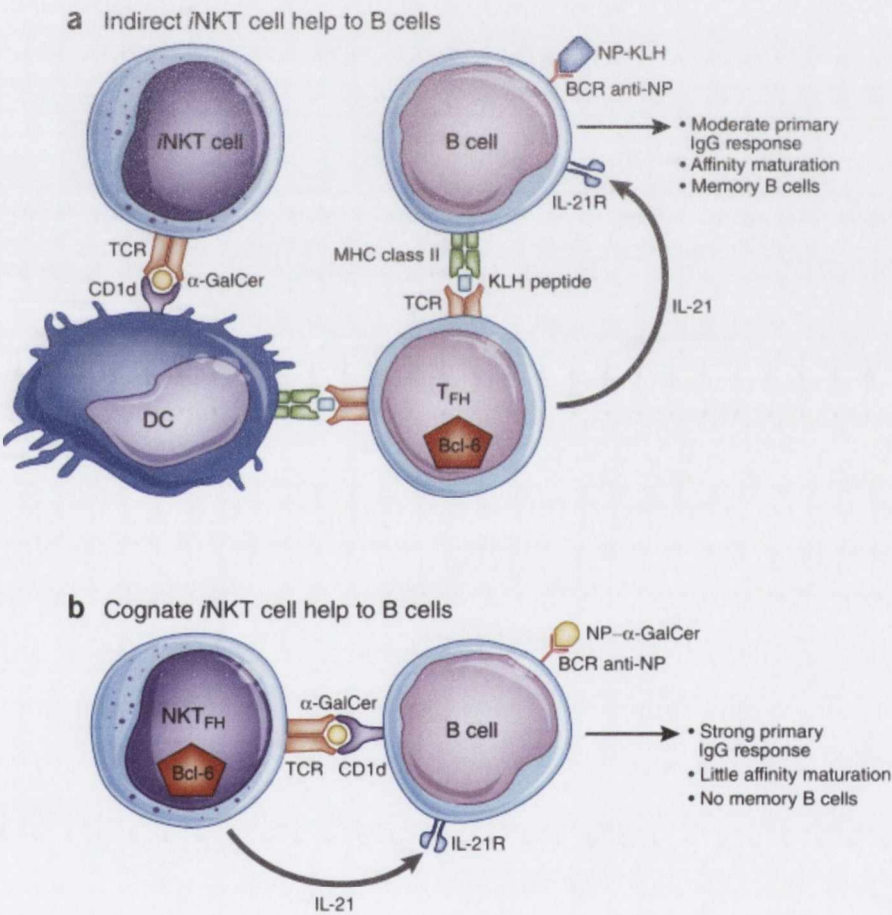


Fig. 1.6. iNKT cell provision of help to B cells. **a.** iNKT cells provide indirect help to B cells by enhancing TD-1 responses. DC present glycolipid Ag (α -GalCer) on CD1d to iNKT cells, which become stimulated and induce the maturation of DC. Matured DC then present peptide Ag on MHC class II to Ag-specific conventional CD4⁺ T cells, which differentiate into T_{FH} cells controlled by the master transcription factor Bcl-6. T_{FH} cells recognise the same Ag presented by B cells and accordingly promote B cell responses through the release of cytokines such as IL-21. **b.** iNKT cells provide help to B cells by making direct contact (cognate interaction) with Ag-specific B cells. Ag-specific B cells present glycolipid Ag (α -GalCer) to iNKT_{FH} cells, stimulate the rapid release of IL-21, which induces rapid Ag-specific B cell responses. This illustration is taken from (Lehuen & Fazilleau 2012). KLH, keyhole limpet haemocyanin.

1.4.7 iNKT cells in mouse models of cancer.

As well as being potent cytokine producers, iNKT cells are major players in cytotoxicity, a process by which cells are targeted for killing (e.g. virally infected or tumour cells). iNKT cells elicit their cytotoxic activity either directly through cognate interaction with the target cell, or via cytokine-mediated trans-activation of other cytotoxic cells such as NK cells and CD8⁺ CTL. Direct cytotoxicity is mediated by the release of cytolytic compounds such as granzyme B and perforin, as well as direct cell contact through FasL and TRAIL (Matsuda et al. 2008).

The role of iNKT cells in immunosurveillance and anti-tumour immunity has been extensively researched since the mid 1990s. Murine iNKT cells are capable of killing a range of tumour cells *in vitro*. The activation of iNKT cells in mice can protect against tumour growth and improve the prognosis for those already bearing tumours. Some of the methods used included IV Injection of α -GalCer and other lipid Ag into tumour-bearing mice, introducing tumours into mice deficient of iNKT cells, and adoptive transfer of iNKT cells into tumour-bearing mice. While CD1d is required for iNKT cells to elicit their anti-tumour activity, they achieve this by indirectly activating other cytotoxic cells (i.e. CTLs and NK cells) (Kobayashi et al. 1995; McEwen-Smith et al. 2015; Bassiri et al. 2014; Tarazona & DelaRosa 2003). Among the iNKT cell subsets in mice, it is the CD4⁻ population that hold the anti-tumour effector functions while CD4⁺ iNKT cells can dampen such responses (Crowe et al. 2005). Vaccines to enhance anti-tumour immunity using tumour Ag combined with TLR agonists such as CpG are under investigation. One study found that α -GalCer loaded CD1d bound to tumour cell mAb significantly enhance anti-tumour immunity when combined with such vaccines, where iNKT cells are thought to act as both an adjuvant and to redirect immune responses to the tumour site (Corgnac et al. 2014).

1.4.8 iNKT cells in human cancers and immunotherapy.

Human iNKT cells are similar to their mouse counterparts in that they are capable of directly killing tumour cells in a CD1d and glycolipid Ag-dependent manner as well as indirect tumour cell killing through IFN- γ and IL-2 mediated activation of NK cells and CTLs. Various studies have demonstrated that iNKT cells are directly

cytotoxic, killing a variety of haematopoietic, pancreatic, colorectal, hepatic, skin, lung, cervical, ovarian, and neurological tumour-derived cell lines when co-culture *in vitro*. For these reasons human iNKT cells are thought to play an important role in tumour immunosurveillance (Kawano et al. 1999; Metelitsa et al. 2001; Bassiri et al. 2014; Vivier et al. 2012).

Clinical studies looking at iNKT cell frequencies in various solid tumour-patient cohorts have shown a reduction in PB iNKT cell frequencies, which have been shown to correlate inversely with tumour load (Berzins et al. 2011). Accordingly, various clinical trials have taken place with the aim of restoring iNKT cell frequencies and anti-tumour function. Following from the successes in mouse models, α -GalCer was given IV to patients with end stage disease (Giaccone et al. 2002). However, no improvement was observed for such patients, unlike the positive outcome observed in mice. Autologous *ex vivo* expanded iNKT cells were given to patients with solid tumours, but with little improvement in prognosis (Motohashi et al. 2006). IV administered α -GalCer-pulsed autologous moDC especially when combined with *ex vivo* expanded iNKT cells showed improvements in iNKT cell and NK cell frequencies as well as IFN- γ production (Kunii et al. 2009; Uchida et al. 2008). However, considering the end stage clinical status of most of the recruited patients, the relatively small proportions of patients showing clinical improvement in these trials is quite significant and very promising. A more detailed account of clinical trials as well as clinical studies on iNKT cells in cancer are outlined in chapter 3.1 and 6.1 respectively.

1.5 Chronic lymphocytic leukaemia.

Haematological malignancies are a heterogeneous group of malignant disorders affecting both haematopoietic tissues including the bone marrow (BM) and blood, and lymphoid tissues including lymph nodes (LNs). Like most malignancies, they manifest as an accumulation of long-lived aberrant cells. Depending on the condition, these cells can range in maturity from haematopoietic precursors to mature cells, and the type of cell can be one of many cell types from either lymphoid or myeloid lineage. In general, the majority of haematological malignancies are classified as either leukaemia or lymphoma, where the former originates in the bone marrow and results in the accumulating cancer cells spilling

into the blood circulation, and the latter originates in peripheral lymphatic tissues (i.e. lymph nodes) resulting in defined tissue masses. Although classified as two separated disorders, there is a significant overlap in the pathology of both conditions. In some cases as the disease progresses, the distinction between the two can be difficult, as leukemia cells can seed lymph nodes and produce lymphomas, and lymphoma cells disseminate into the blood stream and seed the bone marrow to resemble a late leukemia like presentation. The World Health Organisation (WHO) provides a worldwide standardized approach to the diagnosis of haematopoietic and lymphoid malignancies. In accordance with this classification, hematopoietic and lymphoid malignancies include: Mature B cell neoplasms, mature T-cell and NK-cell neoplasms, Hodgkin lymphomas, histiocytic and dendritic cell neoplasms, and post-transplantation lymphoproliferative disorders (PTLDs). Mature B cell neoplasms comprise 39 different conditions including a series of lymphomas, leukaemias, and other diseases such as plasma cell myeloma, amongst others (Jaffe et al. 1999; Byrd et al. 2004; Campo et al. 2011).

1.5.1 Introducing chronic lymphocytic leukaemia (CLL).

CLL, formerly called B-CLL to differentiate it from the T-cell neoplasm T-CLL (now renamed T-cell prolymphocytic leukemia), is the most common leukemia in the western world and is defined as a B cell neoplastic disease affecting the BM, blood, and LNs (Hallek et al. 2008; Caligaris-Cappio & Hamblin 1999). In the UK, based on data collected in 2011, CLL represents 1% of all new cancer cases, and 38% of all new leukemia cases (Cancer Research UK 2011). 90% of CLL cases in Europe and the US are over the age of 50 year with a median age of 65 years, and a male: female ratio of 2: 1 (Jaffe et al. 2001).

1.5.2 Pathology and treatment of CLL.

CLL manifests by the infiltration of BM and blood (i.e. leukemia), with small mature monoclonal B lymphocytes, which are CD5⁺ and CD23⁺. The initial diagnosis requires a B cell count of $> 5 \times 10^9$ B cells / L of blood (Hallek et al. 2008). These cells accumulate in BM affecting normal haematopoiesis, hence resulting in a plethora of secondary conditions including anemia, thrombocytopenia, lymphopenia, lymphadenopathy, hypogammaglobulinaemia, immune cell

dysfunction, and infections. Eventually, the leukemic cells may cause splenomegaly, hepatomegaly, and sometimes infiltrate extra-nodal sites such as skin and breast (Jaffe et al. 2001).

About 25% of CLL patients will have stable disease and do not require treatment. However, those requiring treatment will most likely undergo a chemotherapy regimen potentially in combination with a mAb therapy targeting CLL cells (e.g. anti-CD20). In severe cases or high risk patients, stem cell transplantation may be indicated (Hallek 2005). The current treatments are highly efficacious, but not without the common side effects of chemotherapy.

1.5.3 Role of iNKT cells in CLL.

Clinical research in CLL to date has neglected iNKT cells as a potential target for investigation. Only one study has focused on determining the frequencies and function of iNKT cells from PB of CLL patients (Weinkove et al. 2013). The results from this study, which were published while carrying out our investigation, suggest that iNKT cell frequencies are reduced in CLL patients but are not functionally impaired. Furthermore, a number of studies have reported a reduction in CD1d expression on CLL cells (Weinkove et al. 2013; Zheng et al. 2002; Kotsianidis et al. 2011). However, α -GalCer-pulsed CLL cells were still capable of inducing iNKT cell proliferation *in vitro* (Weinkove et al. 2013).

Further studies are required to confirm the reduction in iNKT cell frequencies in CLL patients, which may in turn be caused by a reduction in CD1d expression on B cells or other APC. Considering the roles that iNKT cells play in anti-tumour immunity, modulating iNKT cell responses *in vivo* might prove a more targeted and more efficient method of treating CLL patients and ultimately improving their prognosis.

1.6 Aims and objectives of the present investigation.

1.6.1 Hypothesis.

iNKT cells have been shown in mice to enhance humoral B cell responses, such investigations in humans are limited. While the majority of studies on iNKT cells in humans do not consider the differential functions of iNKT cell subsets (CD4⁺, CD8⁺, and DN iNKT cell), we hypothesise that iNKT cell subsets may differentially affect B cell functions including Ab production, Ag presentation and subsequent activation of iNKT cells, and differentiation of B cells.

iNKT cells are known for their potent direct cytotoxic killing of tumour cells *in vitro*, and the indirect anti-tumour functions *in vivo*. However, there is a paucity of studies looking at their role and possible potential as therapeutic targets in haematological malignancies and in particular CLL. This study aims to define the frequencies of iNKT cells in CLL patients, comparing them to those recently published by Weinkove et al (2013). As CLL cells are CD1d⁺ B cells, they can be directly targeted by iNKT cells, and we hypothesise that up regulating CD1d expression by CLL cells could potentially enhance iNKT cell cytotoxic killing of CLL cells.

1.6.2 Specific objectives.

- To optimise a method for the *ex vivo* expansion of pure CD4⁺, CD8⁺, and DN human iNKT cell subsets for the ultimate use in cellular immunotherapy.
- To assess the ability of B cells to act as APC to CD4⁺, CD8⁺, and DN iNKT cells with the view of understanding the functional outputs of cognate B cell-iNKT cell interaction.
- To examine the effects of CD4⁺, CD8⁺, and DN iNKT cells on B cell differentiation and function.
- To investigate the potential for iNKT cells as targets for anti-tumour therapy in patients with CLL by examining the responses of CD4⁺, CD8⁺, and DN iNKT cells to glycolipid-pulsed CLL cells. As well as investigating whether modulating CD1d expression on CLL cells affects iNKT cell responses.

Chapter 2 Materials and methods.

2.1 List of materials.

2.1.1 Chemicals and Pharmaceuticals.

A list of the main chemical and pharmaceutical compounds used in this study and their sources are listed in Table 1.

Table 1. Chemicals and pharmaceuticals used in the present investigation.

Product	Company / location	Catalogue #
3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma-Aldrich / Poole, UK	T0440
4 % Paraformaldehyde in PBS	Santa Cruz, CA, USA	281692
Agarose	Sigma-Aldrich / Poole, UK	A9539
All- <i>trans</i> -Retinoic acid (ATRA)	Sigma-Aldrich / Poole, UK	R2625
AM580	Sigma-Aldrich / Poole, UK	A8843
Brefeldin A (BFA)	Sigma-Aldrich / Poole, UK	B7651
Ciprofloxacin	Sigma-Aldrich / Poole, UK	17850
Disodium ethylenediaminetetraacetate dihydrate EDTA	Sigma-Aldrich / Poole, UK	E5134
Disodium phosphate (Na_2HPO_4)	Fisher Scientific / Leicestershire, UK	S/4480/60
Ionomycin calcium salt	Sigma-Aldrich / Poole, UK	I0634
Monopotassium phosphate (KH_2PO_4)	Fisher Scientific / Leicestershire, UK	EC 231-913-4
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich / Poole, UK	P1585
Potassium chloride (KCl)	MERCK / Darmstadt, Germany	104936
Saponin from Quillaja bark	Sigma-Aldrich / Poole, UK	S7900
Sodium azide (NaN_3)	Sigma-Aldrich / Poole, UK	438456
Sodium chloride (NaCl)	Fisher Scientific / Leicestershire, UK	S/3120/65
Sodium hydroxide (NaOH)	Sigma-Aldrich / Poole, UK	S8045
Sulphuric acid (H_2SO_4)	Fisher Scientific / Leicestershire, UK	S/9120/PB17
TRIZMA [®] Hydrochloride	Sigma-Aldrich / Poole, UK	T-3253
Tween [®] 20	Sigma-Aldrich / Poole, UK	P9416

2.1.2 Plastics.

A list of the important plastic consumable materials used in this study are listed in Table 2.

Table 2. Plastics used in the present investigation.

Product	Company / location	Catalogue #
15 ml tubes	Sarstedt / Nümbrecht, Germany	62.554.502
50 ml tubes	Sarstedt / Nümbrecht, Germany	62.547.254
96-well round-bottom TC plates	Corning / MA, UK	3799
Cell strainers, 40uM	Fisher Scientific / Ireland	22363547
Falcon [®] 5ml tubes – sterile	Becton Dickinson / Meylan, France	352063
LS Columns	Miltenyi Biotec / Germany	130-042-401
Minisart [®] Syringe filter (0.2 µm)	Sartorius / Dublin, Ireland	17823
Nalgene [™] Cryo container	Thermo Scientific / NY, USA	5100-0001
Nalgene [™] cryogenic vials (2 ml)	Thermo Scientific / NY, USA	5000-0012
NUNC [®] -Immuno 96-well plate	Thermo Scientific / Roskilde, Denmark	442404
Pre-Separation Filters (30 µm)	Miltenyi Biotec / Bergisch-Gladbach, Germany	130-041-407
Tissue Culture flask - 75 cm ²	Thermo Scientific / Roskilde, Denmark	156499
Universal 30mL Sterilin [®] tube	Thermo Scientific / Upminster, UK	DIS-080-220G

2.1.3 Equipment.

A list of specialized equipment that were used to carry out this study are listed in Table 3.

Table 3. Equipment used in the present investigation.

Product	Company / location	Catalogue #
"The Big Easy" EasySep [™] Magnet	STEMCELL / Canada	18001
AutoMACS Separator	Miltenyi Biotec / Germany	-
BD FACSCanto [™] II	BD Biosciences / US	-
CyAn ADP flow cytometer	Beckman Coulter / UK	-
EasySep [™] Magnet	STEMCELL / Canada	18000
Eppendorf 5331 MasterCycler -	Eppendorf / Hamburg, Germany	-
Gradient Thermal Cycler		
MACS MultiStand	Miltenyi Biotec / Germany	130-042-303
MidiMACS Separator	Miltenyi Biotec / Germany	130-042-302
MoFlo XDP cell sorter	Beckman Coulter / UK	-
Neubauer haemocytometer	Brand / Wertheim, Germany	717805
QuadroMACS Separator	Miltenyi Biotec / Germany	130-090-976
TECAN Sunrise plate reader	TECAN Ltd. / Switzerland	-

2.1.4 Culture media, supplements, and recombinant proteins.

A list of biological compounds used in tissue culture for this study are listed in Table 4.

Table 4. Culture media, supplements, and recombinant proteins used in the present investigation.

Product	Company / location	Catalogue #
2-Mercaptoethanol	Gibco / Paisley, UK	31350-010
Amino acids solution 1X MEM	Gibco / Paisley, UK	11130-036
Bovine Serum Albumin Powder	Fisher Scientific / Leicestershire, UK	12871630
CellTrace™	Thermo Fisher Scientific / Dublin, Ireland	C34557
Cell Proliferation Kit		
Class B CpG oligonucleotide	InvivoGen / Toulouse,	tlrl-2006
(ODN 2006)	France	
DMEM high glucose	Gibco / Paisley, UK	61965-026
GlutaMAX™ supplemented		
DPBS	Gibco / Paisley, UK	14190-094
FUNGIZONE	Gibco / Paisley, UK	15290-026
HEPES	Gibco / Paisley, UK	15630-056
HyClone® / Foetal bovine serum	Thermo Scientific / Northumberland, UK	SV30160.01
L-glutamine	Gibco / Paisley, UK	25030-024
LPS	Sigma-Aldrich / Poole, UK	L5293
Lymphoprep™	Axis-Shield / Oslo, Norway	1114547
Monensin	BioLegend / San Diego, CA, USA	420701
Non-essential amino acids	Gibco / Paisley, UK	11140-035
solution 1X MEM		
Penicillin-Streptomycin solution	Gibco / Paisley, UK	15140-122
Phytohemagglutinin-P (PHA-P)	Sigma-Aldrich / Poole, UK	L1668
Purified NA/LE Mouse Anti-	BD Biosciences / Oxford, UK	555336
Human CD3 (HIT3a)		
rh BAFF / CD257	Immunotools / Friesoythe, Germany	11343436
rh IL-2	Miltenyi Biotec / Bergisch-Gladbach, Germany	130-097-748
rh IL-7	Miltenyi Biotec / Bergisch-Gladbach, Germany	130-095-363
rh sCD40L / CD154	Immunotools / Friesoythe, Germany	11343343
RPMI 1640 GlutaMAX™	Gibco / Paisley, UK	61870-010
supplemented		
Sodium pyruvate	Gibco / Paisley, UK	11360-036
Trypsin-EDTA, phenol red	Gibco / Paisley, UK	25300-054

2.1.5 Antibodies and flow cytometry reagents.

A list of reagents that were used for flow cytometry cell staining in this study are listed in Table 5.

Table 5. Antibodies and flow cytometry reagents used in the present investigation.

Product	Clone	Recom'd (µl / test)	Titrated (µl / test)	Company	Catalogue #
Anti-APC MicroBeads	-	-	-	MB	130-090-855
Anti-iNKT MicroBeads	-	-	-	MB	120-006-764
Anti-PE MicroBeads	-	-	-	MB	130-048-801
APC anti-h BCL-6	BCL-UP	5	5	eBio	17-9880-42
APC anti-h CD14	18D11	-	2	IT	21620146
APC anti-h CD40	HI40a	-	3	IT	21270406
APC anti-h CD69	FN50	-	4	IT	21620696
APC anti-h CD8	HIT8a	5	1	BL	300912
APC anti-h IL-10	JES3-19F1	5	2	BL	506807
APC anti-hTCR Vα24-Jα18	6B11	5	1	BL	342908
APC hCD1d Dextramer [®]	-	10	2	Immudex	XD8002
APC PBS-57-loaded h CD1d Tetramer	-	-	0.05	NIH	-
APC unloaded h CD1d Tetramer	-	-	0.05	NIH	-
APC/Cy7 anti-h CD27	O323	20	5	BL	302816
APC/Cy7 anti-hCD279- (PD1)	EH12.2H7	5	4	BL	329922
APC/Cy7 anti-hCD3	HIT3a	5	1	BL	300318
B Cell Isolation Kit II human	-	-	-	MB	130-091-151
BD [™] CompBeads	-	-	-	BD	552843
CD19 MicroBeads	-	-	-	MB	130-050-301
CD3 MicroBeads	-	-	-	MB	130-050-101
EasySep [™] Human B Cell Enrichment Kit	-	-	-	ST	19054
FITC anti-CD107a	H4A3	5	2	BL	328606
FITC anti-h CD24	SN3	-	5	IT	21270243
FITC anti-h IgM	UCHT1	20	5	BL	314506
FITC anti-hCD4	SK3	5	1	BL	344604
FITC anti-hCD8a	HIT8a	5	1	BL	300906
FITC anti-TCR Vα24	C15	20	4	BC	IM1589

Product	Clone	Recom'd (μ l / test)	Titrated (μ l / test)	Company	Catalogue #
FluoroFix™ Buffer	-	-	-	BL	422101
FVD eFluor® 506	-	-	-	eBio	65-0866-18
Human TruStain FcX™	-	5	-	BL	422302
OneComp eBeads	-	-	-	eBio	01-1111-42
Pacific Blue™ anti-h CD3	HIT3a	5	1	BL	300330
Pacific Blue™ anti-hCD19	HIB19	5	2	BL	302224
PE anti-h CD360 (IL-21R)	2G1-K12	20	10	BL	347806
PE anti-h CD365 (Tim-1)	1D12	5	3	BL	353904
PE anti-h IgD	IA6-2	20	4	BL	348204
PE anti-h IL-21	3A3-N2	5	2	eBio	12-7219-42
PE anti-hCD1d	51.1	5	2	BL	350306
PE anti-hCD31	WM59	5	4	BL	303106
PE anti-hIL-4	8D4-8	5	2	BL	500704
PE anti-TCR V β 11	C21	20	4	BC	IM2290
PE/Cy7 anti-h CD38	HB-7	5	1	BL	356608
PE/Cy7 anti-h CD86	IT2.2	5	3	BL	305422
PE/Cy7 anti-hCD107a- (LAMP-1)	H4A3	5	1	BL	328618
PE/Cy7 anti-hCD3	HIT3a	20	1	BL	300316
PE/Cy7 anti-hCD4	OKT4	5	1	BL	317414
PerCP anti-hCD3	UCHT1	5	1	BL	300428
PerCP.Cy5.5 anti-h HLA-DR	LN3	5	3	eBio	45-9956-42
PerCP.Cy5.5 anti-h IgD	UCHT2	20	3	BL	300622
PerCP.Cy5.5 anti-h IL-17	BL168	5	3	BL	512314
PerCP/Cy5.5 anti-hCD185- (CXCR5)	TG2/CXCR 5	5	2	BL	335001
PerCP/Cy5.5 anti-hCD5	UCHT2	5	3	BL	300620
Rainbow calibration particles-8 peak	-	-	-	BL	422903

BC = Beckman Coulter / High Wycombe, UK

BD = BD Biosciences / Oxford, UK

BL = BioLegend / San Diego, CA, USA

eBio = eBioscience / Hatfield, UK

IT = Immunotools / Friesoythe, Germany

MB = Miltenyi Biotec / Bergisch-Gladbach, Germany

NIH = NIH tetramer core facility / GA, USA

ST = Stemcell Technologies / Grenoble, France

Immudex / Copenhagen, Denmark

2.1.6 Cytokine and immunoglobulin detection kits.

A list of commercial kits for cytokine and immunoglobulin detection that were used in this study are listed in Table 6.

Table 6. Cytokine and immunoglobulin detection kits used in the present investigation.

Product	Company / location	Catalogue #
BD Cytometric Bead Array (CBA) Human immunoglobulin master buffer kit	BD Bioscience / Oxford, UK	558683
Human IFN-gamma DuoSet	R&D Systems / Abingdon, UK	DY285
Human IgA flex set (Bead C9)	BD Bioscience / Oxford, UK	558681
Human IgM flex set (Bead C8)	BD Bioscience / Oxford, UK	558680
Human IL-10 DuoSet	R&D Systems / Abingdon, UK	DY217B
Human IL-4 DuoSet	R&D Systems / Abingdon, UK	DY204
Human total IgG flex set (Bead C6)	BD Bioscience / Oxford, UK	558679

2.1.7 Glycolipids.

A list of iNKT cell glycolipid Ags that were used in this study are listed in Table 7. Structure of glycolipids are shown in Fig. 2.1.

Table 7. Glycolipids used in the present investigation.

Product	Company / location	Catalogue #
Glycosphingolipid-1 (GSL-1)	NIH tetramer core facility / GA, USA	-
KRN700 / α -Galactosylceramide (α -GalCer)	Funakoshi co., ltd. / Tokyo, Japan	KRN7000
OCH	NIH tetramer core facility / GA, USA	-
β -D-glucopyranosyl ceramide (β GlcCer)	Avanti polar lipids / Alabaster, AL, USA	860546
7DW8-5	Funakoshi co., ltd. / Tokyo, Japan	7DW8-5

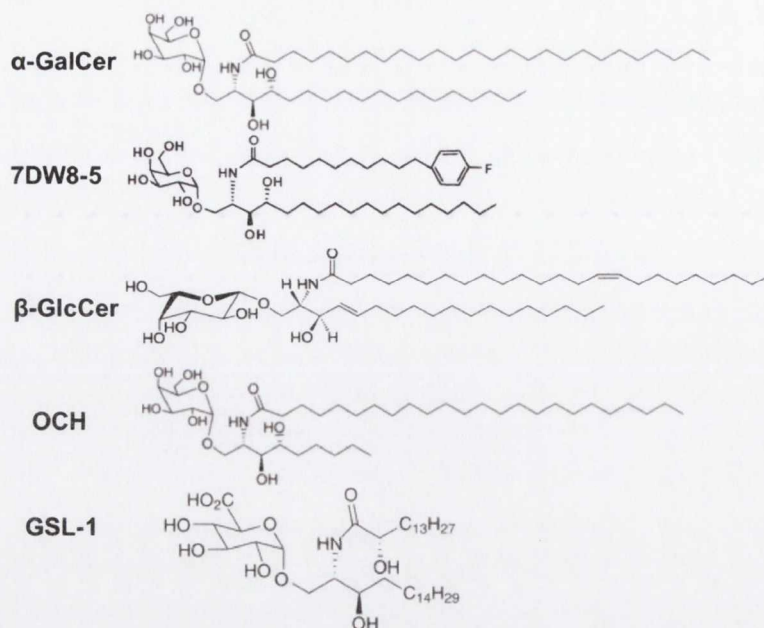


Fig. 2.1. Chemical structures of glycolipid Ags recognized by iNKT cells. α -galactosylceramide (α -GalCer) was isolated from the marine sponge *Agelas mauritanus* and synthesized as KRN7000 (Kobayashi et al. 1995). 7DW8-5 (Li et al. 2010) and OCH (Miyamoto et al. 2001) are synthetic analogues of α -GalCer. β -D-glucopyranosylceramide (β -GlcCer) is endogenously produced by activated cells (Sanderson et al. 2012). GSL-1 is produced by *Sphingomonas* spp. (Long et al. 2007).

2.1.8 PCR reagents.

A list of PCR reagents that were used for mycoplasma detection in this study are listed in Table 8.

Table 8. PCR reagents used in the present investigation.

Product	Company / location	Catalogue #
100bp DNA Ladder	Promega / Madison, USA	G2101
GoTaq [®] GreenMasterMix	Promega / Madison, USA	M7113
Mycoplasma genus specific oligonucleotides: <u>MycoFRW 5'-3'</u> 5'-GGGAGCAAACAGGATTAGATACCCT-3' <u>MycoREV 5'-3'</u> 5'-TGCACCATCTGTCACTCTGTTAACCTC-3'	Eurofins MWG Operon / Ebersberg, Germany	
Nuclease free water	Promega / Madison, USA	P1193

2.2 Buffers and culture media

2.2.1 Phosphate buffered saline (PBS) 10X stock.

A 10X concentrated solution of PBS containing 1.37 M NaCl, 27 mM KCl, 18 mM KH_2PO_4 , and 100 mM Na_2HPO_4 was prepared. Each of the above salts were dissolved in dH_2O and pH adjusted to 6.8. The PBS stock was autoclaved and stored at RT. Before use, the stock was diluted to a 1 X PBS solution and pH adjusted to 7.4. When adjusting the pH NaOH was added to increase the pH and H_2SO_4 was added to decrease the pH.

2.2.2 Foetal calf serum (FCS).

Various batches of FCS obtained from HyClone[®] were tested for optimal iNKT cell expansion and stored at -80°C for further use. Before use the FCS was heat inactivated at 56°C for 30 minutes, and passed through a $0.2\ \mu\text{m}$ sterile nylon filter to remove large protein/fibrin aggregates.

2.2.3 iNKT cell medium (iNKTCM).

T cell medium was prepared using RPMI 1640 supplemented with GlutaMAX[™] as the base medium. RPMI was further supplemented with 10 % FCS (See 2.2.2), 0.02 M HEPES, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Pen Strep), 2 mM L-glutamine, 1X minimum essential medium (MEM) essential amino acids solution, 1X MEM non-essential amino acids solution, 1 mM sodium pyruvate, and $0.1\ \mu\text{M}$ 2-mercaptoethanol. T cell medium was stored at 4°C and warmed to 37°C before use.

2.2.4 Complete RPMI (cRPMI).

cRPMI was prepared using RPMI 1640 supplemented with GlutaMAX[™] as the base medium. RPMI was further supplemented with 10 % FCS (See 2.2.2), 0.02 M HEPES, and 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Pen Strep). cRPMI medium was stored at 4°C and warmed to 37°C before use.

2.2.5 Complete DMEM (cDMEM).

cDMEM was prepared using DMEM supplemented with high glucose GlutaMAX™ as the base medium. DMEM was further supplemented with 10 % FCS (See 2.2.2), HEPES 0.02 M, and 100 units/ml penicillin and 100 µg/ml streptomycin (Pen Strep). cDMEM was stored at 4 °C and warmed to 37°C before use.

2.2.6 Magnetic cell sorting (MCS) buffer.

MCS buffer was prepared using PBS supplemented with 2 mM EDTA and 0.5 % BSA, at pH 7.2. MCS buffer was prepared fresh and kept at 4°C during use.

2.2.7 Ethidium bromide / acridine orange (EB/AO) viability stain.

A stock solution of ethidium bromine (3 mg/ml) and acridine orange (5 mg/ml) in PBS (see 2.2.1) was prepared and stored at 4°C in the dark. A working solution of EB/AO was prepared by diluting the stock 1:100 in PBS. The working solution was stored at RT in the dark.

2.2.8 Tris-acetate-EDTA (TAE) buffer 1X.

A 1X TAE buffer containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA was prepared by dissolving each of the 3 components in dH₂O, and storing at RT.

2.2.9 ELISA wash buffer.

A buffer containing 0.05% Tween® 20 in PBS was prepared, and pH adjusted to 7.2-7.4

2.2.10 ELISA blocking buffer.

A buffer containing 1% BSA in PBS was prepared by dissolving powdered BSA in PBS. The pH was adjusted to pH 7.2-7.4, then filtered using a 0.2 µm filter.

2.2.11 ELISA reagent diluent.

Tris-buffered saline containing 20 mM Trizma base and 150 mM NaCl was prepared by dissolving both compounds in dH₂O. A solution of 0.1% BSA and 0.05% Tween 20 in tris-buffered saline was prepared, pH adjusted to 7.2-7.4, then filtered using a 0.2 µm filter. This buffer was used as a reagent diluent for IFN-gamma and IL-4 ELISA. For IL-10 detection, blocking buffer (section 2.2.10) was used as reagent diluent.

2.3 General tissue culture procedures.

Tissue culture procedures were carried out under strict sterile conditions, in a laminar flow cabinet. All materials were deemed sterile before use; any materials that were not sterile either were autoclaved or filtered (i.e. in the case of solutions) using a 0.2 µm filter before use. 70% ethanol in dH₂O was used to wipe down all surfaces and materials before and after tissue culture procedures. Pipettes were visually inspected before use, for any macroscopic contamination. All cell cultures were incubated in a 5% CO₂ incubator set to 37°C. Solid biological waste was disposed of in yellow incinerable bins, and liquid waste was first decontaminated using an appropriate concentration of Vircon disinfectant, then poured down the sink/sewer. Solid chemical waste (e.g. materials in contact with ethidium bromide) was disposed of in blue bins. Monthly laboratory clean up procedures included cleaning the water bath, centrifuges, bench tops, and incubators if required. Any cultures suspected of containing bacterial growth were disposed of appropriately. Any valuable cell lines tested positive for mycoplasma contamination were appropriately treated using ciprofloxacin (see 2.9.3).

2.4 Maintenance of HeLa cell lines.

HeLa cells transfected with the CD1d gene (CD1d-HeLa) as well as mock transfected (Mock-HeLa) HeLa cells were donated by Prof. Steven Porcelli (Albert Einstein College of Medicine, NY, USA) (Spada et al. 1998). HeLa cells were maintained in 75 cm² flasks in cDMEM medium (see 1.2.5). HeLa cells above 80 % confluency were split and re-seeded in fresh medium. To split HeLa cells, the medium was removed, and cells were washed with 10 ml of PBS. 2 ml of trypsin was then added to the flask and cells were incubated until they detached from the flask wall (up to 2 mins). 20 ml of cDMEM was added to neutralize the trypsin and cells were immediately washed (see 2.7). 10 % of the original cells were then seeded in 25 ml of cDMEM in a 75 cm² flask. Cells were incubated in 5 % CO₂ at 37°C.

2.5 Preparation of peripheral blood mononuclear cells (PBMCs).

PBMCs were isolated from fresh blood or buffy coats from blood pack donations using density gradient centrifugation. Fresh blood samples were obtained in EDTA anticoagulant from healthy consenting-adult donors and buffy coat packs containing Citrate Phosphate Dextrose (CPD) were kindly donated by the IBTS. Each buffy coat containing an average of 10⁹ PBMCs. The blood or buffy coats were diluted 1:1 in PBS then gently layered over Lymphoprep™ (Fig. 2.2). The layered blood was then centrifuged at 400 x g for 25 minutes with brakes tuned off. The PBMC layer just above the lymphoprep™ was gently aspirated using a pastette and washed twice by re-suspending in PBS and centrifuging at 400 x g for 10 minutes. PBMCs were then re-suspended in cRPMI (see 2.2.4) and counted (see 2.6).

2.6 Determination of cell counts.

Cells were counted using an improved Neubauer haemocytometer. Cell suspensions were diluted in a working solution of EB/AO (see 2.2.7) and 10 µl of

the dilution was loaded onto the haemocytometer. Using a UV microscope the number of green viable or orange dead cells were manually counted (Fig. 2.3).

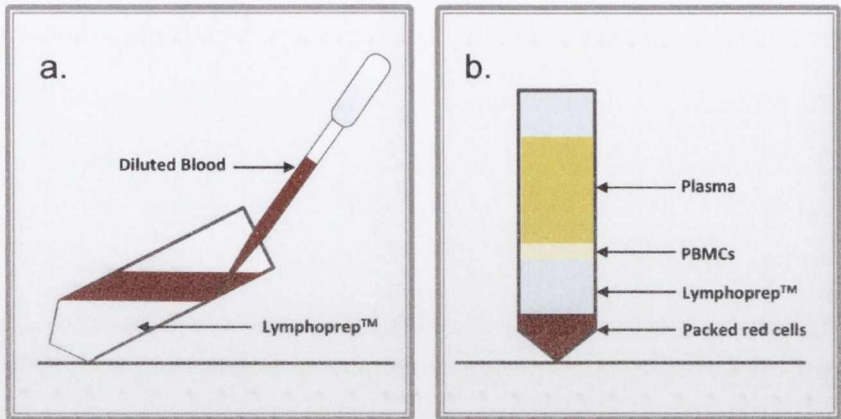


Fig. 2.2. Preparing PBMCs from peripheral blood. (a) Blood was diluted in PBS and gently layered at an angle over Lymphoprep™. (b) After centrifugation the red blood cells and granulocytes form a pellet in the bottom of the tube and the PBMCs form a layer above the Lymphoprep™.

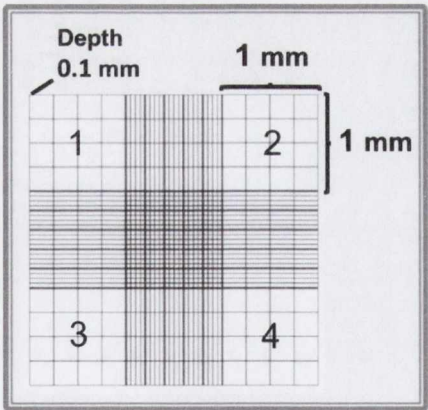


Fig. 2.3. Neubauer haemocytometer counting grid. The grid contains 9 [1 mm x 1 mm] squares each of which is divided into 16 smaller squares. Once the chamber of the haemocytometer is loaded with the cell suspension, the volume above each of the 9 squares is 0.1 mm³. The number of cells in squares 1, 2, 3, and 4 were counted, averaged, multiplied by the dilution factor, and then finally multiplied by 10,000 to obtain the number of cells per ml of original cell suspension.

2.7 Performing cell washes.

Various tissue culture preparation steps as well as post-culture analytical assays require cells to undergo several washing steps. Washing involved the centrifugation of cells in a buffer such as PBS for a specific time and centrifugal force, both determined by the volume of sample, and assay requirements (Table 9). Any assay specific centrifugal requirements are specified in the respective method.

Table 9. Centrifugal force and time used in cell washes for different cell suspension volumes.

Container type	Wash volume	Centrifugal force	Time (minutes)
96-well plate	250 µl	500 x g	3
Micro-tubes	1 ml	500 x g	3
FACS tubes	3 ml	500 x g	5
All other tubes: ¹	15 – 50 ml	400 x g	10
	15 – 50 ml	800 x g	5

¹ MACS® cell separation protocols require more gentle washes at 300 x g for 10 minutes.

2.8 Cell storage: freezing and thawing cells.

iNKT cell lines as well as HeLa cell lines were stored long-term in -80°C or at the preferable -196°C (i.e. liquid nitrogen storage). First cells were slowly brought down in temperature in the presence of DMSO, which reduces the formation of ice crystals that cause cell lysis. DMSO is toxic to cells at temperatures above 4°C, hence all cell processing in the presence of DMSO was carried out on ice. Cells were re-suspended in FCS at a concentration of 2 – 100 x 10⁶ /ml on ice. Ice-cold FCS with 20% DMSO was then added at a ratio of 1:1 to the cells drop-wise. The resulting FCS with 10% DMSO mixture was then aliquoted into pre-labeled cryogenic vials at 1ml / vial. Vials were immediately transferred to a cryo container, making sure the container has sufficient amount of isopropanol.

To thaw out cells for tissue culture, each vial was rapidly heated in a water bath with regular mixing for 1 min. Cells were then immediately added drop-wise to

pre-warmed (37°C) medium; each vial was thawed in 15 ml of medium. Cells were immediately washed (see 2.7) and re-suspended in appropriate cell culture medium. Thawed cells were rested in cytokine-free medium for 24h prior to use in experiments.

2.9 Testing for mycoplasma.

Mycoplasma are the smallest parasitic prokaryotes that lack a cell wall: they include many species. Their size and deformability allows them to pass through standard 0.2 µm filters, along with their ubiquitous environmental presence make them readily able to contaminate tissue cultures. 5 – 35% of tissue cultures are estimated to be contaminated with mycoplasma. Mycoplasma contamination, unlike other bacterial contamination is not obvious and may not affect cell growth, making regular monitoring important. However, contamination can alter almost all aspects of cellular physiology and function, which may lead to erroneous experimental results. Long-term cultures of iNKT cell lines and HeLa cells were tested for the presence of mycoplasma contamination by PCR.

2.9.1 PCR method for mycoplasma detection.

A PCR method for mycoplasma detection was optimised in accordance with previous publications (Young et al. 2010). All aspects of this protocol were carried out in sterile environment and using PCR grade consumables. 100 µl of culture medium was obtained from each cell line; cell lines were not fed for 24h or longer prior to sampling. Where samples were not immediately tested, they were stored at 4°C for up to one month. Samples were centrifuged at 1800 rpm and 1 µl was transferred to a PCR reaction tube. 1 µl of nuclease free water for a negative control, and 1 µl of a known contaminated sample for a positive control, were added to corresponding reaction tubes, always making sure to work with the negative control first and positive last, to avoid cross contamination. A master mix was prepared to include (per reaction): 25 µl GoTaq, 1 µl FWD primer (10 µM), 1 µl REV primer (10 µM) (see table 8), and 22 µl of RNase free water. 49 µl of the master mix was added to each reaction tube. A PCR thermocycler was set deliver 95°C for 5 mins, followed by 40 cycles of: (94°C for 30 secs, 55°C for 30 secs, and 72°C for 1 min), and a final 72 °C for 10 mins. The thermocycler was set to hold

the samples at 4°C for the remainder time. Samples were then subjected to electrophoresis through 2 % agarose gels electrophoresis for DNA band visualization (See 2.9.2). The presence of a 270 bp band is indicative of Mycoplasma contamination. Faint bands between 300 – 500 bp may occur; these were identified as PCR products resulting from primer cross reactivity with *Staphylococcus* spp (Fig. 2.4).

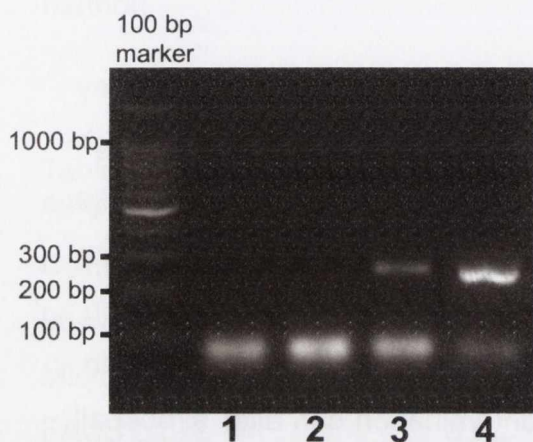


Fig. 2.4. Mycoplasma detection using PCR. Culture medium from CD1d-HeLa (3) and mock-HeLa (2) were tested with a mycoplasma genus specific PCR, along with a negative control (1) and known mycoplasma positive control (4). PCR products were run on a 2% agarose gel, showing the presence of a 270 bp mycoplasma PCR product in CD1d-HeLa sample.

2.9.2 2% Agarose gel electrophoresis.

Agarose to a final concentration of 2 % was thoroughly dissolved in TAE buffer (see 2.2.8) by heating in a microwave. The agarose solution was allowed to cool to 60°C before adding EB to a final concentration of 0.5 µg/ml; necessary precautions were taken when handling EB as it is carcinogenic. The agarose was mixed and immediately poured into a pre-assembled gel casting tray and left to cool. Samples were loaded, and the gel was run using TAE buffer as previously described (Sanderson et al. 2014; Lee et al. 2012).

2.9.3 Treatment of cell lines for mycoplasma contamination.

Cell lines that were identified to be contaminated with mycoplasma were then treated with 10 µg/ml of ciprofloxacin as previously described (Fleckenstein & Drexler 1996). Ciprofloxacin was added to culture medium for a minimum duration of 15 days and until the cell line was mycoplasma free, as determined by PCR (see 2.9.1). iNKT cell lines that were mycoplasma positive were discarded, while HeLa cell lines were treated. A full laboratory de-contamination was carried out post infection.

2.10 Flow cytometric cell analysis.

Flow cytometry was used to analyse changes in cell surface and intracellular protein expression.

2.10.1 Principles of flow cytometry.

Cells were stained with various fluorescent-labeled monoclonal antibodies (Abs) specific for intracellular and/or surface protein antigens. Labeled cells were then acquired by a flow cytometer, where each cell passes past a set of lasers (Fig. 2.5 a) that excite the fluorochromes causing each fluorochrome to emit light with a specific wavelength range. A cell with multiple fluorochromes attached will emit multiple bands of light, which can slightly overlap in wavelength depending on the fluorochromes used (Fig. 2.6). The bands of light emitted from each cell pass through a range of filters and mirrors, which separate the emitted light into single bands with a narrow wavelength range each specific to the fluorochrome excited. Each narrow band is then amplified by passing through a photomultiplier tube (PMT) and finally detected and converted to an electrical pulse that can be read and displayed as an event on a computer (Fig. 2.5 a). Information about the size and granularity of each cell is obtained from forward scatter (FSC) and side scatter (SSC) respectively. FSC and SSC allow the identification of different cell types based on size and complexity without the need for any fluorescent labeling (Fig. 2.5 b).

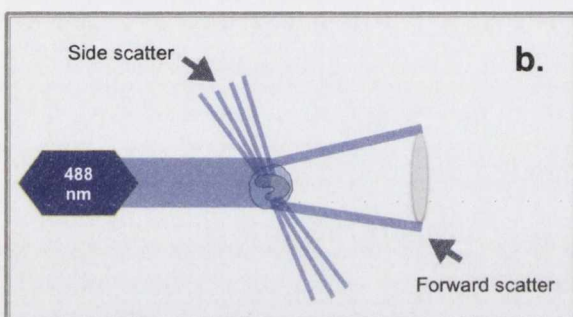
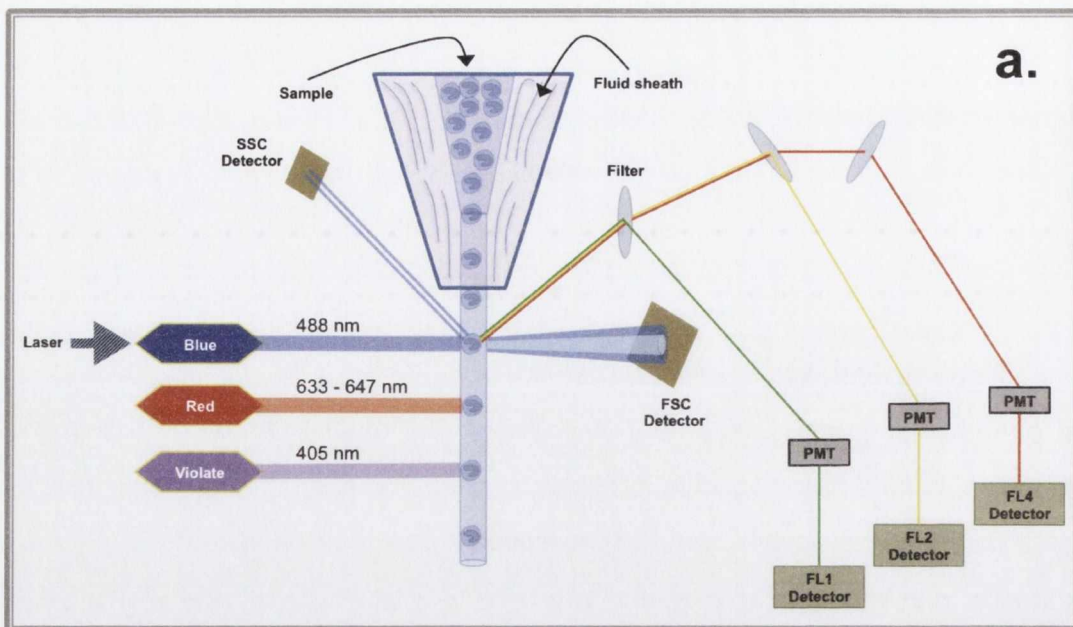


Fig. 2.5. Schematic representation of the components of a flow cytometer. (a)

Three main components make up a flow cytometer: fluidics, optics, and electronics. The sample containing stained cells is taken up by the fluidics system and a buffered fluid sheath surrounds the sample, continuously narrowing the sample stream in a process called hydrodynamic focusing. In this image the sample stream is passed

downwards towards an interrogation point, where each cell passes through a prism-focused laser beam from three different lasers (i.e. Blue, red, and violet laser). Each laser is capable of exciting a set of fluorochromes bound to the cell, which then emit light of higher wavelengths and are captured by a series of filters. Each filter allows specified wavelengths to pass through to the next filter, and reflects the remaining wavelength band towards a photomultiplier (PMT) that amplifies the signal and passes it to a detector that converts the signal to an electric pulse. Each fluorochrome emits a specific wavelength and therefore a specific filter and detector is needed for each fluorochrome. In this image the coloured lines depict the emission spectra of three fluorochromes: green FITC (fluorescein isothiocyanate) that is always detected in the FL1 detector, yellow PE (phycoerythrin) always detected in FL2, and red PerCP (peridinin chlorophyll protein) always detected in FL4. (b) Information about the size and granularity of a cell is obtained from forward scatter (FSC) and side scatter (SSC) respectively. As a cell passes through the blue laser path light waves that make contact with the cell membrane are diffracted and collected by a detector as FSC data. A certain amount of light is absorbed by each cell, gets deflected by subcellular components (e.g. nucleus, organelles, and granules), and is then collected by a detector as SSC data.

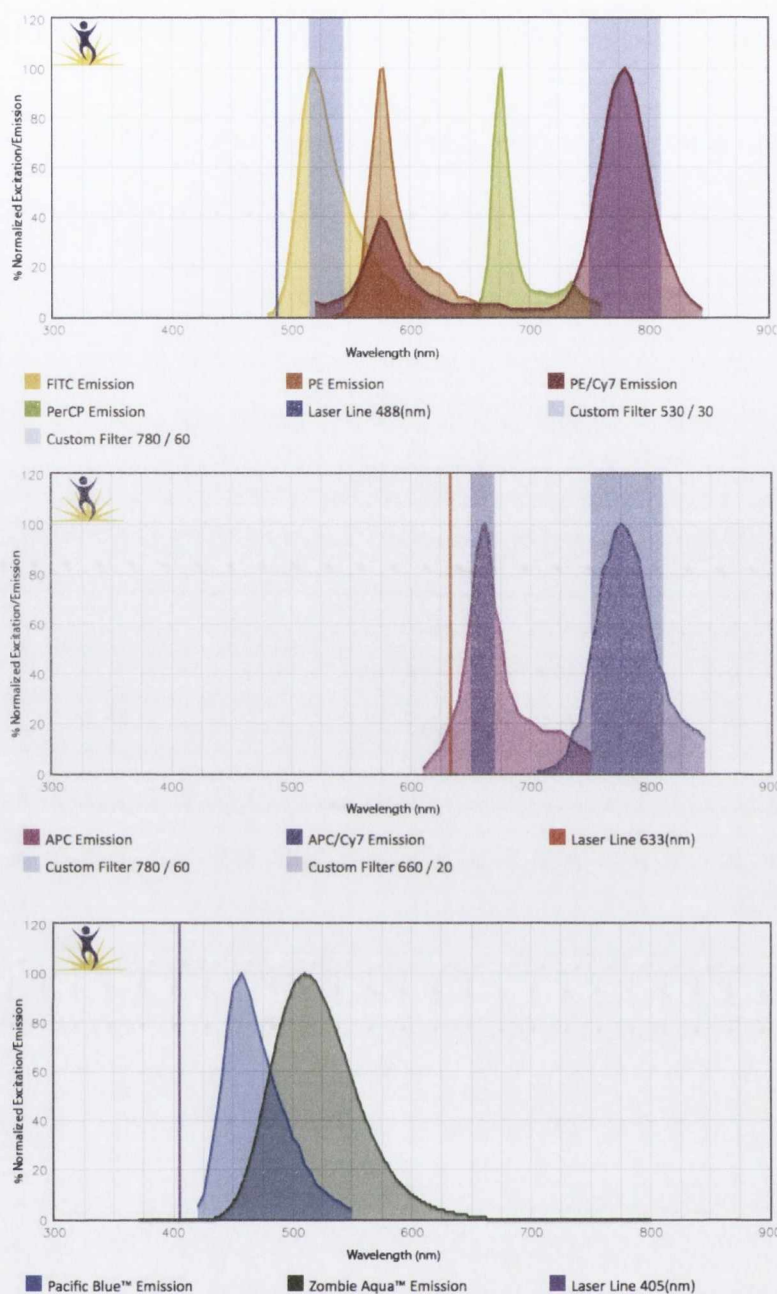


Fig. 2.6. Emission spectral overlap. The wavelength of light emitted by different fluorochromes can overlap. (a) The blue laser emits light at 488 nm (blue line) and excites fluorochromes such as FITC, PE, PerCP, and PE/Cy7, which then emit light of higher wavelengths (yellow, orange, green, and red peaks). (b) The red laser emits light at 633 nm (red line) and excites fluorochromes such as APC and APC/Cy7, which then emit light of higher wavelengths (Pink and purple peaks). (c) The violet laser emits light at 405 nm (purple line) and excites fluorochromes such as Pacific Blue and Aqua, which then emit light of higher wavelengths (blue, and green peaks). Emission spectra from various fluorochromes can overlap (i.e. spectral overlap) and therefore to minimize interference between fluorochromes a tight band (blue shaded band) of each emission spectrum is filtered to the respective detector. The images were created using BioLegend Fluorescence Spectra Analyzer <http://www.biolegend.com/spectraanalyzer>.

Any wavelength overlap between different fluorochromes can cause interferences between the different antigenic readouts and hence spectral overlap was corrected by the use of controls. Controls used included: unstained control, single stained controls, and fluorescence minus one controls (FMOs).

The unstained control is a sample of the target cells, which are left unstained. The unstained control was the first sample to be acquired at the start of every experiment. PMT voltages were adjusted so that the unstained cells were completely visible and positioned at the lowest fluorescence intensity for each fluorochrome. Cells can have a certain amount of auto-fluorescence (i.e. background fluorescence), which differ between cell types and therefore the unstained control was always prepared from the same cells to be analysed. The main controls that allow for compensating spectral overlap is the single stain controls, which were either beads (i.e. synthetic particles that bind Abs) or cells stained with only one fluorochrome. A single stain control for each fluorochrome was prepared, acquired, and recorded on the flow cytometer and digital adjustments were performed to compensate for any spectral overlap. FMOs are controls that take into account antigenic specificity. FMOs were prepared for antigens (Ags) that display a spectrum of positivity on the target cells or for Ags that have a very low target cell frequency of expression (e.g. CD1d on B cells and V α 24-J α 18 on PB T cells respectively). An FMO for Ag [x] was prepared by staining the sample for all other Ags of interest except for Ag [x]. Once acquired on the flow cytometer, the FMO was then used to set a gate beyond which positivity to Ag [x] is determined for every other sample stained for Ag [x] (Fig. 2.7).

Once compensation was carried out to eliminate spectral overlap and a sample was acquired by the flow cytometer, the target cell population for analysis was then selected (i.e. gated) using a general gating strategy (Fig. 2.8). This strategy allows for increased accuracy through the exclusion of elements that can contribute to false results. Such elements include: Cell debris, doublets, and dead cells.

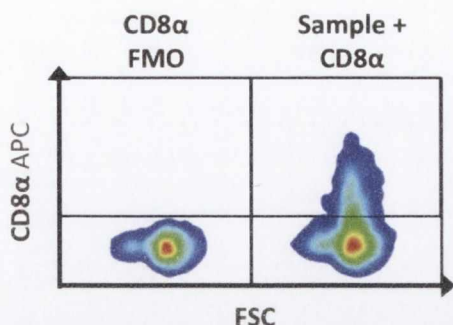


Fig. 2.7. Fluorescence minus one (FMO). iNKT cells were stained for many target Ags including CD8α. The Pseudocolour smooth flow plots show iNKT cells stained with an Ab against CD8α (right), and unstained for CD8α (left) (i.e. CD8α FMO). The black line denotes the threshold set using the FMO, beyond which cells are considered positive for CD8α.

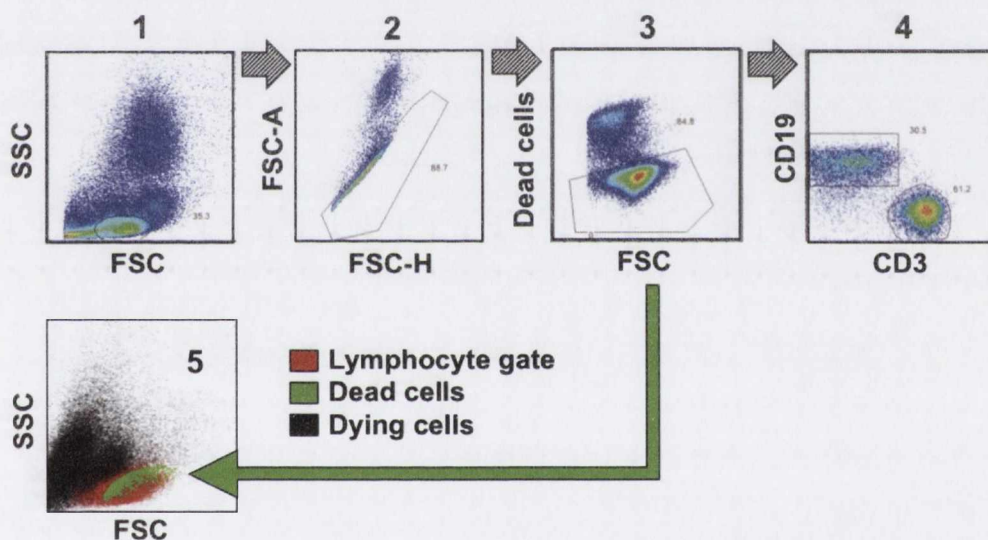


Fig. 2.8. Gating strategy for flow cytometry analysis. Target cell populations were gated based on a general strategy as follows: 1) gate on a specific FCS/SSC population (e.g. lymphocytes), 2) gate on singlets (i.e. exclude doublets that can interfere with mean fluorescence intensity or MFI), 3) gate on live cells (i.e. exclude cells that stain with a dead cell dye as dead cells can bind non-specifically to Abs), 4) gate on the target population based on Ab staining (e.g. CD3⁺ cells). The importance of excluding dead cells is shown in (5) as dead cells (green) can have the same FSC/SSC characteristics as live cells.

In cases where the outcome was not frequency but fluorescence intensity (i.e. FI) in the form of mean FI (MFI) or geometric mean FI (GeoMFI), a method to standardize FI between flow cytometric runs was devised. This method depended on the flow cytometer used. For the CyAn™ ADP, a sample of rainbow beads that contain standard FI values for each fluorochrome were run during cytometer setup at the beginning of each run. Using the beads, PMT voltages were adjusted so that fluorochrome FI values in each run matched the previous runs. When using the BD FACSCanto™ II, a less time consuming process was carried out involving: 1) Running a sample of cytometer setup and tracking (CST) beads before cytometer set up, 2) Setting up an experiment template, and 3) Saving applications settings after all PMT voltages were set up using unstained and SS samples. Every subsequent run involved running CST beads then applying application setting from the previous run to the template, allowing the instrument to automatically make adjustments to standardize FI for the selected fluorochromes. The software used to acquire flow data on the CyAn™ ADP and BD FACSCanto™ II were Summit™ v.4.3 and BD FACSDiva™ respectively. Analysis of all data obtained was carried out using FlowJo 9.5.2 software.

2.10.2 Cells surface staining for flow cytometry.

Changes in cell surface expression of various antigens were measured by flow cytometry. Target populations for analysis included: iNKT cells, B cells, and monocytes. All steps were carried out at room temperature (RT) and protected from light. The first staining step was to stain for viability using Fixable Viability Dye eFluor® 506, referred to in this study as dead cell stain (DCS). This step required the absence of extracellular proteins such as BSA that could quench the DCS. A working solution of DCS (wDCS) was prepared by adding 1 µl of stock to 1 ml of PBS. Cells were washed with PBS then stained with wDCS by adding 1 ml of the stain to 4×10^6 cell and incubating for 10 mins. For B cell staining, a subsequent step to block non-specific binding of Abs to Fc receptors expressed by B cells was carried out using Human TruStain FcX™, incubated with cells for 15 mins. Cells were then re-suspended in PBA (PBS with 1 % bovine serum albumin and 0.02% sodium azide) to a final volume of 50 – 100 µl containing a maximum cell concentration of $(1 \times 10^6 - 4 \times 10^6)$ cells / ml. A predetermined amount (see

Table 10) of each staining mAb was then added to the cells and left to incubate for 15 mins at RT or 30 mins on ice. Cells were washed 1 – 2 times with PBA, then fixed with 1 - 2% paraformaldehyde (PFA) or FluoroFix™ Buffer, washed again, then stored at 4°C in PBA for a maximum of 2 days before analyzing on a flow cytometer. Each experimental output was different and particular Ab panels (see Table 10) were used to achieve these outputs.

2.10.3 Controls for flow cytometry staining.

For each experiment that was analysed by flow cytometry, appropriate flow cytometric controls were incorporated into both cell culture stages, and cell surface staining stages. As explained in (section 2.10.1), the controls included were an unstained sample, single stained samples, and FMO controls. The unstained control was always prepared to include all cell types to be analysed, and was subjected to all culture procedures applied to test samples. The unstained was then excluded from any flow cytometry staining, but was washed along side all test samples; culture medium contains pH indicators that can influence background fluorescence, hence washing allows for the reduction of this background. FMO controls were prepared like the unstained, to contain all cell types, as well the inclusion of any positive stimulations applied to other test samples; stimulating the cells allowed for the maximum expression of surface Ags and other intracellular proteins which were targets for analysis. When staining the FMO controls, all staining steps were applied except for the exclusion of one Ab (e.g. anti-CD24 was excluded from the CD24-FMO). Single stain controls can be prepared using cells, however, in this study, all single stains were prepared using synthetic beads purchased from eBioscience (OneComp eBeads). These beads were a mix of coated coated beads capable of binding to Ig/Abs of mouse, rat and hamster origin, and uncoated beads. To prepare each of the controls, a drop of beads were stained with a single flurochrome labeled Ab from a panel of Abs specifically used in the experiment (see Table 10). The result is one single stain for each of the fluorochromes used. The control samples were always run through the flow cytometer before running the test samples (see .10.1).

Table 10. Antibody panels for cell surface staining. A number of Ab panels were used to stain *ex vivo* and *in vitro* cultured iNKT cells and B cells for flow cytometric analysis. Each panel represents the combination of Abs used to stain a given sample for flow cytometry. PB = Pacific Blue™.

<u>Breg phenotype panel</u>	<u>Breg cytokine panel 1</u>	<u>Breg cytokine panel 2</u>
CD24 (FITC)	CD24 (FITC)	IFN-γ (FITC)
CD1d (PE)	CD1d (PE)	Tim-1 (PE)
CD5 (PerCP.Cy5.5)	CD5 (PerCP.Cy5.5)	IgD (PerCP.Cy5.5)
CD38 (PE/Cy7)	CD3 (PE/Cy7)	CD3 (PE/Cy7)
CD19 (PB)	CD19 (PB)	CD19 (PB)
DCS	DCS	DCS
CD40 (APC)	IL-10 (APC)	IL-10 (APC)
CD3 (APC/Cy7)		CD27 (APC/Cy7)
 <u>B cell subset panel 1</u>	 <u>B cell subset panel 2</u>	 <u>B cell activation panel</u>
IgM (FITC)	IgM (FITC)	IL-21R (PE)
IgD (PE)	IL-21R (PE)	HLA-DR (PerCP.Cy5.5)
CD3 (PerCP)	IgD (PerCP.Cy5.5)	CD86 (PE/Cy7)
CD38 (PE/Cy7)	CD3 (PE/Cy7)	CD19 (PB)
CD19 (PB)	CD19 (PB)	DCS
DCS	DCS	CD69 (APC)
CD40 (APC)	CD40 (APC)	CD3 (APC/Cy7)
CD27 (APC/Cy7)	CD27 (APC/Cy7)	
 <u>CD107a panel</u>	 <u>Tfh panel</u>	 <u>Intacellular cytokine panel</u>
CD4 (FITC)	CD21 (PE)	IFN-γ (FITC)
CD1d (PE)	CXCR5 (PerCP.Cy5.5)	IL-4 (PE)
CD107a (PE/Cy7)	CD3 (PE/Cy7)	IL-17a (PerCP.Cy5.5)
CD19 (PB)	CD19 (PB)	CD3 (PE/Cy7)
DCS	DCS	CD19 (PB)
CD8 (APC)	PD-1 (APC/Cy7)	DCS
CD3 (APC/Cy7)		IL-10 (APC)
 <u>CLL panel 1 (iNKT)</u>	 <u>CLL panel 2 (CD1d)</u>	
CD8 (FITC)	CD1d (PE)	
CD31 (PE)	CD5 (PerCP.Cy5.5)	
CD3 (PerCP)	CD19 (PB)	
CD4 (PE/Cy7)	DCS	
CD19 (PB)	CD14 (APC)	
DCS	CD3 (APC/Cy7)	
6B11 (APC)		

2.11 Magnetic bead cell separation.

Mixed cell populations were separated using a technique that relies on the selective binding of mAbs to a target cell (e.g. anti-CD19 to B cells); mAbs are were either directly or indirectly bound to magnetic beads. In the presence of the target cell population, the mAb-bound beads specifically bind the cells and are then held in a magnetic field while other cell populations are washed away. Where a single target cell population was magnetically labeled, the technique used was positive selection; if a single target cell population was the only unlabeled population, the target cells were untouched, and the technique was negative selection.

2.11.1 Positive selection of lymphocyte populations.

In this study, 5 different cell populations were individually isolated from PBMCs using the MACS[®] technology for cell separation by Miltenyi Biotec; populations isolated were CD19⁺, CD3⁺, V α 24-J α 18⁺ (TCR), CD4⁺, and CD8⁺ cell populations. Instructions provided on MACS[®] cell separation data sheets were followed. Briefly, cells (e.g. PBMCs) were incubated at 4°C (not on ice) with the recommended amount of MicroBeads in MCS buffer (see 2.2.6); MicroBeads are coated with antibodies against the target cell population (e.g. anti-iNKT MicroBeads). Cells were washed, re-suspended in 1 ml MCS buffer, and passed through the manual MACS[®] Cell Separation apparatus: a pre-separation filter on top of a MACS[®] LS column held in a MACS[®] separator magnet. Several washes were applied to the column and the elution containing the unbound cells was collected. The LS column was then removed from the magnet and using a plunger, the bound cells (i.e. target cells) were collected. Cells were then placed in iNKTCM (see 2.2.3) for further manipulations. In rare cases the automated (semi-sterile) autoMACS[®] Separator was used instead of the manual apparatus, to allow for a faster separations. When isolating iNKT cells before FACS sorting using the anti-iNKT cell MicroBeads, where more than 10⁸ starting PBMCs were used, up to 10X less MicroBeads than recommended for this number of cells was used. For CD4⁺ and CD8⁺ cell separations, a previous cell surface-staining step was carried out using anti-CD4 or anti-CD8 (PE) Abs, following which anti-PE MicroBeads were used to isolate the target cells as described above.

2.11.2 Negative selection of lymphocyte populations.

Negative selection is an indirect labeling system that allows for the isolation of untouched target cells. In this study only one target was isolated using this method; B cells were isolated for the purpose of comparison between different methods for B cell isolation. Two kits were used to negatively select B cell: B cell isolation kit II human kit by miltenyi biotec, and EasySep™ Human B Cell kit by stemcell technologies.

2.11.2.1 B Cell Isolation using MACS® cell separation.

PBMCs were labeled with a cocktail of biotin-conjugated Abs and anti-biotin MicroBeads to label all cells other than B cells, which are then retained in the MACS® column, while B cells get eluted. Instructions provided on MACS® cell separation data sheets were followed. Manual MACS® Cell Separation steps were carried out as previously described (see 2.11.1).

2.11.2.2 EasySep™ B Cell Enrichment.

The EasySep™ Human B Cell Enrichment Kit by STEMCELL™ Technologies was used to isolate untouched B cells as per instructions manual. Briefly, PBMCs were incubated for 10 mins at RT with a B cell enrichment Ab cocktail of tetrameric Ab complexes (TAC); TAC are conjugates of anti-dextran and other lineage specific Abs. Dextran-coated magnetic particles were then added for 5 mins to allow binding with TACs containing the anti-dextran Abs. The tube containing labeled PBMCs was then placed in a magnetic field allowing for non-B cells to be retained close to the tube wall, while unbound B cells are decanted from the tube.

2.12 Flow cytometric cell sorting.

Flow cytometry cell sorting is a method used to sort or separate mixed populations of cells into pure populations with defined phenotypes, using a specialized flow cytometer. Fluorescently labeled cells pass through the cytometer module, as previously described (see 2.10.1), where the fluorescence from each cell is identified. The fluid stream containing the identified cells is disturbed to give rise to single cell-containing droplets. Based on the fluorescence reading, each droplet is then given a corresponding charge by an electrode immediately downstream from the interrogation point (i.e. where fluorescence was identified). Electrically charged plates then guide the droplets based on their charge to up to 4 collection tubes, allowing for up to 4 populations to be sorted at the same time.

In this study, cell sorting was carried out in a semi sterile MoFlo XDP cell sorter by a qualified technician (Ann Atzberger, Barry Moran, or Dr Eamon Breen). The populations of interest that were sorted using this method were $V\alpha 24^+V\beta 11^+$, $V\alpha 24^-V\beta 11^-$, $V\alpha 24^+V\beta 11^+CD4^+CD8^-$, $V\alpha 24^+V\beta 11^+CD8^+CD4^-$, and $V\alpha 24^+V\beta 11^+CD4^-CD8^-$. The first 2 populations (i.e Total iNKT cells and non-iNKT cells) were sorted first using part of the starting sample, then the rest of the sample was sorted to obtain the 3 iNKT cell subset populations (i.e. $CD4^+$, $CD8^+$, and DN iNKT cells). The samples to be sorted were prepared using standard aseptic technique. PBMCs were prepared as previously described (see 2.5), and enriched for iNKT cells using magnetic bead separation (see 2.11.1). Enriched PBMCs were then washed in PBS with 2% FCS, resuspended at a maximum density of 50×10^6 / ml, and stained using appropriate cell surface staining mAbs: $V\alpha 24$ (FITC), $V\beta 11$ (PE), $CD8$ (APC), and $CD4$ (PE-Cy7), for 30 mins at $4^\circ C$ protected from light. Cells were then washed and re-suspended in 0.5 ml of iNKTCM (see 1.2.3), and placed in sterile FACS tubes on ice until sorted. An unstained sample and single stained beads were prepared for the cytometer set up. In addition, a collection tube for each sorted cell population was prepared by adding 0.5 ml of iNKTCM to sterile FACS tubes.

2.13 Generation of iNKT cell-lines.

iNKT cells represent 0.02 – 0.8% of all CD3⁺ cells in the peripheral circulation (Lynch et al. 2009). To carryout *in vitro* functional work on iNKT cells, expansion of pure iNKT cells lines in culture was necessary. iNKT cell lines were generated by expanding pure populations (> 97%) of iNKT cells obtained through by flow cytometry cell sorting, in the presence of feeder cells, cytokines, and appropriate stimulant. To obtain starting iNKT cells of appropriate purity and numbers from cell sorting, PBMCs were first enriched to obtain iNKT cell purities of > 2 % of total PBMCs. Enriching iNKT cells was carried out using one of two methods depending on the starting number of PBMCs. Where > 0.5 X 10⁹ PBMCs were available, magnetic bead enrichment (see 2.11.1) was the method of choice to positively select out iNKT cells from PBMCs before flow cytometry cell sorting; where < 0.5 x 10⁹ PBMCs were available, due to the limited numbers of iNKT cells, a method was devised to increase the frequency and absolute numbers of iNKT cells from PBMCs in culture, before flow cytometry cell sorting. As a result, two different methods for expanding iNKT cells were employed in this study, depending on the starting numbers of PBMCs: 1) expansion of iNKT cells from large PBMC samples, and 2) expansion of iNKT cells from small blood samples.

2.13.1 Expansion of iNKT cells from large PBMC samples.

Buffy coat packs were kindly donated by the Irish Blood Transfusion Service (IBTS). Each pack contained (0.5 – 1) x 10⁹ PBMCs in CPD anticoagulant. PBMCs were isolated from each pack as previously described (see 2.5). The PBMCs were enriched for iNKT cells using magnetic bead separation, where 6B11⁺ cells were physically pulled out using magnetic beads (see 2.11.1). PBMCs containing 20 – 80 % iNKT cells were then sorted using flow cytometry cell sorting (see 2.12) to obtain total iNKT cells, CD4⁺, CD8⁺, and DN iNKT cell subsets of > 97% purity. Irradiated (25 Gy) buffy coats, from 2 different donors, were kindly donated by the IBTS; irradiated PBMCs (feeders) were isolated as previously described (see 2.5). Feeders from both donors (1:1) were suspended at a final concentration of 10⁶ /ml in iNKTCM (see 2.2.3) supplemented with 100 IU/ml of IL-2 and 10 ng/ml of IL-7. Feeders were then pulsed with α -GalCer, which was prepared (see 2.17) and added to feeders at a final concentration of 100 ng/ml.

Sorted iNKT cells were added to feeders at ratios of 1 iNKT cells to 200 or 2000 feeder cells, and plated in a 96-well round bottom plate, with a volume of 200 μ l per well. iNKT cells were expanded in culture for 30 days, with regular medium and cytokine supplementation (see 2.16).

2.13.2 Expansion of iNKT cells using PHA.

Using the T cell mitogen phytohaemagglutinin PHA to expand iNKT cells is a non-specific method that relies heavily on the purities of iNKT cells prior to expansion. The protocol was carried out as previously described (O'Reilly et al. 2011). Pure populations of iNKT cells were isolate from PBMCs, added to feeder cells, and plated in 96-well round bottom plates as previously described (see 2.13.1). In place of α -GalCer 1 μ g/ml of PHA and 250 IU/ml of IL-2 were added to the iNKT cells and feeders. Culture medium was changed at days 1 and 2 in order to dilute out PHA, by replacing with fresh IL-2-containing iNKTCM (see 2.2.3).

2.13.3 Expansion of iNKT cells from small blood samples.

PB samples were taken from CLL patients and healthy control subjects. 1.5 – 20 ml of blood was collected in EDTA anticoagulated collection tubes, and processed within 24h of collection. PBMCs were isolated from each sample as previously described (see 1.5). The PBMCs were enriched for iNKT cells in culture; PBMCs were re-suspended at 10^6 /ml in iNKTCM (see 2.2.3) supplemented with 100 IU/ml of IL-2 and 10 ng/ml of IL-7. α -GalCer was processed (see 2.15) and added at a final concentration of 100 ng/ml. PBMCs were then plated in 96-well round bottom plates and maintained in culture (see 2.14) for 2 – 3 weeks, to enrich for iNKT cells. iNKT cell enriched PBMCs were then sorted using flow cytometry cell sorting (see 2.12), and stimulated in culture with feeder cell, IL-2 and α -GalCer as previously described (see 2.13.1).

2.14 Maintenance of iNKT cell lines.

iNKT cell lines were cultured in 96-well round bottom plates, suspended in 200 – 250 μ l/well of iNKTCM (see 2.2.3) supplemented with 100 IU/ml of IL-2 and 10 ng/ml of IL-7. Twice a week, the plates were gently handled so to not disturb cell pellets, and 100 μ l of medium was removed; the pipette tip was angled 45° just above the cells and resting on the wall of the well while gently aspirating the medium. 110 μ l of fresh (warm) medium was then added to each well. Cells were incubated in 5 % CO₂ at 37°C.

2.15 Addition of glycolipids to cell cultures.

In order to assess iNKT cell responses to CD1d⁺ APCs, various glycolipid Ags were loaded onto CD1d⁺ HeLa cells, and B cells then co-cultured with iNKT cells. Prior to the addition of any glycolipid Ag to cells in culture, first they were processed to ensure the appropriate concentration was achieved. Glycolipids used included α -GalCer, 7DW8-5, OCH, β -GlcCer, and GSL-1 (Fig. 2.1). All glycolipids were stored as 1 mg/ml stocks in DMSO. Each glycolipid was first brought to RT then heated to 80°C for 2 mins, sonicated for 10 minutes in a water bath sonicator, and vortexed for 1 min. Any required dilutions were carried out at this point in warm medium or DMSO, heating at 80°C for 2 mins, sonicating for 5 mins, and vortexing after every dilution. Glycolipids were added to CD1d⁺ cells immediately after preparation for analysis of iNKT cell responses. Any dilutions in DMSO were stored in -20°C for re-use. Glycolipids were processed in this manner prior to each use.

2.16 Setting up iNKT cell and Hela cell co-cultures.

iNKT cells were co-cultured with CD1d-HeLa and mock-HeLa to either test the functionality of iNKT cell lines, or to test the ability of various glycolipids to activate iNKT cells. HeLa cells at 80% confluency or higher were split as previously described (see 2.4). HeLa cells in DMEM were then seeded at 50 x 10³ cells / well / 200 μ l in a 96-well round bottom plate. Appropriate glycolipids were processed (see 2.15) then added to both CD1d-HeLa and mock-HeLa cells, and incubated

overnight at 37°C. DMSO was used in place of the glycolipids as a negative control (vehicle). iNKT cells were rested in cytokine-free iNKTCM O/N at 37°C. 175 µl of DMEM was then removed from all HeLa cell wells, and iNKT cells in fresh iNKTCM were added to the HeLa cells at 0.1×10^6 /well in 200 µl. PMA (50 ng/ml) and ionomycin (1 µg/ml) (P/I) were added at this stage to control wells (i.e. positive control). Co-cultures were left to incubate: a) over night if extracellular cytokines were measured by ELISA, b) 4h if a CD107a assay was carried out, in the presence of anti-CD107a and monensin (see 1.18), c) 5h in the presence of BFA (10 µg/ml) if intracellular cytokine staining was to be carried out (see 2.20). After the appropriate incubation period, supernatants were carefully transferred to a new plat and stored at -20°C for analysis, and cells to be analysed by flow cytometry were stained for surface (see 2.10.2) and intracellular markers (see 2.20).

2.17 Setting up iNKT cell and B cell co-cultures:

iNKT cells were co-cultured with CD19⁺ B cells from healthy donors control subjects or patients with CLL, in order to study the responses of B cells to iNKT cells *in vitro*, and to look at the reciprocal ability of B cells to modulate iNKT cell responses in the presence and absence of exogenous glycolipid Ags. CD19⁺ B cells were isolated from PBMCs as previously described (see 2.11.1); B cells were > 90% pure as determined by flow cytometry. CD19⁺ cells in iNKTCM were plated at $0.1 - 0.2 \times 10^6$ cells in 200 µl in a well of a 96-well round bottom plate. Appropriate glycolipids were processed (see 2.15) then added to CD19⁺ cells, and incubated overnight at 37°C. DMSO was used in place of the glycolipids as a negative control (vehicle). Expanded iNKT cells (total or CD4⁺, CD8⁺, and DN fractions) as well as CD3⁺ non-iNKT cells were rested in cytokine-free iNKTCM overnight at 37°C. 175 µl of medium was removed from all CD19⁺ cells, and T cells in fresh iNKTCM were added to the B cells at a 1:1 ratio in a total volume of 200 µl/well. PMA (50 ng/ml) and ionomycin (1 µg/ml) (P/I) were added at this stage to control wells (i.e. iNKT cell positive control). Where B cells were analysed for cytokine production or activation markers, a positive control was set up where B cells were stimulated with 10 ng/ml of BAFF and 1 µM CpG. Other controls that were included in some experiments included anti-hCD3, sCD40L, rhIL-2, rhIL-4,

and rhIL-15. Once all the appropriate stimulations were added to the co-cultures, the cells were incubated at 37°C for a time appropriate to the experimental output/readout.

- **Immunoglobulin secretion:**

Co-cultures were incubated for 8 days, then supernatants were removed and analysed using the cytometric bead array (CBA) kit (see 2.22). Supernatants were stored at -20°C, where analysis was not carried out immediately.

- **Cytotoxic degranulation:**

Co-cultures were incubated for 4 h in the presence of anti-CD107a and monensin, and cells were stained and analysed by flow cytometry (see 2.19).

- **Intracellular cytokine detection:**

Where IL-10 production by B cells was investigated, co-cultures were incubated for 72 h, then all co-cultures were split two ways, one half receiving 10 µg/ml of BFA, and the other half receiving BFA and 50 ng/ml of PMA and 1 µg/ml of ionomycin. Co-cultures were incubated for a further 15 h, before staining for intracellular cytokines (see 1.20) and analysing by flow cytometry. Where cytokine production by iNKT cells was the readout, co-cultures were incubated for 5 h in the presence of 10 µg/ml of BFA. Cells were then stained for intracellular cytokines (see 1.19) and analysed by flow cytometry.

2.18 Testing the effects of retinoic acid (RA) on CD1d expression on B cells.

All-*trans* retinoic acid (ATRA) and AM580, an analogue of RA, are classed as retinoids or vitamin A derivatives. Both bind to the retinoic receptor (RARα), and have been reported to induce CD1d expression on tonsillar B cells (Allan et al. 2011). To test the ability of ATRA and AM580 to upregulate CD1d expression of PB B cells from healthy controls and CLL patients, CD19⁺ B cells were isolated from both patient and control PBMCs as previously described (see 2.5); B cells were > 90% pure as determined by flow cytometry. CD19⁺ cells were plated at 0.1 - 0.2 X 10⁶ cells in a volume of 200 µl of iNKTCM in wells of a 96-well round

bottom plate. ATRA or AM580 were added to the appropriate wells containing CD19⁺ cells. Cells were incubated for the appropriate time (1-3 days) at 37°C. Levels of CD1d on B cells were then measured by flow cytometry (see 2.10). Treated B cells were then co-cultured with iNKT cells as previously described (see 1.16). The effect of ATRA and AM580 on the susceptibility of CLL cells to iNKT cell cytotoxicity was tested (see 1.19).

2.19 Analysis of cytolytic degranulation: CD107a assay.

CD107a was used as a sensitive marker for cytotoxic degranulation activity (Aktas et al. 2009). CD107a or lysosome-associated membrane protein-1 (LAMP-1) is found on lysosomal membranes. A cell primed for cytotoxicity releases the contents of lysosomes through the fusion of these vesicles with the plasma membrane. Upon fusion, CD107a becomes temporarily exposed to the extracellular environment. Fluorescently tagged Abs can then bind CD107a before the vesicles are recycled back into the cell. The addition of monensin, a protein transport inhibitor, prevents CD107a recycling, allowing its detection by flow cytometry. iNKT cells were co-cultured with target cells (see 1.16-17) in the presence of anti-CD107a Ab. Co-cultures of iNKT cells (10^6 / ml), CD1d-HeLa, mock-HeLa, or B cells (10^6 / ml), were set up in 96-well round bottom plates, with 200 μ l of cells per well, and anti-CD107a Ab was added directly to each well. After 1h of incubation at 37°C, monensin was added to each well at a final concentration of 1X, and incubated for a further 3h. Cells were then washed and stained for further surface markers as previously described (see 2.10.2).

2.20 Intracellular cytokine detection by flow cytometry.

Changes in cytokines such as IFN- γ , IL-4, IL-10, IL-17a, and IL-21 production by iNKT cells and/or B cells, after stimulation in culture, were measured by flow cytometry. Cells were first stained for surface markers as previously described (see 2.10.2). After fixation with 1 – 4% of PFA, the cells were re-suspended in 0.2% saponin, appropriate amounts of cytokine staining Abs added, and incubated

at RT protected from light for 20 mins or 40 minutes at 4°C. Cells were then washed 2 – 3 times, fixed with 1 - 2% paraformaldehyde (PFA) or FluoroFix™ Buffer, washed again, then stored at 4°C in PBA for a maximum of 2 days before analyzing on a flow cytometer. For each experiment that was analysed by flow cytometry, appropriate unstained, single stains, and FMO controls were prepared (see 2.10.3).

2.21 Detection of extracellular cytokines using enzyme-linked immunosorbent assay (ELISA).

Cytokine secretion by B cells and/or iNKT cells was measured quantitatively using ELISA; cytokines included IFN- γ , IL-4, and IL-10. Each ELISA was carried out as per manufacturers instructions, with the exception of adding only half the volume of each reagent per ELISA well; this reduced the overall assay volume but maintained relative concentration. Briefly, ELISA plate was coated with 50 μ l per well of capture Ab diluted to the recommended concentration in PBS, and incubated overnight at RT. Plate was washed 3 times with wash buffer (see 2.2.9), then blocked by adding 300 μ l of blocking buffer (see 2.2.10) for 2h or overnight at 4°C. Plate was washed 3 times, and 50 μ l of each sample to be tested was added to corresponding wells; a provided cytokine standard with a known concentration was serially diluted in reagent diluent (see 2.2.11) and 50 μ l of each standard added in duplicate to wells of the ELISA plate, and incubated for 2h at RT or 1h at 37°C. The plate was washed 3 times, then 50 μ l of detection Ab diluted to the recommended concentration in reagent diluent (see 2.2.11) was added for 2h at RT or 1h at 37°C. The plate was washed 3 times, and 50 μ l of Streptavidin-HRP diluted to the recommended concentration in reagent diluent was added for 20 mins at RT. The plate was washed 3 times, then 50 μ l of TMB substrate was added for 20 mins at RT. The reaction was stopped by adding 25 μ l of 2N H₂SO₄. Plate was read at 450 nm with a 540 nm correction on the TECAN Sunrise plate reader.

2.22 Extracellular immunoglobulin detection using a cytometric bead array (CBA).

iNKT cells were co-cultured with autologous or allogeneic B cells for 8 days at 37°C. Supernatants were removed and stored at -20°C before analysis. The secretion of total IgG, IgM, and IgA by B cells were quantitatively measured using the BD™ cytometric bead array (CBA). The CBA assay utilises beads coated with antibodies against the immunoglobulin of interest, which bind to respective immunoglobulins present in the supernatants; a fluorescently tagged secondary Ab binds to Igs, giving the beads a fluorescent output for detection by flow cytometry. For quantification, a standard curve was obtained using a known standardized sample of Ig, and the concentration of Ig in each test sample was calculated by referencing the sample MFI to the standard curve.

IgA and IgM detection assays were multiplexed, but IgG was carried out separately, as specified by manufacturer (BD bioscience, UK). Ig standards were prepared to the appropriate concentration; the same standard was used to prepare a dilution series for all Igs. One dilution series was carried out for IgA and IgM multiplex, and a different dilution series was performed for total IgG assay. Only half the volume of each reagent and samples were added per test; results obtained by using half the volumes was comparable to using the full amount. 25 µl of each standard and neat samples were added to labeled 5 ml tubes. 25 µl of capture beads, which have been diluted to the specified concentration using provided reagents, was added to each test. All tubes were gently mixed and incubated for 1 h at RT. Capture beads were washed by adding 1 ml of a provided wash buffer to each tube, and centrifuged at 200 x g for 5 minutes. 25 µl of PE labeled detection beads, which have been diluted to the specified concentration using provided reagents, was added to each test. All tubes were gently mixed and incubated for 2 h at RT. All tests were washed as above. 60 µl of FluoroFix™ Buffer was added to all tests and stored at 4°C; this step was added to allow for storage of samples before analyzing by flow cytometry.

2.23 Patients and healthy subjects.

2.23.1 Patients with chronic lymphocytic leukaemia.

Blood samples were obtained from patients diagnosed with CLL, who attended the haematology clinic run by Prof Elisabeth Vandenberghe in St. James's hospital, Dublin. Patients with no previous treatment history, as well as those not receiving treatment for more than one year, were identified by the clinical team during the course of the clinical examination, informal consent was obtained, and samples were taken. Written ethical approval from each of the patients was also obtained prior to recruiting them to the study. A total of 9 ml of blood was collected per patient, 3 ml of which was given to me by Dr Tony McElligott who kindly collected the samples from the clinic, and 6 ml kept by Dr McElligott for other research investigations. Obtaining the patient samples was challenging and infrequent, with an average of one sample per month.

2.23.2 Healthy control subjects.

All blood samples from healthy subjects were taken after obtaining informal consent. Most healthy subjects were recruited from the institute of molecular medicine, St. James's hospital. For the purpose of age matching with CLL patients, healthy staff members from the clinical pathology laboratory in St. James's hospital, were recruited and blood samples taken after obtaining informal and written consent. Another sample type is a buffy coat, which constitutes the main source of immune cells for this study. Buffy coats are blood packs enriched with immune cells, and were donated by the Irish Blood Transfusion Service (IBTS). Standard policies for blood donations and blood pack handling mean that each buffy coat does not come devoid of any identifiers including sex and age.

2.24 Ethical statement.

Ethical approval for the CLL study was granted by St. James's Hospital and Adelaide and Meath incorporating the National Children's Hospital Ethics Committee. All patients and healthy controls enrolled in the CLL study provided written informed consent. All other healthy donors provided verbal informed consent.

2.25 Statistical analysis.

Prism 5.0c for Mac OS X was used to carryout all statistical analysis. Mann Whitney test was used to compare two unpaired/unmatched groups. Kruskal-Wallis test was used to compare multiple unpaired/unmatched groups with post hoc Dunns multiple comparison tests. Wilcoxon signed rank test was used to compare two paired/matched groups. Friedman test was used to compare multiple paired/matched groups with post hoc Dunns multiple comparison tests. All tests carried out in this study were nonparametric. Statistical significance was recorded as: * $P \leq 0.05$, ** $P \leq 0.01$, or *** $P \leq 0.001$.

**Chapter 3 Generation and Characterisation of
iNKT Cell Lines.**

3.1 Introduction.

Invariant natural killer T cells are innate-like lymphocytes that express a semi-invariant T cell receptor capable of recognizing glycolipid antigens presented to them in the context of a CD1d molecule by APCs. iNKT cells in humans are defined as having a $V\alpha 24^+$ $V\beta 11^+$ TCR (Godfrey et al. 2004). Upon stimulation, iNKT cells can release a variety of cytokines including IFN- γ and IL-4, which then act on other cells to induce various effector or regulatory functions. iNKT cells also release cytotoxic molecules such as granzyme and perforin that cause target cell death. iNKT cell cytotoxicity has been demonstrated extensively *in vitro* using a variety of human and murine tumour cell lines, and *in vivo* studies in tumour mouse models indicate that murine iNKT cells play a central role in tumour immunosurveillance (Matsuda et al. 2008)(Berzins et al. 2011).

In the past two decades our knowledge on iNKT cells has dramatically increased and so has the number of studies carried out looking at iNKT cells in human disease. The precise roles that iNKT cells may play in the pathogenesis of various diseases have yet to be revealed. Various studies have found changes in iNKT cell frequency and function in a number of diseases. While such changes in iNKT cells may be a consequence of the disease, they may equally be a cause of pathology in such diseases. Patients with infectious diseases, such as hepatitis C virus (HCV) viremia (Lucas et al. 2003) and active TB (Sutherland et al. 2009) have significant reductions in iNKT cell frequencies in PB. iNKT cells have been shown in some autoimmune diseases to be numerically and/or functionally impaired (e.g. in multiple sclerosis – MS) (Voskuyl et al. 2001; Gausling et al. 2001), but unimpaired in others (e.g. Insulin Dependent Diabetes Mellitus – IDDM) (Lee, Putnam, et al. 2002). In malignancy, studies have shown that iNKT cell frequencies are reduced in a number of different cancers, and in some cases their frequencies correlate inversely with tumour load and proportionally with good prognosis (Yanagisawa et al. 2002; Tachibana et al. 2005; Schneiders et al. 2012). While most clinical studies investigating iNKT cells in disease have reported on iNKT cell frequencies, only a fraction of such studies include data on iNKT cell function, mainly looking at *ex-vivo* IFN- γ and IL-4 production (Dhodapkar et al. 2003; Tahir et al. 2001). Hence, more in depth work is needed to study the functional characteristics of iNKT cells in various diseases.

One of the main and well established roles for iNKT cells is in direct and indirect (i.e. NK and CD8⁺ T cell transactivation) cytotoxicity (Bassiri et al. 2014). The anti-tumour functions of iNKT cells have already been utilised in many clinical trials for cancer treatment. A better knowledge of iNKT cell function, and in particular looking at the differential functions of CD4⁺, CD8⁺, and DN iNKT cells, will help improve on iNKT cell targeted therapies.

iNKT cells have been found to be reduced in the PB of various cancer patients. A number of clinical trials aimed at increasing circulating iNKT cell numbers in order to improve prognosis have been carried out. One approach given by the example of a phase I trial carried out by Motohashi et al, tested the effects of IV infusions of autologous PBMCs that have been enriched for iNKT cells (~ 20%). PBMCs were treated with the glycolipid α -GalCer for 2 weeks then given to patients with non-small cell lung cancer, but no tumour regression was observed (Motohashi et al. 2006). Another approach taken was to infuse patients with autologous APCs (e.g. monocyte derived DCs - moDCs) that are loaded with α -GalCer (i.e. α -GalCer-moDCs) with the view of expanding and activating iNKT cells *in vivo*. Such studies found that α -GalCer-moDCs caused an increase of circulating V α 24⁺ V β 11⁺ cells, increased IFN- γ producing cells, and increased NK cell activity and frequency (Chang et al., 2005; Ishikawa et al., 2005; Nieda et al., 2004; Okai et al., 2002; Uchida et al., 2008). One study found that combining *in vitro* expanded V α 24⁺ V β 11⁺ cell infusions with α -GalCer loaded APCs results in a greater increase in IFN- γ producing cells compared to mono-therapy (Kunii et al. 2009; Uchida et al. 2008). As shown in Table 11, the improvement in clinical outcome for patients after targeting iNKT cell function are modest, with the most promising study showing partial remission in 3 out of 8 patients. There are many factors that may be hindering the effectiveness of such therapeutic approaches, such as: small cohort size, heterogeneity of cancer types and stages within the cohort, advanced stage/untreatable cases, and the therapies being systemic and not targeted. There are also a number of factors that need to be considered regarding the specific use of iNKT cells for immunotherapy to treat cancers and other diseases.

- 1) The susceptibility of the target tissue or disease to iNKT cell action (e.g. CD1d expression and glycolipid presentation).
- 2) The heterogeneity of iNKT cells as a whole population in terms of the functional difference that can be attributed to

specific subset populations of iNKT cells. However, such modest improvements in cancer patient prognosis hold promise for the future of iNKT cell targeted therapy for such patients.

Table 11. Summery of iNKT cell-based phase I/II clinical trials. This summery table was taken from a review article entitles “Invariant NKT Cells as Novel Targets for Immunotherapy in Solid Tumors” by (Pilonos et al. 2012).

Tumor	Number of patients	Treatment regimen	Summary of study results	Reference
(A) Direct α -galactosylceramide injection				
Solid tumors	24	α -Galactosylceramide i.v. (40–4800 $\mu\text{g}/\text{m}^2$)	(1) No dose-limiting toxicity (2) Increase serum TNF- α and GM-CSF in 5 of 24 patients (3) Response dependent on pre-existing number of NKT cells which were significantly lower in cancer patients	Giaccone et al., 2002
(B) Infusion of ex vivo expanded NKT cells				
Nonsmall cell lung cancer	6	V α 24NKT cells restimulated with α -galactosylceramide-pulsed PBMCs	(1) No adverse effects (2) Highest dose induced expansion of NKT cells (in 2 of 3 patients) and IFN- γ producing cells (in 3 of 3 patients)	Motohashi et al., 2006
(C) Ex vivo generated DC loaded with α -GalCer				
Nonsmall cell lung cancer	11	α -Galactosylceramide-pulsed dendritic cells	(1) No severe toxicities (2) Increase NKT cells in 1 of 11 patients (3) No partial or complete response seen	Ishikawa et al., 2005
Head and neck cancer	9	α -Galactosylceramide-pulsed APC	(1) No serious toxicities (2) Increase NKT cells in 4/9 patients (3) Increase NK activity in 8/9 patients	Uchida et al., 2008
Nonsmall cell lung cancer	23	α -Galactosylceramide-pulsed PBMC cultured with IL-2/GM-CSF	(1) No severe toxicities (2) Stable disease in 5 (3) Increased IFN response correlated with survival	Motohashi et al., 2009
Nonsmall cell lung cancer	4	α -Galactosylceramide-pulsed APC	(1) Increase in NKT cells in lung tumors (2) Highest IFN- γ production following in vitro restimulation of TILs with α -galactosylceramide	Nagato, K et al 2012
(D) Combination strategies				
Head and neck squamous cell cancer	8	In vitro expanded NKT + α -galactosylceramide-pulsed APC	(1) Transient and mild adverse effects (2) Increased NKT cells and IFN- γ secretion in 7 of 8 patients (3) Partial response in 3 patients, stable disease in 4, progressive disease in 1	Kunii, N et al, 2009

Previous studies suggest that CD4⁺ iNKT cells are more efficient at activating B cells, produce more IL-4, and are phenotypically more immunosuppressive, while CD8⁺ iNKT cells are more inclined to be cytotoxic, produce more IFN- γ , trans activate CD8⁺ T cells, and potentially carry out a more pro-inflammatory role. DN iNKT cells appear to mediate similar functions to those of CD8⁺ iNKT cells (Lin, Nieda, Rozenkov, et al. 2006; O'Reilly et al. 2011; Lin, Nieda, Hutton, et al. 2006). Tailoring iNKT cell immunotherapy to the disease in hand is important, for example CD8⁺ iNKT cells could be a more beneficial tool for cancer immunotherapy. While CD4⁺ iNKT cells may provide a more immunosuppressive environment needed to ameliorate proinflammatory conditions, but at the same time may promote Th2 driven autoimmune conditions.

Several studies in mouse models have demonstrated how different synthetic glycolipids can induce either a Th1 or Th2 biased *in vivo* response (Mallevaey & Selvanantham 2012). However, advances in the development of synthetic glycolipids that can induce such immune biases in humans is limited. Such targeted glycolipids could potentially be coupled with moDCs for a more tailored immunotherapy. In the absence of glycolipids that favor a specific Th response, selecting a functionally appropriate iNKT cell population for immunotherapy is a viable alternative. Using this strategy to treat cancer patients, immunotherapy using CD8⁺ and DN iNKT cells could yield more positive prognostic outcomes when compared to the previously trialed mixed iNKT cell immunotherapies.

Whether for *in vitro* characterisation or for immunotherapy, iNKT cells need to be expanded *ex vivo* in order to obtain large numbers for manipulation. The main source for obtaining iNKT cells is human PB due to the ease of access to such samples. The larger the volume of blood, the more starting number of iNKT cells, the easier and quicker it is to expand an iNKT cell line. However, it is important to be able to expand iNKT cells from samples with very low starting iNKT cell numbers. Low starting numbers can be caused by a reduced sample size (e.g. 2-3 ml of PB or a tissue biopsy) or due to intrinsically low iNKT cell frequencies (e.g. PB from CLL patients).

Most approaches for *in vitro* expansion of iNKT cells, including those used to expand autologous iNKT cells for immunotherapy, have used IL-2 and α -GalCer-pulsed APCs to expand iNKT cells in culture from PBMCs (Kunii et al. 2009; Motohashi et al. 2006; Exley et al. 2010; S. M. A. Tahir et al. 2001). Others used IL-2, phytohaemagglutinin (PHA), and irradiated allogeneic PBMCs to expand sorted iNKT cells in culture (Bricard et al. 2010; Exley et al. 2010; O'Reilly et al. 2011). In our laboratory, the latter method was used and as published by O'Reilly et al., CD4⁺ iNKT cells were preferentially expanded *in vitro* using this method. Other variations on the above protocols include the use of cytokines such as IL-7 and IL-15, in combination and with or without IL-2 (Lin, Nieda, Rozenkov, et al. 2006; Exley et al. 2010). Previous studies have suggested that IL-7 and IL-15 promote CD8⁺ T cells growth and survival (Diab et al. 2005; Wallace et al. 2006), as well as IL-7 and IL-15 promoting the proliferation of total V α 24-V β 11⁺ iNKT cells (Nishi et al. 2000; Dunne et al. 2001; van der Vliet et al. 2001). However, the use

of specific cytokines to differentially enhance or promote the expansion of CD4⁺, CD8⁺, or DN iNKT cells has not been previously reported.

3.2 Objectives.

- To optimize methods for the detection of human iNKT cells by flow cytometry.
- To examine the frequencies as well as CD4 and CD8 expression on circulating iNKT cell in healthy subjects.
- To optimize a protocol that efficiently expands CD4⁺, CD8⁺, and DN iNKT cell subsets, testing mode of stimulation, cytokines and growth factors, and media composition.
- To optimize a method for expanding iNKT cell subsets from small volumes of blood.
- To examine the kinetics of expanding iNKT cells, looking at cell turnover, markers of exhaustion and senescence.
- To test the functionality of generated iNKT cell lines, looking at IFN- γ , IL-4, and CD107a upregulation upon glycolipid stimulation.

3.3 Results.

3.3.1 Identification of iNKT cells by flow cytometry.

iNKT cells can be identified by flow cytometry in three different ways: the first exploits the iNKT cell specific interaction between its T cell receptor (TCR) and a glycolipid loaded CD1d tetramer molecule. Here iNKT cells were identified as CD3⁺ cells that bind to fluorescently tagged PBS-57 loaded human CD1d (hCD1d) tetramers (Fig. 3.1). The second method involved staining iNKT cells with an Ab against the specific iNKT cell TCR α -chain V α 24-J α 18 (Fig. 3.1). The third method involved staining iNKT cells with an Ab against the TCR V α 24 chain and an Ab against the TCR V β 11 chain, where iNKT cells will appear double positive for both stains (Fig. 3.1). The phenotyping in this project was carried out by gating on iNKT cells using either of the first 2 methods due to their superior specificity (Berzins et al. 2011; Montoya et al. 2007; Lee, Putnam, et al. 2002) and technical convenience.

3.3.2 iNKT cell frequencies in fresh peripheral blood mononuclear cells (PBMCs).

Invariant natural killer T (iNKT) cells reside in many tissues of the human body, such as the liver, spleen, omentum, and blood (Johnston 2015). The proportions of iNKT cells as percentages of total CD3⁺ cells vary between different tissues. In the peripheral circulation iNKT cells generally represent (0.02 – 0.8%) of all CD3⁺ cells (Lynch et al. 2009). Peripheral blood samples were obtained from healthy donors aged 20 – 70 and investigated for the frequencies of iNKT cells using flow cytometry (see 3.1). Analysis of iNKT cell frequencies revealed that the average iNKT cell frequency in our cohort was 0.1% of CD3⁺ cells, which was found to decrease with age (Fig. 3.2). The absolute number of iNKT cells per ml of blood also decreased with age (Fig. 3.2).

3.3.3 CD4 and CD8 expression by iNKT cells in fresh PBMCs.

iNKT cells like other T lymphocytes can express CD4, CD8, neither (i.e. double negative / DN), or both (i.e. double positive / DP). Using flow cytometry the frequencies of each of these four iNKT cell subsets in 13 healthy donors were analysed (Fig. 3.3). The proportion of iNKT cells that are DN was higher than the other iNKT cell subsets averaging at around 45 % of total iNKT cells. DP iNKT cells represented the lowest proportion with percentages ranging from (0 – 3) % of total iNKT cells. While CD4⁺ and CD8⁺ iNKT cells were similarly represented, accounting for an average 30 % of total iNKT cells (Fig. 3.3).

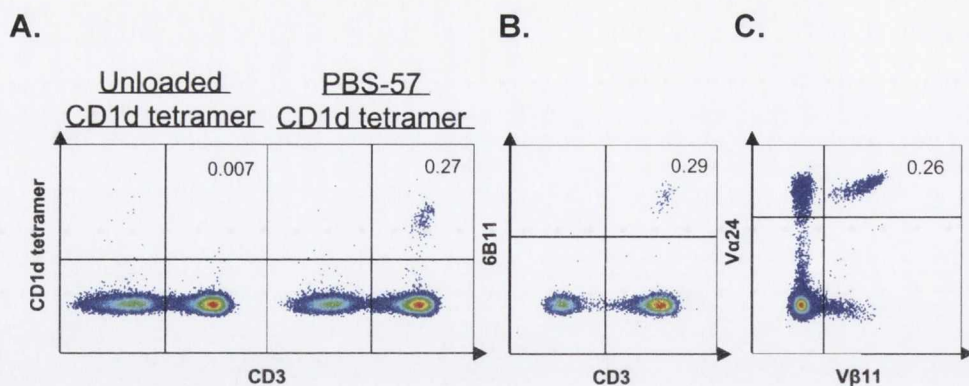


Fig 3.1. Identifying iNKT cells by flow cytometry. PBMCs from a healthy adult donor were isolated from an EDTA anticoagulated venous blood sample. PBMCs were stained with an anti-CD3 mAb in combination with; human CD1d tetramer loaded with PBS-57 or unloaded (A), 6B11 Ab against the Va24/Ja18 TCR (B), and anti-Va24 with anti-Vb11 (C), and analysed by flow cytometry. Pseudocolour dot plots of each staining method are shown in A, B, and C.

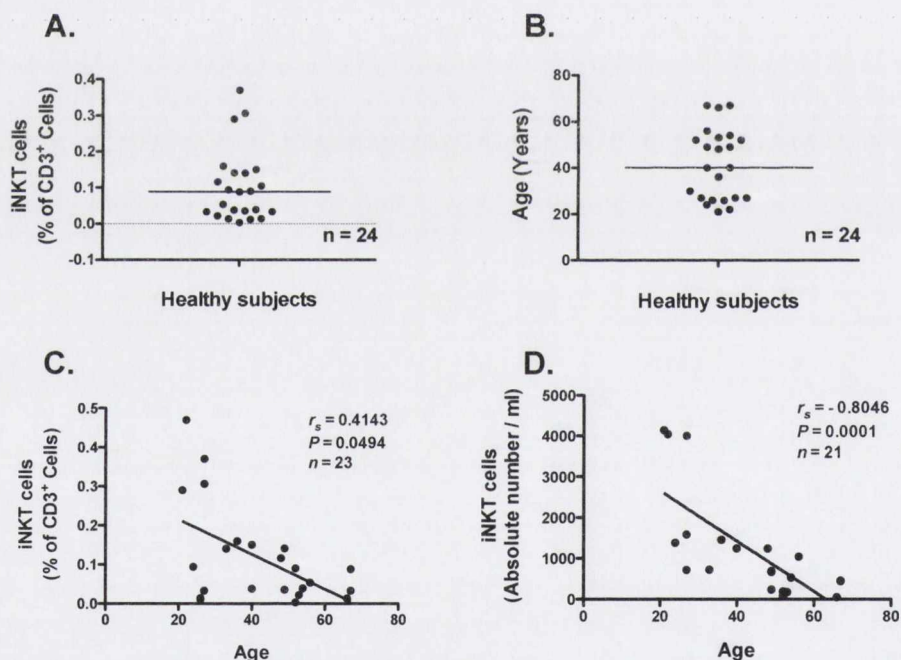


Fig 3.2. iNKT cell frequencies in PBMCs of healthy adults. EDTA anticoagulated venous blood was obtained from 24 healthy adult donors. PBMCs were isolated and stained with an anti-CD3 mAb and a PBS-57 loaded CD1d tetramer, and analysed by flow cytometry. **A**, Scatter plot showing iNKT cell frequencies as percentages of CD3⁺ PBMCs. **B**, Scatter plot showing the ages of the healthy adult donors. Correlation plots between age and iNKT cells as a percentage of CD3⁺ PBMCs (C) or as cell numbers per ml of blood (D) are shown. Each data point represents one donor in each of the three graphs. Horizontal lines show the median. Statistical analysis was carried out using Spearman correlation analysis.

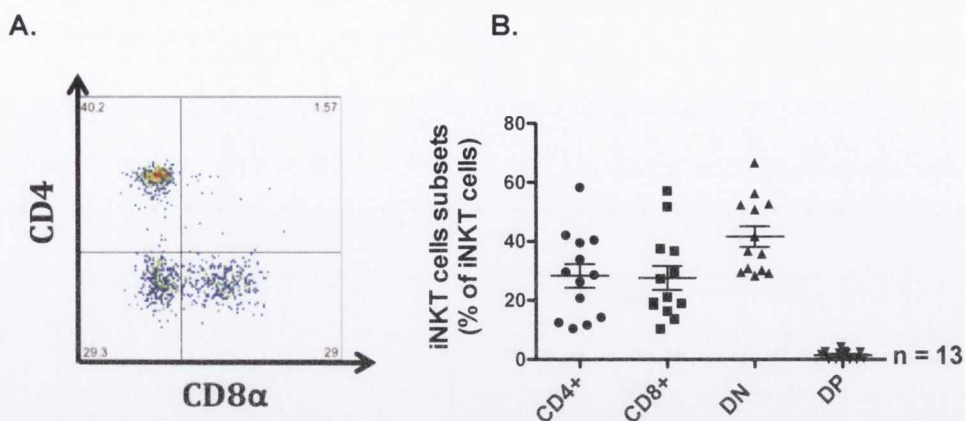


Fig. 3.3. Frequencies of circulating iNKT cell subsets based on CD4 and CD8 expression. PBMCs were isolated and stained for flow cytometry analysis. iNKT cells were gated and analysed for CD4 and CD8 expression. **A**, Flow cytometric pseudocolour dot plot of gated iNKT cells showing their expression of CD4 and CD8. **B**, Scatterplot showing the frequencies of each iNKT subset (i.e. CD4⁺, CD8⁺, DN, and DP) as percentages of total iNKT cells from 13 healthy donors. Each data point represents one donor and is plotted with mean ± SEM.

3.3.4 Methods for expanding iNKT cell lines *in vitro*.

Ex vivo expansion of iNKT cells is required to obtain sufficient numbers of cells for *in vitro* experiments. Two strategies for the generation of iNKT cell lines, based on previously reported protocols (Bricard et al. 2010; Exley et al. 2010; S. M. A. Tahir et al. 2001; O'Reilly et al. 2011) were used in the present investigation. PBMCs were isolated from buffy-coat packs. PBMC yields of $0.5 \times 10^9 - 1 \times 10^9$ were typically obtained. PBMCs were enriched for iNKT cells by magnetic bead separation using iNKT cell MicroBeads. Enriched iNKT cells were then stained with an anti-V α 24 and anti-V β 11 mAb and sorted on a MoFlo™ XDP Cell Sorter to obtain a pure population ($\geq 97\%$) of iNKT cells. Sorted iNKT cells were then expanded by co-culturing with allogeneic irradiated PBMCs in the presence of either PHA or the glycolipid α -GalCer for 30 days. To maintain cell growth and survival throughout the 30 days, the cells were maintained in the presence of 250 IU/ml of IL-2. Fig. 3.4 outlines the two possible methods for preparing iNKT cell lines: 1) Using the T cell mitogen PHA and IL-2. 2) Using the iNKT cell adjuvant α -GalCer and IL-2.

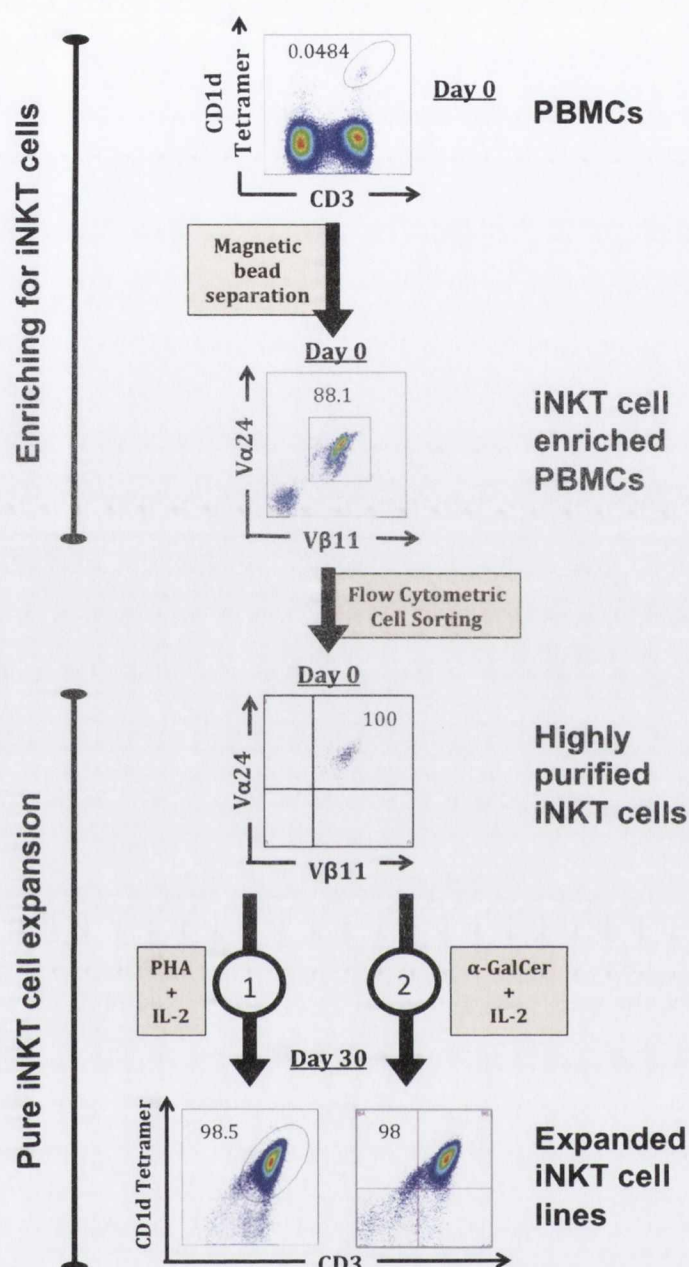


Fig. 3.4. Schematic representation showing two methods of iNKT cell line generation. Peripheral blood PBMCs containing iNKT cells (CD1d tetramer⁺) at 0.05 % of CD3⁺ cells were enriched using magnetic bead separation to obtain iNKT cells (Vα24⁺Vβ11⁺) that are 88.1 % of CD3⁺ cells. MoFlo™ XDP Cell Sorter was used to obtain a pure population of iNKT cells (98.5 %), which were then co-cultured with allogeneic irradiated (25 Gy) PBMCs (i.e. feeders). Appropriate stimuli were also added: approach (1) using IL-2 (250 IU/ml) and PHA (1 μg/ml), and approach (2) using IL-2 (250 IU/ml), and the glycolipid α-GalCer (100 ng/ml). After 30 days in culture the iNKT cells were expanded in numbers and a pure iNKT cell line was obtained (~ 98 %).

3.3.5 IL-2, IL-7, and IL-15 differentially affect iNKT cell expansion.

Previous studies have shown that iNKT cell expansion in the presence of IL-2 results in preferential expansion of CD4⁺ over CD8⁺ iNKT cells (O'Reilly et al. 2011). Cytokines such as IL-7 and IL-15 have previously been shown to favour the survival of CD8⁺ non-iNKT cells (Diab et al. 2005; Wallace et al. 2006). To investigate whether IL-7 or IL-15 can enhance the yields of CD8⁺ iNKT cells, iNKT cells were expanded from PBMCs in the presence of these cytokines. PBMCs were stimulated with α -GalCer for 10 days *in vitro* in the presence of IL-7 (10 ng/ml), IL-15 (10 ng/ml), and/or two concentrations of IL-2 (100 or 250 IU/ml). After 10 days in culture, the PBMCs were counted, stained, and analysed by flow cytometry. The presence of IL-7 with IL-2 resulted in an increase in total iNKT cell numbers in 3 out of the 5 donors when compared to either concentration of IL-2 alone (Fig. 3.5.A). In one donor, the presence of IL-15 with IL-2 resulted in a greater increase in total iNKT cells compared to IL-7 with IL-2 (Fig. 3.4.A). iNKT cell numbers were significantly ($P \geq 0.05$) increased when PBMCs were cultured in the presence of IL-2 compared to PBMCs cultured with no cytokines (Fig. 3.5.B).

Using flow cytometry the percentage of CD4⁺, CD8⁺, DN, and DP iNKT cells that were present after expansion with α -GalCer in the presence of various cytokines were determined (Fig. 3.6). IL-2 combined with IL-7 or IL-15 gave the highest yields of CD8⁺ iNKT cells. IL-2 alone, IL-2 with IL-7, and IL-2 with IL-15 all resulted in a significant increase in CD4⁺ iNKT cells. IL-2 with IL-7 resulted in a significant increase in DN iNKT cells (Fig. 3.6.A). Although IL-7 and IL-15 alone did not increase the total yield of iNKT cells when compared to IL-2, IL-15 resulted in an increase in the percentage of CD8⁺ iNKT cells and IL-7 caused a decrease in DN iNKT cells compared to all other cytokine treatments (Fig.3.6.B).

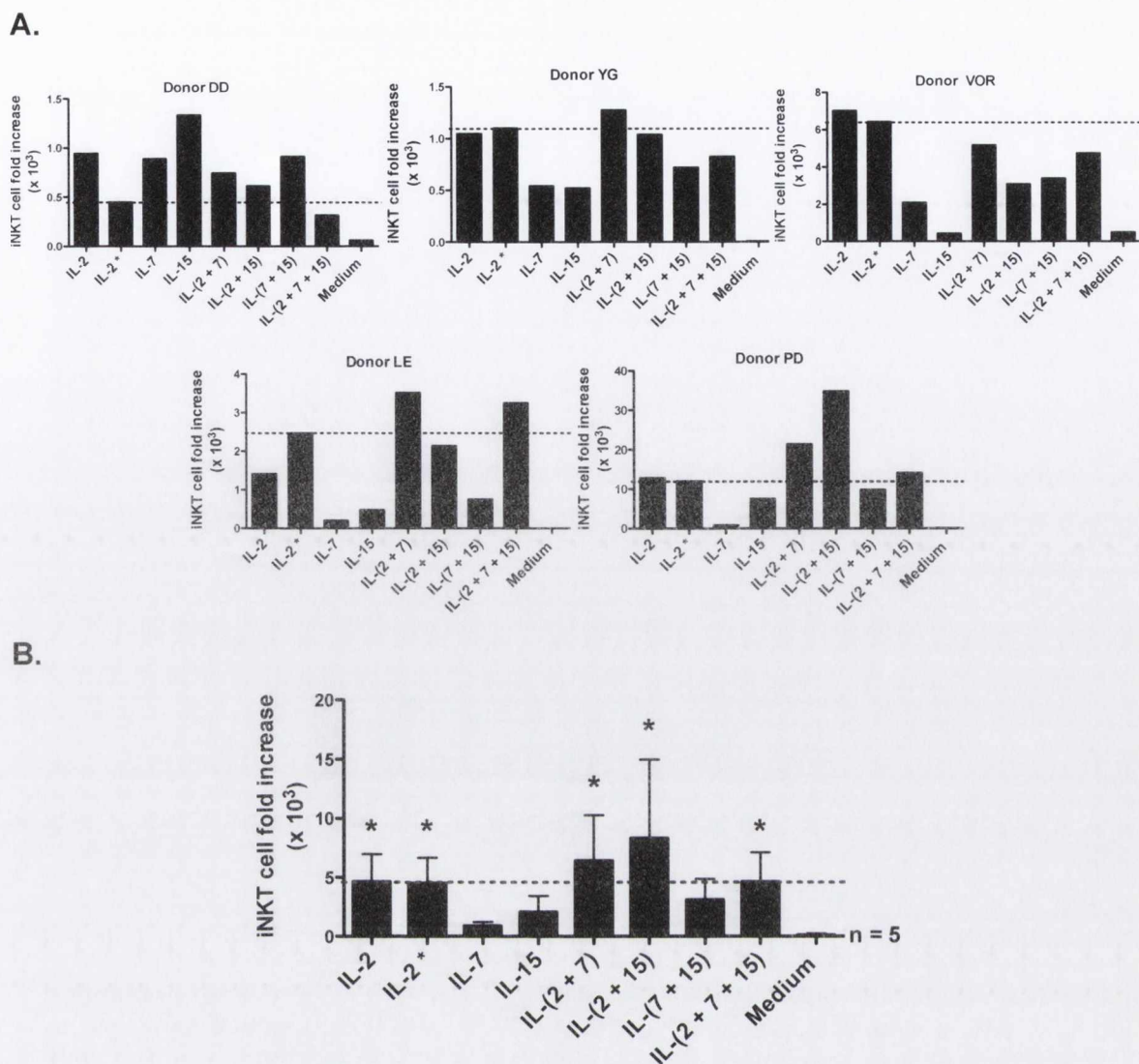
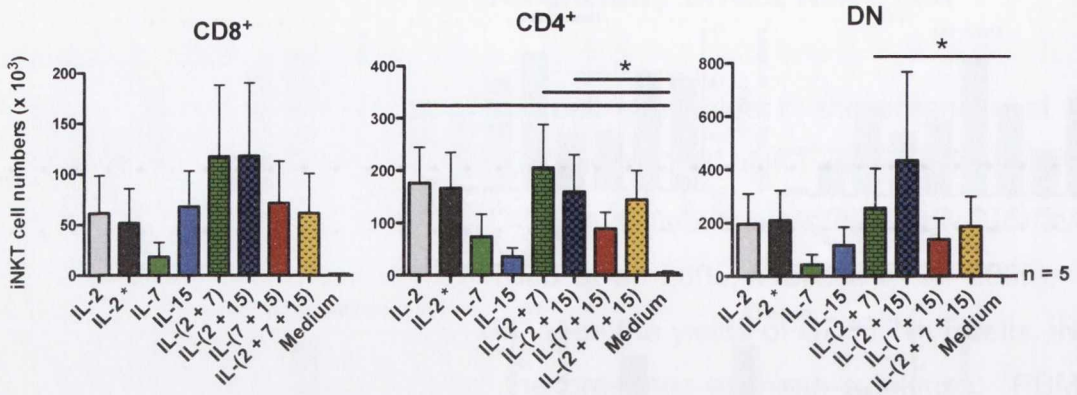


Fig. 3.5. Expansion of iNKT cells using IL-2, IL-7, and IL-15. . PBMCs were incubated for 10 days with the iNKT cell adjuvant α -GalCer in the presence or absence of IL-2 (100 IU/ml), IL-2* (250 IU/ml), IL-7 (10 ng/ml), IL-15 (10 ng/ml), and combinations of IL-7, IL-15, and IL-2 (100 IU/ml). **A.**, Five separate bar charts, each showing the magnitudes of iNKT cell expansion from individual donors in the presence of the different cytokines. **B.**, Bar chart showing the average numbers of iNKT cells after 10 days in culture with the various cytokines. The dashed line shows the expansion in the presence of 250 IU/ml of IL-2. Data was plotted as one single donor per graph (**A**) or as mean $\pm$ SEM from 5 healthy donors (**B**). * $P \leq 0.05$ compared to cells cultured in medium alone. Statistical analysis was carried out using the non-parametric Kruskal-Wallis test with post hoc Dunns multiple comparison tests.

A.



B.

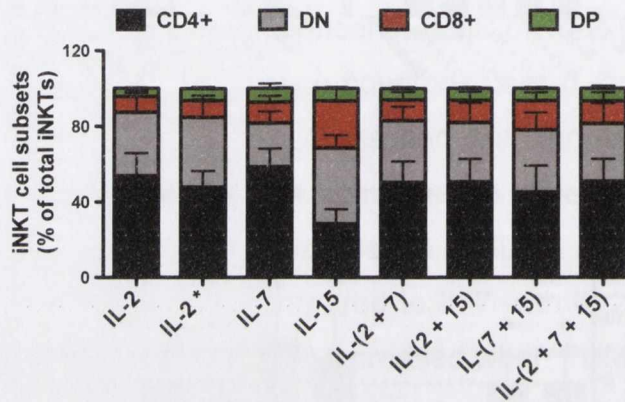


Fig. 3.6. The differential effect of IL-2, IL-7, and IL-15 on iNKT cell subset expansion *in vitro*. PBMCs were incubated for 10 days with the iNKT cell agonist glycolipid α -GalCer in the presence or absence of IL-2 (100 IU/ml), IL-2* (250 IU/ml), IL-7 (10 ng/ml), IL-15 (10 ng/ml), and combinations of IL-7, IL-15, and IL-2 (100 IU/ml). PBMCs were analysed by flow cytometry to assess iNKT cell expression of CD4 and CD8. **A**, Absolute number of CD8⁺, CD4⁺, and DN iNKT cell subset after 10 days culture in the presence of each of the above cytokines. **B**, Proportion of CD4⁺, CD8⁺, DN, and DP iNKT cell as a percentage of the total iNKT cell population after 10 days culture in the presence of each of the above cytokines. Data in both graphs are from 5 healthy donors and plotted as mean $\pm$ SEM. * $P \leq 0.05$ compared to cells cultured in medium alone. Statistical analysis was carried out using the non-parametric Kruskal-Wallis test with post hoc Dunns multiple comparison tests.

3.3.6 iNKT cell medium (iNKTCM) and the anti-microbial Fungizone (Amphotericin B) inversely affect iNKT cell expansion *in vitro*.

Fungizone is a commercial name for the anti-fungal drug Amphotericin B, whose primary target is ergosterols in fungal cell membranes (Anderson et al. 2014). Fungizone binds to these sterols and forms trans-membrane channels that contribute to the target cell death. Fungal and mammalian cells have similar cell membrane composition and therefore cross-reactivity can occur resulting in cytotoxicity. Fungizone is used in combination with other antimicrobials for long-term primary tissue culture. To investigate if Fungizone affects the expansion of iNKT cell lines *in vitro*, PBMCs from 3 healthy donors were stimulated with α -GalCer and IL-2 and cultured for 30 – 40 days in the presence or absence of Fungizone in either a basic medium of cRPMI or the iNKT cell medium (iNKTCM) normally used for iNKT cell expansion (see 2.13.1). Total cell counts and flow cytometric analysis was performed. Although not significant, there was a reduction in the percentages and absolute numbers of iNKT cells when cultured in the presence of Fungizone (Fig. 3.7.A/B). iNKTCM appeared to increase the yields of iNKT cells when compared to cRPMI (Fig. 3.7.A/B). Fungizone caused a reduction in total PBMCs when cultured with both cRPMI and TCM (Fig. 3.7.C).

3.3.7 Yield and purities of iNKT cell lines expanded using PHA or α -GalCer.

iNKT cells were expanded *in vitro* using either PHA and IL-2 or α -GalCer with IL-2 as shown in Figure 3.7. Both methods have previously been described and used to obtain iNKT cell lines (Exley et al. 2010; O'Reilly et al. 2011). PBMCs enriched for iNKT cells from 3 healthy donors were sorted using a MoFlo™ XDP Cell Sorter into CD4⁺ CD8⁺ and DN iNKT cell populations ($\geq 97\%$). Each population was then expanded *in vitro* for 18 days in the presence of irradiated feeder cells and IL-2 (250 IU/ml) and either PHA (10 μ g/ml) or α -GalCer (100 ng/ml). iNKT cell lines were then counted and analysed by flow cytometry to obtain yields and purities. All subsets of iNKT cells stimulated with PHA gave higher yields compared to those obtained using α -GalCer stimulation (Fig. 3.8.B). However, α -GalCer maintained better iNKT cell purities compared to PHA (Fig. 3.8.A) where there was a higher proportion of other CD3⁺ cells.

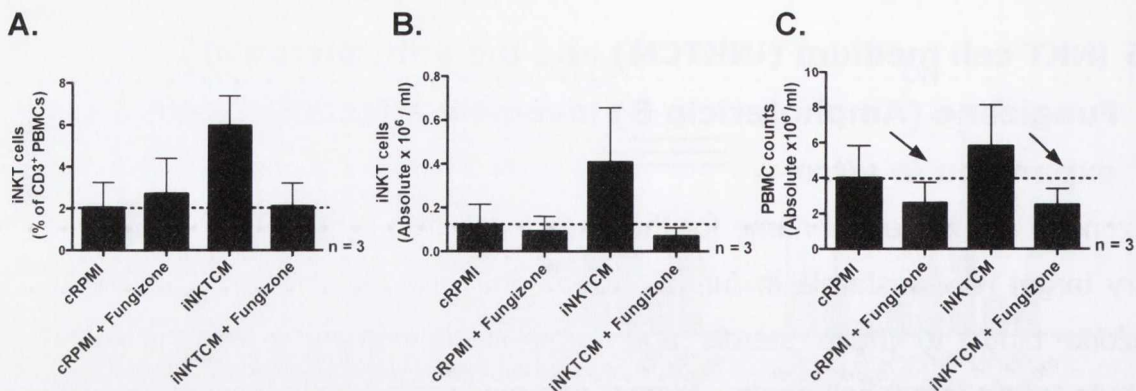


Fig. 3.7. The effect of culture media and Fungizone (Amphotericin B) on iNKT cell expansion *in vitro*. PBMCs were stimulated with α -GalCer (100 ng/ml) and IL-2 (100 IU/ml) for 30 days in cRPMI or iNKTTCM with or without Fungizone. After 30 days the percentage and absolute numbers of iNKT cells in the PBMCs was obtained. **A**, iNKT cells as a percentage of CD3⁺ PBMCs. **B**, Absolute numbers of iNKT cells in the PBMC culture. **C**, Absolute counts of PBMCs after 30 days in culture. Data is plotted as mean $\pm$ SEM from 3 healthy donors.

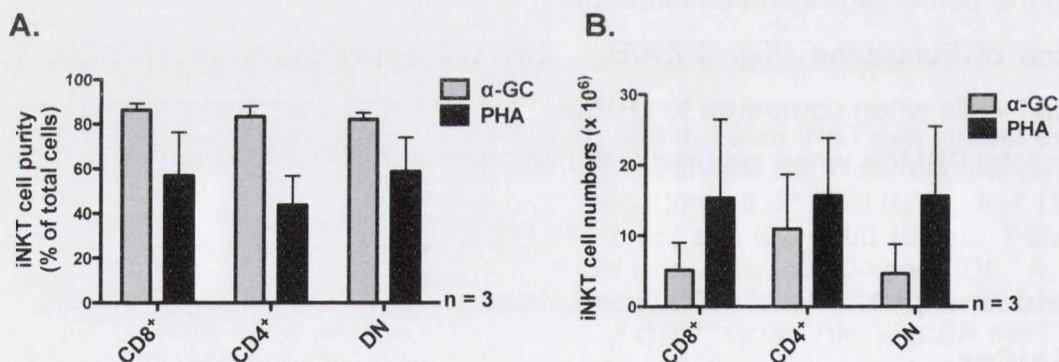


Fig. 3.8. The effect of PHA and α -GalCer on the yields and purities of CD8⁺, CD4⁺, and DN iNKT cell lines. FACS purified iNKT cell subsets from 3 healthy donors were stimulated *in vitro* for 18 days with either PHA or α -GalCer, in the presence of IL-2 and irradiated feeder PBMCs. Cell counts were obtained and cells were analysed by flow cytometry for iNKT cell purities. **A**, Percentage purities of CD8⁺, CD4⁺, and DN iNKT cell lines. **B**, Absolute numbers of iNKT cells from each of three iNKT cell subset lines. Data are plotted as mean $\pm$ SEM from 3 healthy donors.

3.3.8 iNKT cells can be expanded from small blood samples.

The success of expanding iNKT cell lines using the methods described in (3.3.4) depends on a large starting number of PBMCs (i.e. $[0.5 - 1] \times 10^9$). When such numbers of PBMCs are enriched for iNKT cells using MoFlo™ XDP cell sorting, a pure population of iNKT cells ranging in numbers from $(0.01 - 1) \times 10^6$ cells were obtained. These are then expanded *in vitro* for 30 days to obtain an iNKT cell line. Considering that healthy blood PBMCs are present at $\sim 1 \times 10^6$ cell / ml of blood, and considering that as little as 3 ml of blood is obtained from clinical patients and healthy controls, an alternative method was required to expand iNKT cell lines from such samples without the need for cell sorting iNKT cells from 3 ml of blood. PBMCs were prepared from 3 ml of blood, given 100 ng/ml of α -GalCer, and cultured for 20 days in the presence of 100 IU/ml of IL-2 and 10 ng/ml of IL-7. PBMCs enriched for iNKT cells were then subjected to MoFlo™ XDP cell sorting to obtain a pure iNKT cell population. iNKT cells were then further expanded *in vitro* by co-culturing with irradiated feeder PMBCs (1:200), α -GalCer, IL-2, and IL-7 as above for 30 days. All iNKT cell lines prepared from small fresh peripheral blood samples were processed in this manner. A schematic flow chart with representative flow cytometry plots at various stages of expansion as shown in (Fig. 3.9).

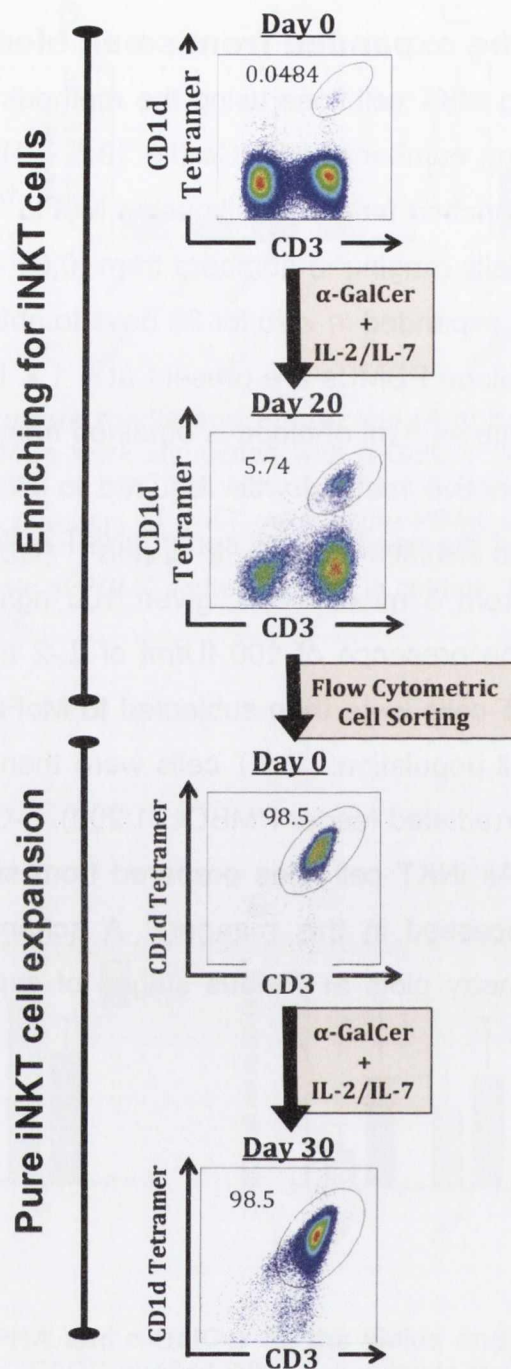


Fig. 3.9. Expansion of iNKT cells from small amounts of blood. PBMCs were isolated from 3 ml of blood, containing (0.05 %) iNKT cells and were enriched *in vitro* by pulsing with α -GalCer (100 ng/ml), IL-2 (100 IU/ml), and IL-7 (10 ng/ml) for 20 days to obtain (5.74 %) iNKT cells. PBMCs enriched for iNKT cells were then sorted using a MoFlo™ XDP cell sorter to a purity of (98.5 %). iNKT cells were then expanded using α -GalCer (100 ng/ml), IL-2 (100 IU/ml), and IL-7 (10 ng/ml) for 30 days. iNKT cell maintained a purity of (98.5 %) after expansion.

3.3.9 The kinetics of iNKT cell expansion using α -GalCer.

iNKT cells must be expanded *in vitro* to obtain adequate numbers of cells for *in vitro* manipulation. Peripheral blood from healthy donors usually contain a few hundred iNKT cells / ml of blood, which can become a few million iNKT cells / ml of blood after expanding for 30 days. Understanding the kinetics of iNKT cell expansion is important when determining the amount of time sufficient to obtain a desired cell yield or enrichment of iNKT cells within PBMCs before MoFlo™ XDP Cell Sorting. Changes in iNKT cell numbers and percentages in PBMCs stimulated for 30 days were monitored. The rate of proliferation beyond the 30 days, where the iNKT cells were sorted and re-stimulated for a further 12 days was also examined. PBMCs from 16 healthy donors were stimulated *in vitro* for 30 days with α -GalCer (100 ng/ml), IL-2 (100 IU/ml), and IL-7 (10 ng/ml). iNKT cells were then isolated using a MoFlo™ XDP Cell Sorter, re-stimulated for 12 days with α -GalCer, IL-2, IL-7, and irradiated feeder PBMCs. The total cell counts and percentage of iNKT cells were obtained at day 0, 10, 20, and 30 as well as day 12 after re-stimulation (i.e. day 42). The percentage of iNKT cells within total CD3⁺ PBMCs was significantly increased at day 10 and continued to increase until day 20 of stimulation (Fig. 3. 10. A). iNKT cell numbers were significantly higher at day 10 and continued to increase until day 30 of stimulation (Fig. 3. 10. B). The absolute numbers of iNKT cells after 30 days of expansion correlated with the starting iNKT cells stimulated at day 0 (Fig. 3. 10. C).

The number of rounds of cell division (i.e. population doubling (PDL)) significantly increased after 10 days of expansion. PDL continued to increase over the 42 days, however PDL at day 30 was not significantly higher than that at day 10, nor was PDL at day 42 significantly higher than that at day 30 (Fig. 3.10.C).

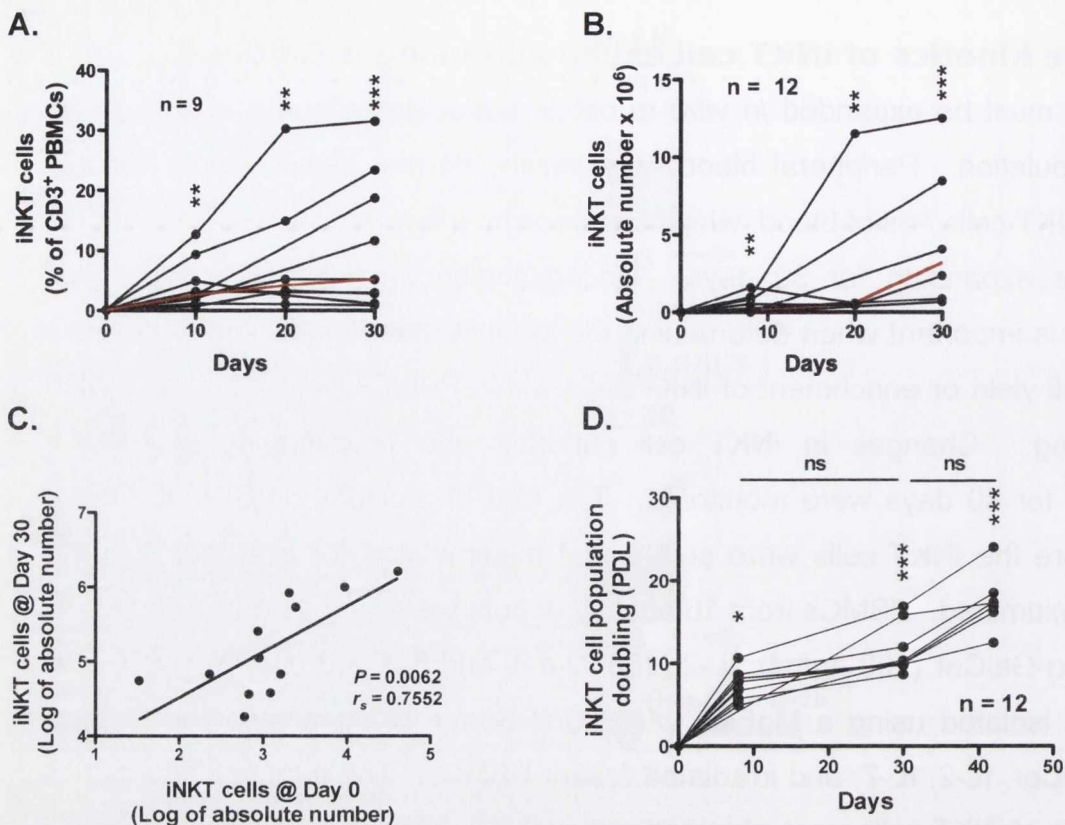


Fig. 3.10. Changes in the percentages and absolute numbers of iNKT cells in α -GalCer stimulated PBMCs over time. PBMCs were stimulated with 100 ng/ml of α -GalCer in the presence of 100 IU/ml of IL-2 and 10 ng/ml of IL-7 for 30 days. iNKT cells were then isolated using the MoFloTM XDP Cell Sorter then re-stimulated as above for a further 12 days in the presence of irradiated feeder PBMCs. Cell counts were performed and the percentage of iNKT cells within CD3⁺ PBMCs were obtained using flow cytometry. **A**, Percentage of iNKT cells from 9 healthy donors at days (0, 10, 20, and 30) post stimulation. **B**, Absolute counts of iNKT cells from 12 healthy donors at days (0, 8, 20, and 30) post stimulation. **C**, Correlation plot between starting iNKT cell numbers and numbers after 30 days of expansion from 12 healthy donors. **D**, iNKT cell population doubling from 12 healthy donors at days 8 and 30 post stimulation, and day 12 post re-stimulation. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared to day 0. Horizontal lines (**D**) compare days 10 and 30, and days 30 and 42. Statistical analysis was carried out using the non-parametric Kruskal-Wallis test with post hoc Dunns multiple comparison tests (**A**, **B**, **D**), and Spearman correlation analysis (r_s = Spearman correlation coefficient).

3.3.10 CD4 and CD8 expression by expanding iNKT cells.

iNKT cells can be subdivided based on the expression of CD4 and CD8 into 4 subsets: CD4⁺, CD8⁺, DN (double negative), and DP (double positive). O'Reilly and coworkers (2011) demonstrated that CD4⁺ iNKT cells expand more readily than CD8⁺ and DN iNKT cells when cultured with PHA and IL-2 (250 IU). To investigate whether iNKT cell subsets are differentially expanded using the new method of expansion, PBMCs from 10 healthy donors were cultured in the presence of α -GalCer, IL-7, and IL-2 (100 IU/ml) for 30 days then measured the percentage of each subset by flow cytometry. There was a significant decrease in the proportion of CD4⁺ and a significant increase in DP subsets relative to the total iNKT cell populations. There was also a trend toward increase CD8⁺ and DN cells based on the median change after 30 days expansion (Fig. 3.11).

CD8⁺ and DN iNKT cells are hard to definitively separate using MoFlo™ XDP Cell Sorting due to the lack of a clear separation between the two populations based on CD8 expression (Fig. 3.12.A). When iNKT cells were sorted into the different subsets there is always a 3 – 5 % impurity particularly in the DN and CD8⁺ iNKT cell populations. The long-term effect of culturing MoFlo™ XDP Cell Sorted iNKT cell subsets on their purity was investigated. Three of the iNKT cell lines previously enriched from PBMCs were sorted into CD4⁺, CD8⁺, and DN iNKT cell populations (each > 95 % pure), which were then expanded for 30 days using α -GalCer (100 ng/ml), IL-2 (100 IU/ml), and IL-7 (10 ng/ml). At day 20 and day 40 the purities of each line was measured by flow cytometry. The purities of CD4⁺ iNKT cells did not change over 40 days of culture, except for a slight rise in DP iNKT cells. The purities of DN iNKT cell lines did not change, where contaminating CD8⁺ iNKT cells remained at < 15 % in all lines. However, the purities of CD8⁺ iNKT cell lines did show a decline between 20 and 40 days in culture, where DN iNKT cells increased from around 10 % to over 20 % of total CD8⁺ iNKT cell lines (Fig. 3.12.B).

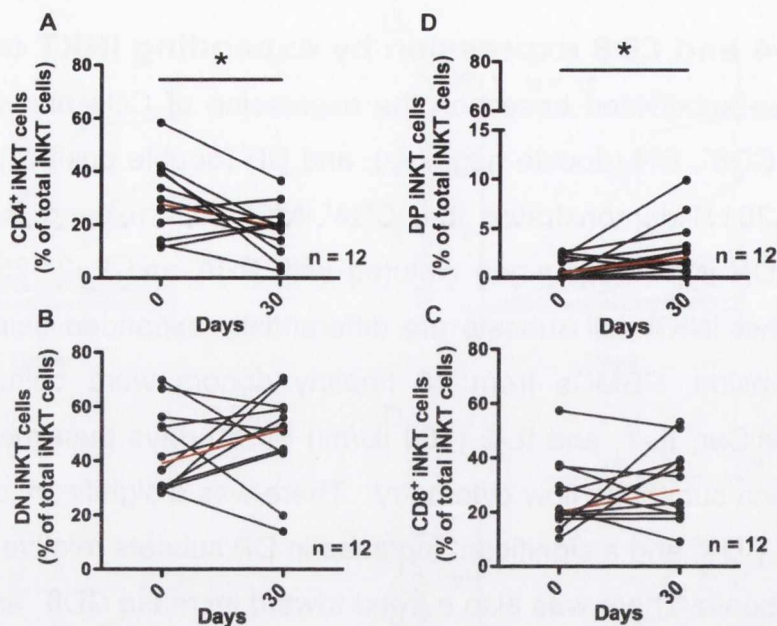
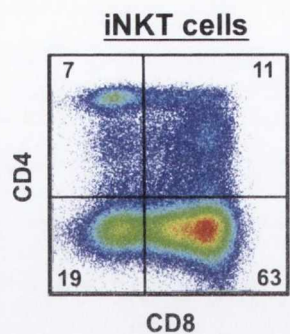


Fig 3.11. Changes in iNKT subset proportions in PBMCs after 30 days expansion. PMBCs were cultured for 30 days with α -GalCer, IL-7, and IL-2. The percentage of each iNKT cell subset was measured by looking at CD4 and CD8 expression on iNKT cells by flow cytometry. Displayed are four graphs showing the percentage of CD4⁺ (A), DN (B), CD8⁺ (C), and DP (D) iNKT cells as a percentage of total iNKT cells at day 0 and day 30 of expansion. Each donor is represented by two connected dots. The median result from all 12 donors is graphed as a red line.

A.



B.

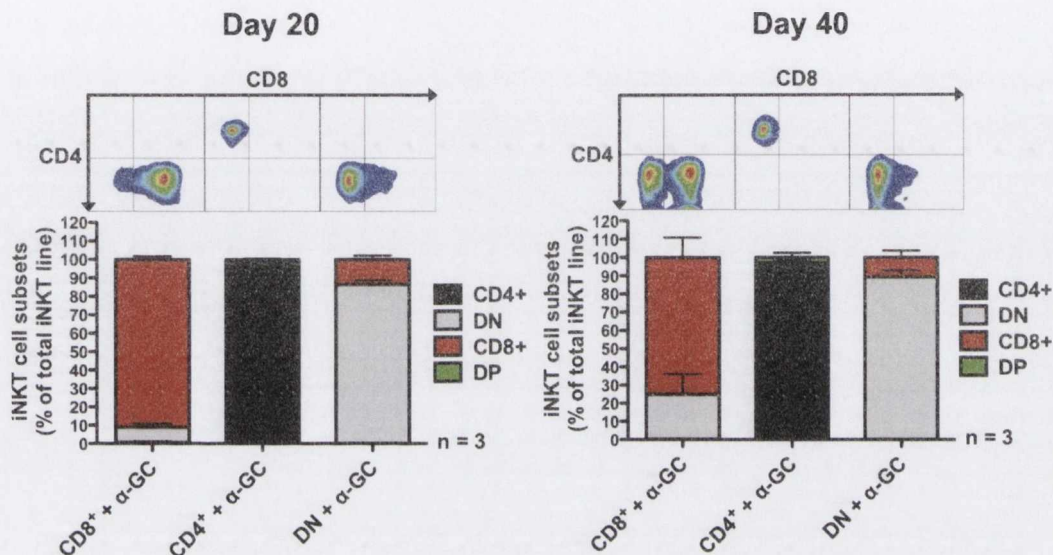


Fig 3.12. Changes in the purity of expanded CD8⁺, CD4⁺, and DN iNKT cell lines. Total iNKT cells were enriched from PBMCs then sorted into pure populations of CD8⁺, CD4⁺, and DN iNKT cells using MoFlow™ Cell Sorting. Sorted cells were expanded in culture for 40 days. The purity of each subset line was measured by flow cytometry at day 20 and day 40 of expansion. **A**, Flow cytometry dot plot of expanded iNKT cells showing CD4 and CD8 expression. **B**, Two bar charts showing the purities of CD8⁺, CD4⁺, and DN iNKT lines at day 20 and day 40. Each column represents one iNKT cell subset line and is composed of stacked bars representing the subset constituents of each line. Data are plotted as mean ± SEM from 3 healthy donors.

3.3.11 PD-1 and CD31 expression by expanding iNKT cells.

PD-1 is an inhibitory molecule that is expressed on activated T cells and can be upregulated on exhausted cells (Schietinger & Greenberg 2012). CD31 is a marker that has been shown to correlate with the presence of TRECs in T cells and can be used as an indicator for residual replicative potential (Junge et al. 2007). To examine the long-term effects of expanding iNKT cells in culture on PD-1 and CD31 expression, iNKT cell lines from 11 healthy donor were expanded using α -GalCer (100 ng/ml), IL-2 (100 IU/ml), and IL-7 (10 ng/ml). iNKT cells were sorted and re-stimulated at days 30 and day 75 from the initial day 0 stimulation. The expression of CD31 and PD1 was measured by flow cytometry at various time points throughout a 100-day period from the initial stimulation. CD31 expression was dramatically decreased over the first 60 days of stimulation from about 70 % to 10 % of total iNKT cells (Fig. 3.13.A). PD-1 maintained a steady level of expression throughout the full 100 days in culture, with the exception of a spike in expression 10 days after the initial stimulation (Fig. 3.13.B).

3.3.12 Testing the functionality of iNKT cell lines: IFN- γ and IL-4 secretion.

The functionality of generated iNKT cell lines was tested by examining IFN- γ and IL-4 secretion upon stimulation with α -GalCer. iNKT cells (0.25×10^6 /ml) from 14 iNKT cell lines were co-cultured for 24 hours with either CD1d-HeLa cells (i.e. HeLa cells transfected with the CD1d gene) or Mock-HeLa (i.e. HeLa cells transfected with an empty vector) pulsed overnight with either α -GalCer (100 ng/ml) or DMSO vehicle control. IFN- γ and IL-4 levels in the supernatants were measured using ELISA. The levels of both cytokines were increased upon activation with α -GalCer loaded CD1d-HeLa cells when compared to the unloaded CD1d-HeLa cells (Fig. 3.14.). No cytokines were detected in the supernatants of the mock-HeLa co-cultures (data not shown).

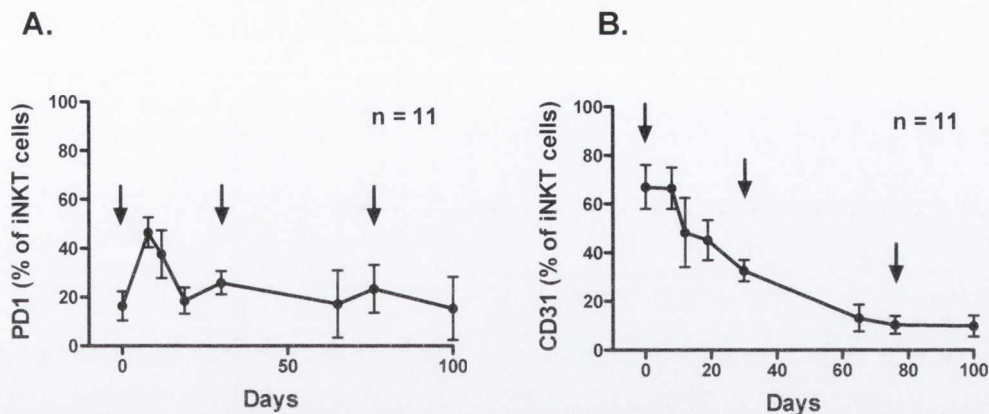


Fig 3.13. Changes in CD31 and PD1 expression on expanding iNKT cells over time. PBMCs from 11 healthy donors were stimulated with α -GalCer (100 ng/ml), IL-2 (100 IU/ml), and IL-7 (10 ng/ml) for 30 days. iNKT cells were isolated using MoFloTM cell sorting and re-stimulated with α -GalCer, IL-2, IL-7, and irradiated feeder cells for 45 days, and again re-stimulated for another 25 days. Throughout the 100 days in culture the iNKT cells were monitored by flow cytometry for CD31 and PD1 cell surface expression. **A**, Percentage of iNKT cells expressing PD1 at various time points during expansion. **B**, Percentage of iNKT cells expressing CD31 at various time points during expansion. The arrows delineate the time of stimulation. Data are plotted as mean $\pm$ SEM from 11 healthy donors.

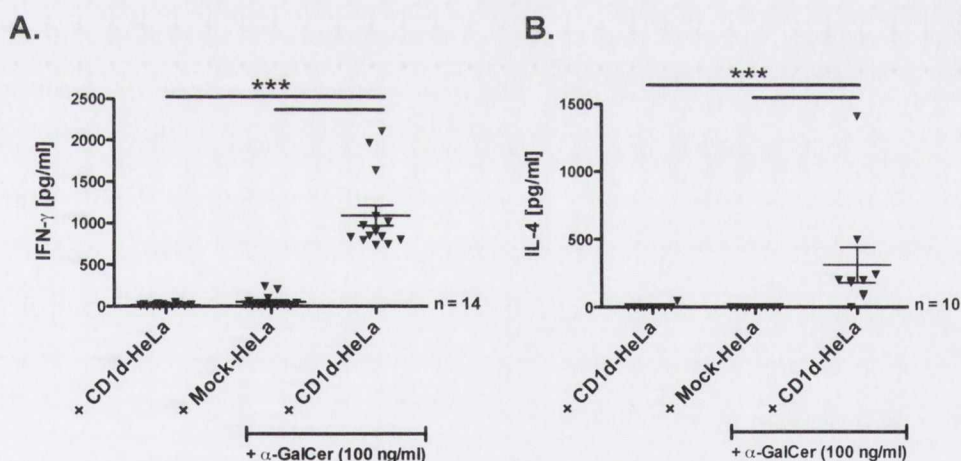


Fig 3.14. IFN- γ and IL-4 secretion by α -GalCer activated iNKT cell lines. iNKT cells were rested in cytokine free medium for 24h then co-cultured with either Mock-transfected HeLa cells or CD1d-transfected HeLa cells that were previously pulsed with α -GalCer (100 ng/ml) or DMSO vehicle control for 24 hours. Supernatants from the co-cultures were assayed by ELISA for the presence of IFN- γ and IL-4. **A**, IFN- γ levels from 14 iNKT cell lines. **B**, IL-4 levels from 10 iNKT cell lines. *** $P \leq 0.001$ comparing α -GalCer loaded CD1d-HeLa with either unloaded CD1d-HeLa or Mock-HeLa cells. Statistical analysis was carried out using the non-parametric Kruskal-Wallis test with post hoc Dunns multiple comparison tests.

The α -GalCer derivative (7DW8-5) is marketed as a more potent iNKT cell agonist. The potency of 7DW8-5 and α -GalCer were compared in terms of their ability to induce cytokine production. Three different iNKT cell lines were co-cultured with Mock-HeLa or CD1d-HeLa cells in the presence of various concentrations of 7DW8-5 or α -GalCer. IFN- γ and IL-4 secretion was assessed after 24 hours in culture. 10 ng/ml of both α -GalCer and 7DW8-5 was sufficient to significantly increase IFN- γ and IL-4 production in the presence of CD1d-HeLa cells but not Mock-HeLa, hence showing no difference in potency between the two glycolipids (Fig. 3.15. A/B).

3.3.13 Testing the functionality of expanding iNKT cell lines: CD107a expression.

The ability of iNKT cell lines to kill CD1d⁺ target cells presenting the glycolipid ligand α -GalCer was investigated. CD107a is an effector cell marker that correlates with target cell killing (Aktas et al. 2009). CD107a surface expression was detected by flow cytometry as shown in (Fig.3.16.A). Mock-HeLa or CD1d-HeLa cells were labeled with α -GalCer (100 ng/ml) or vehicle (DMSO) control and co-cultured with equal numbers of iNKT cells for 4 hours. Cells were stained for CD107a, CD4, and CD8 (see 2.19). CD107a was upregulated on iNKT cells in the presence of CD1d-HeLa cells presenting α -GalCer (Fig. 3.16.B). CD8⁺ and DN iNKT cell subsets showed significant increases in CD107a expression in the presence of CD1d-HeLa presenting α -GalCer compared to vehicle control (Fig. 3.16.C). CD107a expression by CD4⁺ and DP iNKT cells was only moderately upregulated in the presence of α -GalCer.

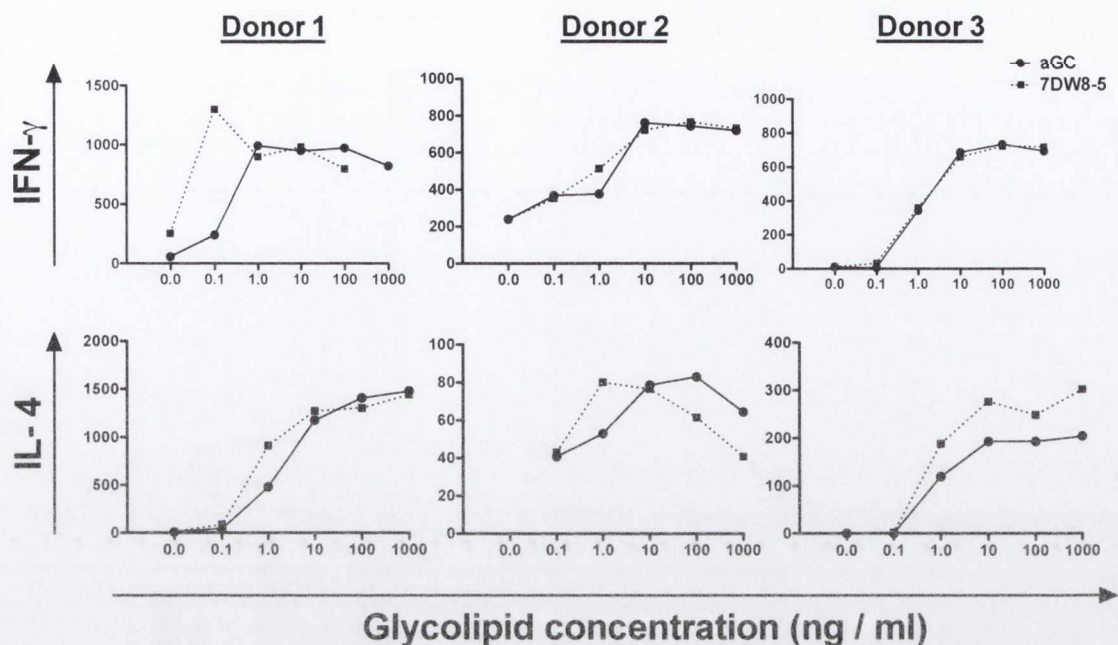


Fig 3.15. IFN- γ and IL-4 secretion in response to various concentrations of α -GalCer and 7DW8-5. iNKT cells were rested in cytokine free medium for 24h then co-cultured with CD1d transfected HeLa cells in the presence of various concentrations (0 – 1000 ng/ml) of α -GalCer (—) or 7DW8-5 (---). Supernatants were tested by ELISA for the presence of IFN- γ and IL-4. Each iNKT cell line (i.e. donor) is plotted as a separate graph.

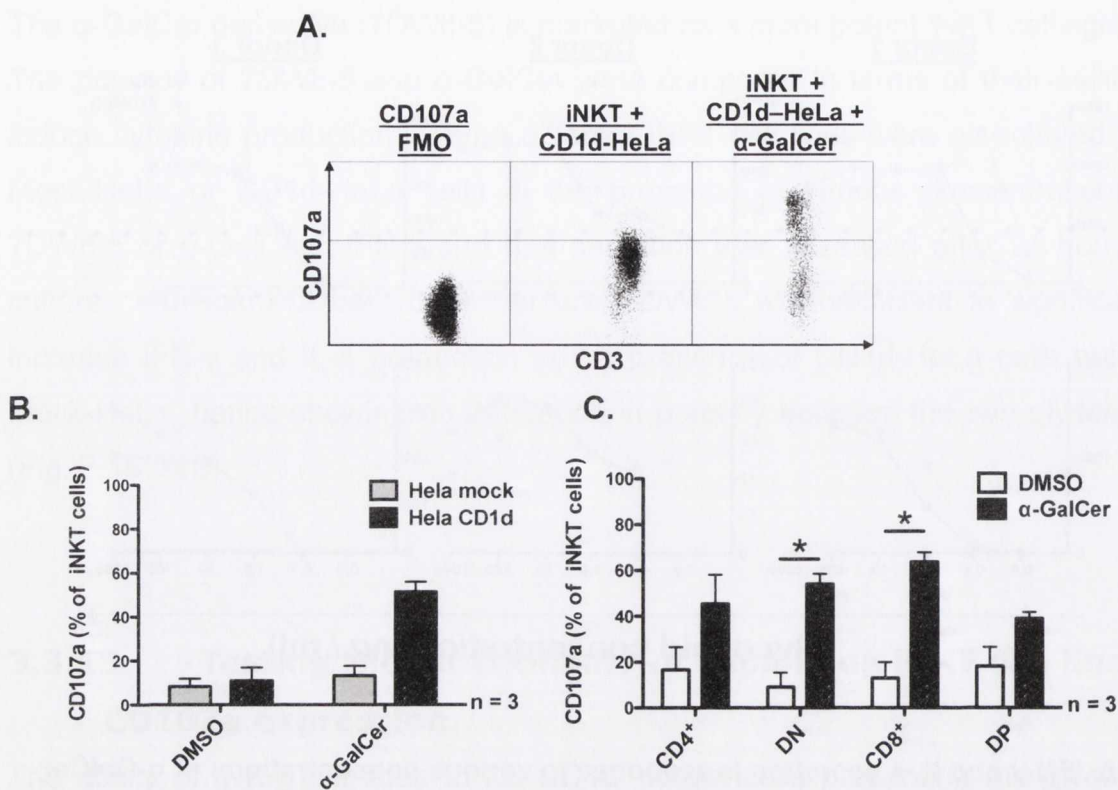


Fig 3.16. CD107a expression by iNKT cells stimulated with α -GalCer. iNKT cells were co-cultured with mock transfected HeLa cells or CD1d transfected HeLa cells in the presence of vehicle or α -GalCer for 4 hours in the presence of a CD107a staining Ab and the protein transport inhibitor monensin. iNKT cells were stained for CD4 and CD8 and analysed by flow cytometry. **A**, Flow cytometry dot plots showing iNKT cells in the absence of the CD107a mAb (CD107a FMO), and in the presence of HeLa-CD1d and anti-CD107a Ab with and without α -GalCer. **B**, Bar chart showing the percentage expression of CD107a by total iNKT cells. **C**, Bar chart showing percentage expression of CD107a by CD4⁺, DN, CD8⁺, and DP iNKT cells. Data are plotted as means $\pm$ SEM from 3 iNKT cell lines. * $P \leq 0.05$ comparing CD1d-HeLa and its corresponding Mock-HeLa stimulated iNKT cells, using the non-parametric Kruskal-Wallis test with post hoc Dunns multiple comparison tests.

3.4 Discussion.

iNKT cells are innate-like T lymphocytes that are defined in humans as CD3⁺ cells expressing a V α 24⁺ V β 11⁺ TCR that recognizes the Ag presenting CD1d molecule found on APCs. The canonical Ag that is used to identify and stimulate iNKT cells is the synthetic glycolipid α -GalCer, which is presented in the context of a CD1d molecule. iNKT cells in humans represent a small proportion of CD3⁺ cells that vary in frequency from one tissue to another, and from one person to another (Berzins et al. 2011).

In peripheral blood (PB) iNKT cells represent < 1 % of CD3⁺ cells but can reach up to 10% in omental tissue (Lynch et al. 2009). A cohort of healthy control subjects (n = 24) with a median age of 40 were recruited. PBMCs were isolated and stained with a PBS-57-loaded CD1d tetramer and analysed by flow cytometry to enumerate iNKT cells. As shown in Fig. 3.2 iNKT cells were found to represent a median of 0.1 % of total CD3⁺ cells. The proportion of iNKT cells in this cohort significantly decreased with age, contrary to a recent publication that reported iNKT cells did not change in frequency from birth through adulthood (Bienemann et al. 2011). However, a past report using only V α 24 as an iNKT cell marker reported a significant decrease in such cells in elderly subjects (DelaRosa et al. 2002). It is important to note that Bienemann et al, studied adults up to the age of 45, while the results shown in the present study include ages up to 70. The data obtained from healthy individuals (n = 23) gave a significant negative correlation between iNKT cell frequencies and age (P = 0.05), and between iNKT cell numbers age (P = 0.0001).

iNKT cells are generally categorized phenotypically based on their expression of CD4 and CD8: CD4⁺, CD8⁺, CD4⁻CD8⁻ (DN), and CD4⁺CD8⁺ (DP). Flow cytometric analysis of CD3⁺ 6B11⁺ and CD3⁺ PBS-57 CD1d tetramer⁺ cells from 13 healthy PBMC samples showed DN iNKT cells to be the most abundant subset (median > 40 %), followed by CD4⁺ and CD8⁺ iNKT cell subsets (median ~ 30 %). Previously O'Reilly et al. found that DN iNKT cells (~ 50 %) are significantly more prevalent than CD4⁺ iNKT cells (< 20 %), with CD8⁺ iNKT cells averaging at around 30 %. Another study found that CD4⁺ iNKT cells make up ~ 40% of iNKT

cells from PBMCs compared to 90% from cord blood and thymus where they make up the majority of iNKT cells (Baev et al. 2009).

There are a number of possible factors that might influence the difference seen in CD4 and CD8 expression between different healthy cohorts, including the staining method used for iNKT cell identification, age, and total iNKT cell frequency. Montoya and colleagues showed slightly higher frequencies of CD8⁺ and lower frequencies of CD4⁺ iNKT cells when staining with 6B11 Ab compared to PBS-57 loaded CD1d tetramer (Montoya et al. 2007). This finding might partly explain why there was an increase in CD4⁺/CD8⁺ ratio when PBS-57 loaded CD1d tetramer was used (Fig. 3.3) compared to the findings in O'Reilly et al, where 6B11 was the staining method of choice. Age was excluded as a factor influencing subset distribution, as no correlation was found between age and the percentage of each iNKT cell subset (data not shown). Montoya et al, also demonstrated in their study that frequencies of iNKT cells as a percentage of CD3⁺ cells correlate positively with DN iNKT cell frequency and negatively with CD4⁺ iNKT cell frequency. However, no such correlation was found in the current study (data not shown).

Peripheral blood is the most abundant and easily accessible human specimen. However, with iNKT cells only making up 0.01 – 0.1 % of total PBMCs (Berzins et al. 2011), 1ml of blood contains only 100 – 1000 iNKT cells. Such small numbers of iNKT cells requires *in vitro* expansion to obtain enough cells for *in vitro* analysis. Two general methods for iNKT cell expansion have been described by others (Bricard et al. 2010; Exley et al. 2010; S. M. A. Tahir et al. 2001; O'Reilly et al. 2011). Both involve enriching iNKT cell from PBMCs and then stimulating them to proliferate using either α -GalCer with IL-2 or PHA with IL-2 for 20 to 30 days in culture (Fig. 3.4). Using each of the two methods pure populations of iNKT cells (> 97 % of PBMCs) were obtained as demonstrated by flow cytometry.

A number of clinical trials that focus on harnessing the direct and indirect cytotoxic effects of autologous iNKT cells are ongoing for humans with cancer. One strategy involved removing PBMCs from the patient, stimulating them *ex vivo* with α -GalCer and IL-2 to expand iNKT cells, and returning them to the patient. This was found to be safe and caused no adverse clinical effects (Kunii et al. 2009; Motohashi et al. 2006). In the present study, I optimised a method utilizing α -

GalCer and IL-2 for generating iNKT cell lines from buffy coat packs, and this was further refined to generate iNKT cell lines from as little as 2 ml of blood. Methods were optimized to give high iNKT cell yields containing CD4⁺, CD8⁺, and DN subsets, as well as for creating separate cell lines of each iNKT cell subset.

To produce an iNKT cell line, PBMCs were first enriched for iNKT cells then sorted into pure iNKT cell populations. The type of enrichment depended on the starting number of PBMCs. Larger number of PBMCs allowed for *ex vivo* iNKT cell enrichment using anti-6B11 coated beads. Smaller numbers of PBMCs meant an *in vitro* culturing step was needed, where PBMCs were stimulated with α -GalCer and IL-2 for a minimum of one week to expand iNKT cell numbers and frequency. Cell sorting was then used to isolate pure population of iNKT cells, which were then further expanded *in vitro* using α -GalCer and IL-2.

To make sure the cells identified and isolated for expansion can be accurately labeled as iNKT cells, the appropriate markers were chosen to label iNKT cells from PBMCs. There has been some debate in the literature as to the most accurate method to phenotypically identify iNKT cells. V α 24 usage is not restricted to iNKT cells as there are V α 24⁺ non-iNKT cells that are not CD1d restricted, and in some cases may co-express the V β 11 TCR chain (Montoya et al. 2007). Although α -GalCer-loaded CD1d tetramer staining identifies all iNKT cells, it also stains some non-iNKT cells, where 0.01 – 0.05 % of CD1d tetramer⁺ cells are V α 24⁺ (Lee et al. 2002; Gadola et al. 2002). For this reason combining α -GalCer-loaded CD1d tetramer staining with V α 24 staining is more accurate for iNKT cell identification (Lee et al. 2002). The 6B11 mAb, which is specific for the V α 24-J α 18 CDR3 loop, which is responsible for recognizing α -GalCer loaded CD1d is highly specific for iNKT cells when used alone or in combination with an anti-V β 11 Ab. However, the 6B11 mAb can fail to detect iNKT cells that have an altered CDR3 region due to a single amino acid (aa) substitution (Exley et al. 2008). Keeping all this in mind, the method described in this study involves the sorting of V α 24⁺V β 11⁺ T cells and subsequent stimulation with APCs loaded with α -GalCer, causing the expansion of V α 24⁺V β 11⁺ iNKT cells that are CD1d restricted. However, if 6B11 enrichment is used prior to expansion, rare iNKT cell clones with CDR3 alterations may be excluded. Hence, if the objective is to

generate lines of iNKT cells clones, it may not be advisable to pre-select/enrich with an anti-6B11 mAb.

CD4⁺, CD8⁺, and DN iNKT cells are functionally distinct subsets of iNKT cells. O'Reilly and colleagues previously demonstrated that expanding iNKT cells *in vitro* using PHA and IL-2 for 30 days resulted in a preferential expansion of CD4⁺ iNKT cells (O'Reilly et al. 2011). In the present study a method for expanding iNKT cells was optimized to give high iNKT cell yields while reducing the bias towards CD4⁺ iNKT cell expansion. Since IL-7 and IL-5 promote CD8⁺ T cell growth and survival (Diab et al. 2005; Wallace et al. 2006), we tested the ability of combinations of IL-2, IL-7, and IL-15 to enhance CD8⁺ iNKT cell expansion. PBMCs were stimulated with 100 ng/ml of α -GalCer for 10 days in the presence of IL-7 (10 ng/ml), IL-15 (10 ng/ml), and/or two concentrations of IL-2: IL-2 (100 IU/ml) or IL-2* (250 IU/ml). IL-7 in combination with IL-2 led to increased total iNKT cell yields in 4 out of the 5 donors when compared to IL-2* alone. Although not statistically significant, there was better CD8⁺ iNKT cell expansion with IL-7 and IL-2 compared to IL-2 alone. IL-15 with IL-2 also promoted expansion of both total iNKT cells and CD8⁺ iNKT cells, but the results were more variable from one donor to the next. In order to confirm the comparative findings that these cytokine cocktails improve CD8⁺ iNKT cell expansion a larger cohort of donors are needed, and expansion time should be increased. IL-2 and IL-7 were chosen in combination to expand iNKT cell lines for this study, and as shown in Fig. 3.12. expanding iNKT cells in this way caused a significant reduction in the percentage of CD4⁺ iNKT cells, while there was an increase seen in all other subset percentages.

Amphotericin B (AmB), an anti-fungal compound is used to treat systemic and deep-seated fungal infections in humans. AmB has a wide range of toxic side effects that are mainly attributed to its ability to cross-react with cholesterol and therefore causing host cell damage. A wide range of immune-modulatory and immune-inhibitory effects of AmB have been reported in animal models, which in turn may contribute to a wide range of systemic side effects (Chapman et al. 2011). NK cell activity including ADCC were reduced in PBMCs pretreated with AmB (Nair & Schwartz 1982). AmB was also reported to decrease T cell proliferation in response to PHA (Tärnvik & Ansehn 1974; Stewart et al. 1981) and ConA but not PMA/I stimulation (Kumar & Chakrabarti 2000). All of the above

effects of AmB were observed at concentrations similar to those recommended for use in tissue culture (~ 2.5 µg/ml). Fungizone, a tissue culture marketed AmB, was utilized as part of culture medium for *in vitro* expansion of iNKT cells (O'Reilly et al. 2011). However, no study has looked the effects of Fungizone on iNKT cell expansion using α-GalCer. The effects of Fungizone, as well as the use of iNKTCM (i.e. fortified cRPMI medium – see methods 3.x.x) compared to cRPMI medium on iNKT cell expansion were investigated. PBMCs from 3 healthy donors were stimulated with α-GalCer and cultured for 30 days in cRPMI or iNKTCM containing IL-2, with or without Fungizone (2.5 µg/ml). As expected, total PBMC numbers were higher when grown in iNKTCM compared to cRPMI (Fig. 3.7.C), as this medium is designed to support T cell growth. Interestingly, iNKTCM caused a noticeable increase in both percentage and absolute number of iNKT cells compared to PBMCs cultured in cRPMI alone. However, no such increases were observed in the presence of 2.5 µg/ml of Fungizone. The numbers of both iNKT cells and total PBMCs were reduced when Fungizone was present. The results indicate that the addition of EAA, NEAA, sodium pyruvate, 2-mercaptoethanol, and L-glutamine to cRPMI are important in selectively promoting iNKT cell expansion. The use of Fungizone in iNKTCM reduced both yields and purities of iNKT cells expanded using α-GalCer from PBMCs. For these reasons iNKTCM containing no Fungizone was subsequently used for iNKT cell tissue culture.

Both PHA and α-GalCer can be used to expand iNKT cells. We compared the capacity of these stimuli to expand CD4⁺, CD8⁺, and DN iNKT cells. PBMCs from 3 healthy donors were subjected to MoFlo™ cell sorting to isolate CD4⁺, CD8⁺, and DN iNKT cells. Each population was then stimulated with either PHA or α-GalCer in the presence of IL-2 and irradiated PBMC feeder cells for 18 days. Although there was no statistically significant difference, α-GalCer gave better iNKT cell purities compared with PHA (Fig. 3.8.A). The observed expansion of non-iNKT cells in the PHA stimulated population indicate that the 1-3 % of residual non-iNKT cells expanded more efficiently in response to PHA, causing the reduction in total iNKT cell purity. Our findings suggest that α-GalCer is a better stimulus for maintaining good iNKT cell purities, but PHA gave slightly better iNKT cell yields. In cases where a highly pure starting iNKT cell population is guaranteed, PHA could be a better stimulus for iNKT cell expansion.

So far, it has been shown that stimulating sorted iNKT cells with irradiated feeder cells and α -GalCer and culturing them in iNKTCM containing IL-2 and IL-7 without Fungizone is optimal for generating lines of iNKT cells and for increasing CD8⁺ and DN iNKT cell yields. Following from these findings the method for expanding iNKT cells from PBMCs was carried out as per Fig. 3.4. In order to expand iNKT cells from small starting numbers of total PBMCs, iNKT cells were first expanded in numbers within PBMCs using α -GalCer, IL-2 and IL-7 for up to 20 days. Once a large enough population of iNKT cells were obtained, MoFloTM cell sorting was used to isolate a pure iNKT cell population(s). Sorted iNKT cells were then expanded in culture for 30 days as described above. This pre-enrichment step was required when dealing with small blood samples of 2-3 ml (e.g. from CLL patients), but also when PBMCs are depleted of iNKT cells due to a predisposing condition is the patient cohort (e.g. CLL patients have reduced iNKT cells numbers).

We next examined the kinetics of iNKT cell expansion using this method in order to determine the time required to generate significant numbers of iNKT cells, changes in subset composition after expansion, and the capacity for multiple rounds of expansion. Using 16 donor PBMCs the purities and absolute numbers of iNKT cells were determined 10, 20, and 30 days post stimulation with α -GalCer. The most dramatic increases were observed 10 days post stimulation. iNKT cells continued to double in population size for 30 days after the first stimulation, and again when measured 12 days after a second stimulation at day 42 indicating that 10 days is enough to expand iNKT cells and that they can be restimulated to achieve further expansion.

There was a large variability in iNKT cell percentages and yields from different donors after expansion. PDL also varied between donors at the different time points. Correlation analysis revealed a positive correlation between the starting numbers of iNKT cells and yields after 30 days, which suggests that the variability observed between donor yields is relative to the starting number of iNKT cells and not due to other donor specific factors (e.g. age). Furthermore, there was no correlation found between PDL and age or PDL and *in vivo* percentages of iNKT cells (data not shown), suggested variability in PDL is independent of age and PB frequency of iNKT cells. Previous studies have suggested that the proliferative

potential (i.e. the number of PDLs a cell undergoes before reaching senescence) of T cells can decrease with age (Junge et al. 2007; Kimmig et al. 2002; Kushner et al. 2010), and has been measured to be a mean value of 41 PDLs for T cells (Effros & Pawelec 1997). However, because the type of cell can bear effect on residual replicative potential (Effros & Pawelec 1997), iNKT cells may differ from other T cells in their residual replicative potential. Residual proliferative potential of the expanded iNKT cell lines could not be evaluated as 42 days in culture was not enough time to observe proliferative senescence.

As previously mentioned one of the aims was to establish a method for expanding iNKT cells that did not cause a significant outgrowth of CD4⁺ iNKT cells and a concomitant reduction in CD8⁺ iNKT cells. After 30 days post α -GalCer stimulation there was a statistically significant decrease in CD4⁺ and an increase in DP iNKT cells, while CD8⁺ and DN iNKT cells showed a slight upward trend. These results demonstrate that expanding iNKT cells using α -GalCer and IL-2 and IL-7 improves the yields of CD8⁺ and DN iNKT cell subsets. Since CD8⁺ and DN iNKT cells appear to have the most potent anti-tumour potential, this method may ultimately be useful for selectively expanding anti-tumour iNKT cells.

The next step was to expand each iNKT cell subset in isolation after MoFlo™ cell sorting to obtain large numbers of pure iNKT cell subsets for *in vitro* manipulation. CD8⁺ and DN iNKT cells are hard to definitively separate because of the wide spectrum of CD8 expression. CD4⁺ iNKT cells on the other hand are phenotypically distinct and were easy to sort from other subsets, and as a result CD4⁺ iNKT cells often maintained their purity after expansion. As for sorted DN and CD8⁺ iNKT cells, the purity of each respective subset tended to diminish (Fig. 3.13.B) with expansion time, and further sorting was required prior to subsequent rounds of re-stimulations. The observed changes in subset composition in each expanded iNKT cell line could be attributed to differences in the proliferative potential of one subset over the other, which allows contaminating subsets (e.g. DP iNKT cells) to flourish within the primary subset line (e.g. CD4⁺ iNKT line). Another explanation is the possibility that iNKT cells can change in CD4 and CD8 expression in culture as a function of differentiation or maturation. A study showing that CD4⁺ iNKT cell clones maintained for up to 8 months did not lose CD4 expression would suggest that CD4 expression is determined from the

thymus and fixed throughout the iNKT cell's life span (Baev et al. 2009). However, there has been suggestions that CD4⁺ iNKT cells may undergo a process of differentiation that results in CD4 loss (Sandberg et al. 2004). More in depth studies are required to unravel the dynamics of CD4 and CD8 expression in human peripheral iNKT cells.

An important consideration when repeatedly stimulating cells is the possibility of inducing T cell exhaustion or dysfunction. PD-1 is an inhibitory molecule that binds to PD-L1 and can dampen T cell proliferation and function (Binné et al. 2012). PD-1 expression has been associated with T cell exhaustion, however PD-1 is also upregulated by activated T cells and has recently been shown to be a phenotypic marker of effector memory T cells with no bearing on their functional capacity (Duraismamy et al. 2011). iNKT cells from 11 donors were stimulated at day 0, 30, and 75 with α -GalCer in the presence of IL-2, IL-7, and irradiated feeder PBMCs. PD-1 expression peaked at 10 days post stimulation but leveled off by day 20. Overall PD-1 expression did not change over 100 days of culture, including 3 rounds of stimulation, indicating that iNKT cells did not show signs of dysfunction as measured by PD-1 expression.

Replicative senescence was first discovered by Hayflick and Moorhead in 1961, where diploid cells were found to have finite life spans (Hayflick & Moorhead 1961) and the number of PDLs a cell undergoes in its life span is dubbed the Hayflick limit (Pazolli & Stewart 2008; Hayflick & Moorhead 1961; Hayflick 1965). Telomere shortening occurs with each round of replication, and has been considered a mitotic indicator for replicative senescence. However, telomere shortening is not a perfect indicator as some cells can constitutively express telomerase an enzyme that maintains telomere lengths. T cells can induce telomerase expression upon activation (Park & Angeles 1996; Hodes 1999). Although telomere length decreases with PDLs (Park & Angeles 1996), and telomere shortening is associated with the development of replicative senescence, there is no specific telomere length or threshold beyond which senescence is inevitable (Weng et al. 1995). TCR excision circles (TRECs) are fragments of genomic DNA that are excised during VDJ recombination and remain in the mature naïve T cells as they exit the thymus into the periphery. TRECs cannot be replicated and hence become diluted upon T cell division, making TREC

measurement an indicator of post-thymic cell division (Hazenberg et al. 2001). CD31 also known as platelet/endothelial cell adhesion molecule 1 (PECAM1) is an adhesion molecule expressed on various cells including T cells, with an emerging understanding of its role as a regulatory or T cell responses (Marelli-berg et al. 2013). CD31 expression has been associated with naïve CD45RA⁺ T cells with high TREC content, while naïve CD45RA⁺ T cells with low TREC content, that have presumably expanded in the periphery, are CD31 negative (Kimmig et al. 2002; Junge et al. 2007). These CD31⁺CD4⁺CD45RA⁺ cells also showed a significantly higher telomere lengths and telomerase activity compared to CD31⁻ counterparts (Junge et al. 2007). CD31 expression has been shown to positively correlate with the presence of TRECs (Junge et al. 2007) and inversely with rounds of cell division (Demeure et al. 1996) but no correlation was found between telomere length and TRECs in these T cells (Junge et al. 2007). Hence, CD31 expression on T cells is used as a measure of replicative history and an indicator or residual replicative potential. In the present study CD31 expression by expanded iNKT cells gradually dropped over 100 days and 3 rounds of stimulation from 70 % to 10 %. However, further monitoring of these lines beyond the 100 days is required to determine the point of senescence and how CD31 expression can help predict iNKT cell senescence and residual replicative potential. CD31 expression on iNKT cells could potentially be used as a marker to predict the potential of *ex vivo* expanded iNKT cells to further replicate and function *in vivo* when used for cellular immunotherapy.

Cytokine production is one of the primary means by which iNKT cells exert their immune regulatory effects. As iNKT cells functionally mature and proliferate in the periphery they become potent cytokine producers (Baev et al. 2009). IFN- γ and IL-4 are important cytokines but only 2 of many that are produced by iNKT cells when activated (Matsuda et al. 2008). iNKT cells are also professional killer cells that are capable of directly targeting other cells for apoptosis in a CD1d dependent manner which is augmented in the presence of glycolipid Ag. Activated iNKT cells produce IL-2 and IFN- γ through which they trans-activate cytotoxic NK cells, hence indirectly targeting cells for killing (Metelitsa et al. 2001). iNKT cells may also perform cytotoxic killing independent of CD1d through the engagement of the cell surface stress-ligand receptor NKG2D (Kuylenstierna et al. 2011). Cytotoxic molecules such as perforin and granzyme B are released by the activated iNKT

cell and cause the targeted cell to undergo cell death. CD107a has been described as a marker of cytotoxic cell degranulation and a sensitive marker for cytotoxic activity (Aktas et al. 2009).

In order to ensure that the iNKT cell lines were functional, cytokine production (i.e. IFN- γ and IL-4) and cytotoxic activity (i.e. CD107a surface expression) were assessed. 14 iNKT cell lines were co-cultured with CD1d⁺ HeLa cells pulsed with α -GalCer or as controls in the absence of Ag and with CD1d⁻ HeLa cells. All of the lines tested showed significant IFN- γ and IL-4 secretion in response to CD1d and Ag stimulation. IFN- γ and IL-4 secretion was Ag concentration-dependent. Two glycolipid antigens were tested (i.e. α -GalCer and its derivative 7DW8-5) and 10 ng/ml based on the three donors gave optimal cytokine response.

CD107a expression was assessed in a similar manner using CD1d⁺ HeLa cells and α -GalCer. Three donors were tested and an average of 50 % of all activated iNKT cells showed cytotoxic activity. With the aid of flow cytometry gating, iNKT cell subsets were analysed for CD107a expression. As expected and as previously suggested (O'Reilly et al. 2011) CD8⁺ iNKT cells showed the most potent cytotoxic activity. However, DN iNKT cells showed similar CD107a positivity to CD8⁺ iNKT cells, indicating a lack of a clear functional and phenotypic (i.e. CD8 expression) distinction between the two subsets. Both CD4⁺ and DP iNKT cells showed similar cytotoxic activity upon stimulation but to a lesser extent than CD8⁺ and DN subsets. These results show that iNKT cell lines generated by our method display normal functional characteristics as defined by cytokine production and cytotoxic activity.

iNKT cells are found in low numbers in human PB. To be able to expand these iNKT cells *ex vivo* for *in vitro* studies or to use as part of a patient targeted cellular immunotherapy is an invaluable tool. Although iNKT cells have previously been expanded *in vitro*, the present study has optimized the mode of stimulation, and cytokines for expanding iNKT cells from small and large blood samples. Our method utilised α -GalCer as the stimulus and the cytokines IL-2 and IL-7 to improve yields and obtain better CD4⁺ iNKT cell yields. The resulting iNKT cells showed no sign of exhaustion as indicated by basal levels of PD-1 expression. Although CD31 expression dramatically dropped over 2 months of expansion,

iNKT cells continued to expand and showed no signs of senescence. All resulting iNKT cells were fully functional as measured by IFN- γ and IL-4 secretion as well as cytotoxic activity as indicated by CD107a expression. Further work is needed to look at the functional distinctions between iNKT cell subsets, the differential capacity of each to replicate and respond *in vivo* if used for immunotherapy, and finally to tease out ways by which distinct functional outputs can be triggered using different iNKT cell stimuli or different iNKT cell subsets.

**Chapter 4 Influence of B cells on iNKT cell
function.**

4.1 Introduction.

iNKT cells express a semi-invariant T cell receptor, which recognizes glycolipid antigens presented to them in the context of a CD1d molecule by APCs (Godfrey et al. 2004). Upon activation, iNKT cells can produce a number of cytokines such as: IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IFN- γ , TNF- α , TGF- β , and GM-CSF (Matsuda et al. 2008). iNKT cells are subdivided based on CD4 and CD8 expression into CD4⁺, CD8 α ⁺, and double negative (DN) iNKT cells. Functionally, the three subsets are distinct but partially overlap. Functionally, CD4⁺ iNKT cells tend to produce Th1 and Th2 cytokines, while CD8 α ⁺ and DN iNKT cells tend to display predominant Th1 cytokine profile (Takahashi et al., 2002; O'Reilly et al., 2011).

B cells are one of a number of professional APC that express CD1d (Exley et al. 2000), and as a result are capable of presenting lipid Ag to iNKT cells, causing their activation (Allan et al. 2011; Bosma et al. 2012). B cells have been shown to be required for normal iNKT cell growth and proliferation (Allan et al. 2011; Bosma et al. 2012). A specific subset of B cells called marginal zone B cells (MZB cells) express high levels of CD1d, and in mice they have been shown to preferentially induce the proliferation of iNKT cells in response to lipid Ag presentation (Leadbetter et al. 2008; Barral et al. 2008).

Mouse studies have revealed that iNKT cells play a major role in antibody production by B cells, and that cognate interaction between iNKT cells and B cells are required for lipid-specific antibody responses (Leadbetter et al. 2008). A subset of iNKT cells dubbed follicular helper (NKT_{FH}) iNKT cells, express PD-1 and the chemokine CXCR5, and provide cognate help to B cells (King et al. 2011). IL-21, a cytokine produced by iNKT cells, happens to be one required for B cell differentiation and function. IL-21 production is thought to be an important mechanism by which iNKT_{FH} cells provide B cell help (Bryant et al. 2007; Coquet et al. 2007).

In order for iNKT cells to be activated, they require the recognition of endogenous or exogenous glycolipid Ag presented to them by APC. Since its discovery in 1995, the glycolipid α -GalCer has been the most commonly used glycolipid Ag for iNKT cell stimulation *in vivo* and *in vitro*. Various studies have demonstrated the

potency of α -GalCer using CD1d transfected cell lines such as CD1d-HeLa cells and CD1d-C1r cells (O'Reilly et al. 2011), and dendritic cells (Porubsky et al. 2012). Recently a synthetic glycolipid analogue of α -GalCer (7DW8-5) was shown to be more potent at activating iNKT cells when compared to α -GalCer (Li et al. 2010). So far, there have been no studies to compare the potency or effectiveness of α -GalCer and 7DW8-5, as well as other glycolipids such as β -GlcCer and GSL-1, using peripheral blood B cells as APC.

4.2 Objectives.

The general aim of this part of the study is to analyse the effectiveness of human peripheral blood B cells as APC for iNKT cell activation, as measured by cytokine production and cytotoxic degranulation. Also, to compare the stimulatory capabilities of various glycolipids presented by B cells for iNKT cells. Further understanding of how B cells contribute to iNKT cell function is essential if iNKT cell responses are to be exploited for therapeutic benefits.

- To examine at the levels of CD1d expression by peripheral blood B cells.
- To determine the optimal concentration of glycolipid Ag for iNKT cell stimulation by B cells.
- To compare the responses of iNKT cells to B cells pulsed with various glycolipid Ags.
- To compare the responses of CD4⁺, CD8⁺, and DN iNKT cells to α -GalCer pulsed B cells.
- To determine if B cells can induce the differentiation of iNKT_{FH} cells *in vitro*.

4.3 Results.

4.3.1 B cells express low levels of CD1d.

In humans, B cells express CD1d, are capable of presenting α -GalCer and activating iNKT cells, and have been shown to be required for normal iNKT cell growth and proliferation (Allan et al. 2011; Bosma et al. 2012). Levels of CD1d expression on peripheral blood CD19⁺ B cells were investigated and compared to those on CD14⁺ monocytes and CD1d transfected HeLa cell line provided by Prof. Steven Porcelli (Spada et al. 1998). PBMC were isolated from 10 healthy donors, and along with a sample of CD1d-HeLa, were surface stained for flow cytometric analysis using mAb against CD1d, CD14, and CD19. CD19⁺ B cells populations all showed cell surface CD1d expression, although at much lower densities than on CD14⁺ monocytes (Fig. 4.1. A and C). In comparison, HeLa cells expressing transfected CD1d (CD1d-HeLa) showed the highest levels of expression of CD1d, when compared to B cells, monocytes, and mock transfected HeLa cells (mock-HeLa) (Fig. 4.1. B and C). CD1d was found to be expressed by all healthy donor CD19⁺ PBMC, suggesting these cells to be capable of presenting glycolipid Ags to iNKT cells.

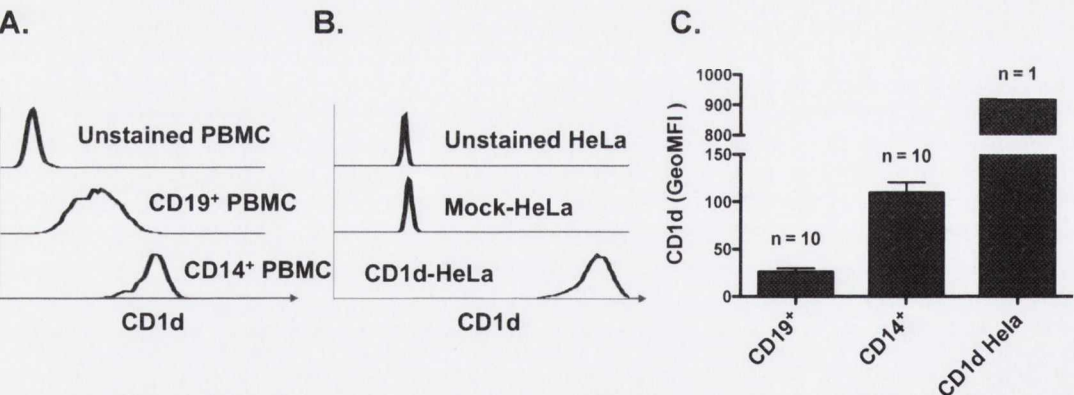


Fig. 4.1. CD1d expression on human peripheral blood B cells, monocytes, and CD1d transfected HeLa cells. PBMC from 10 healthy donors as well as a CD1d-transfected and mock-transfected HeLa cell line were stained using mAb against CD1d, and analysed by flow cytometry. A and B, Representative histograms showing relative levels of CD1d expression by CD19⁺ and CD14⁺ PBMC (A) and HeLa cells (B). C, Bar chart showing levels of CD1d expression on CD19⁺ B cells, CD14⁺ monocytes, and CD1d-HeLa cells. GeoMFI: geometric mean fluorescence intensity.

4.3.2 Cytokine production by total, CD4, CD8, and DN iNKT cells upon stimulation with α -GalCer-pulsed B cells.

CD4⁺, CD8⁺, and DN iNKT cell subsets when co-cultured with CD1d-HeLa cells or DC pulsed with 10-100 ng/ml of α -GalCer are capable to producing a variety of cytokines, with redundancy between the iNKT cell subsets (O'Reilly et al. 2011). Using these APC, CD4⁺, CD8⁺, and DN iNKT cells produce Th1 cytokines such as IFN- γ , and Th2 cytokines such as IL-4 in response to stimulation with α -GalCer. However, greater proportions of CD4⁺ iNKT cells produce Th2 cytokines, whereas greater proportions of CD8⁺ iNKT cells produce Th1 cytokines, with DN iNKT cells showing intermediate responses (O'Reilly et al. 2011). In the present study cytokine produced by CD4⁺, CD8⁺, and DN iNKT cell subsets in response to α -GalCer-pulsed B cells were investigated. CD4⁺, CD8⁺, DN, as well as total iNKT cells, and CD3⁺ non-iNKT cells were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. CD19⁺ B cells were purified from 4 donors fresh PBMC, then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. Each T cell lines was cultured alone or co-cultured with the Ag pulsed B cells at a 1:1 ratio for 72 hours. Brefeldin A was then added to co-cultures, which were then split; one half was re-stimulated with PMA/I and the other left unstimulated. Cells were finally stained with mAb against CD3, CD19, IFN- γ , IL-4, and IL-10, and analysed by flow cytometry. After 3 days of co-culture with allogeneic B cells, there were no significant changes to IFN- γ , IL-4, or IL-10 production by iNKT cells (Fig. 4.2 – 4.4). Pulsing B cells with 100 ng/ml of α -GalCer did not improve cytokine production by iNKT cells (Fig. 4.2 – 4.4). However, modest reductions in IL-4 and IL-10 production by all iNKT cell subsets in the presence of allogeneic B cells were observed (Fig. 4.3 and 4.4). These results suggest that cytokine production by iNKT cells is not affected after 3 days of co-culture with allogeneic B cells, regardless of the presence or absence of 100 ng/ml of α -GalCer.

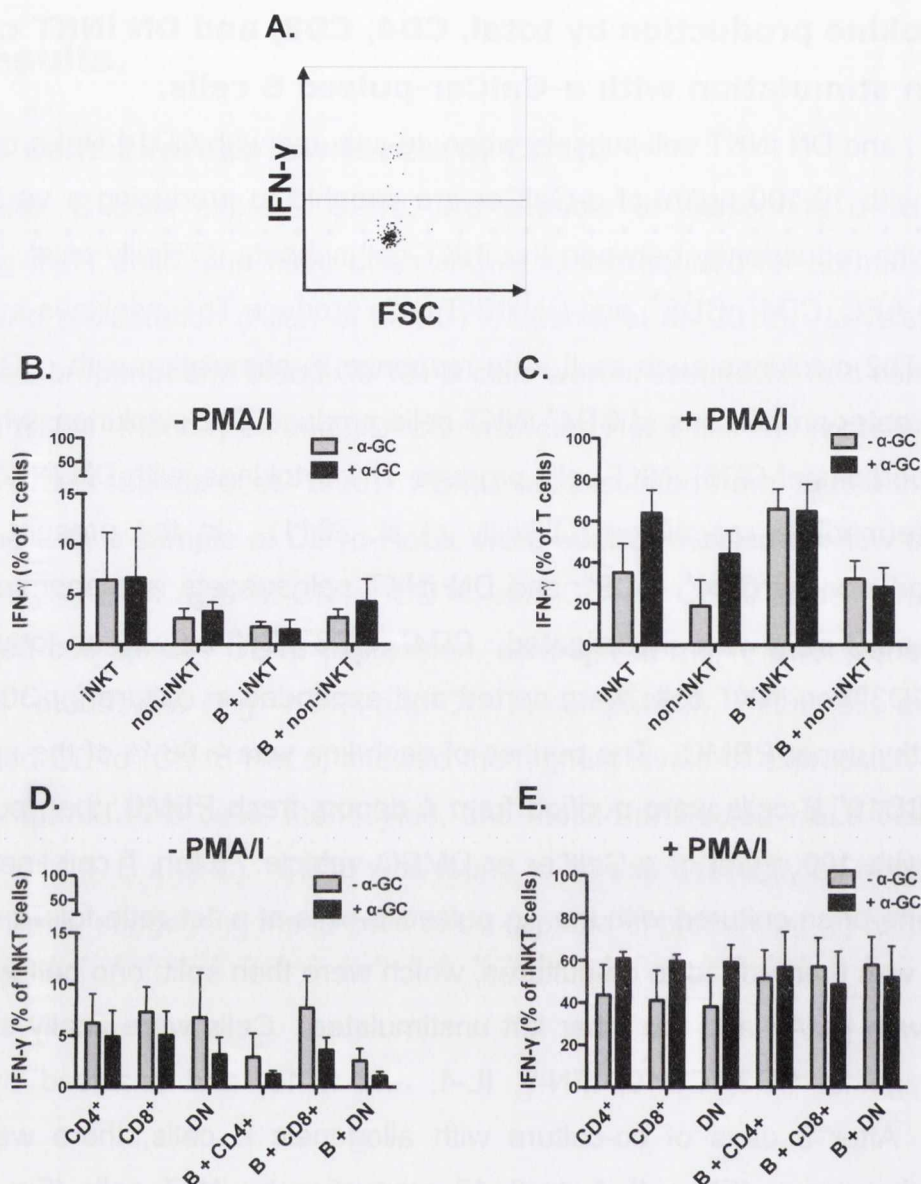


Fig. 4.2. B cells pulsed with 100 ng/ml of α -GalCer do not induce IFN- γ production by autologous CD4 $^+$, CD8 $^+$, and DN iNKT cells. iNKT cell lines (total, CD4 $^+$, CD8 $^+$, and DN), as well as CD3 $^+$ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19 $^+$ cells were isolated from fresh PBMC from the same 4 donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer over night at 37°C. The autologous iNKT cells were then cultured alone or co-cultured with B cells that that had been cultured in the absence or presence of α -GalCer for 72 hours. After 72 hours brefeldin A was added to the co-cultures and they were re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated for 15 hours. Cells were then stained for intracellular flow cytometry analysis using a mAb against IFN- γ . **A**, Representative dot plot showing flow cytometric gating on IFN- γ producing iNKT cells. **B** and **C**, Frequencies of iNKT cells and non-iNKT cells expressing IFN- γ after co-culturing with autologous B cells, in the absence (**B**) or presence (**C**) of PMA/I re-stimulation (n = 4). **D** and **E**, Frequencies of CD4 $^+$, CD8 $^+$, and DN iNKT cells expressing IFN- γ after co-culturing with autologous B cells, in the absence (**D**) or presence (**E**) of PMA/I re-stimulation (n = 4). Data points are plotted as mean $\pm$ SEM.

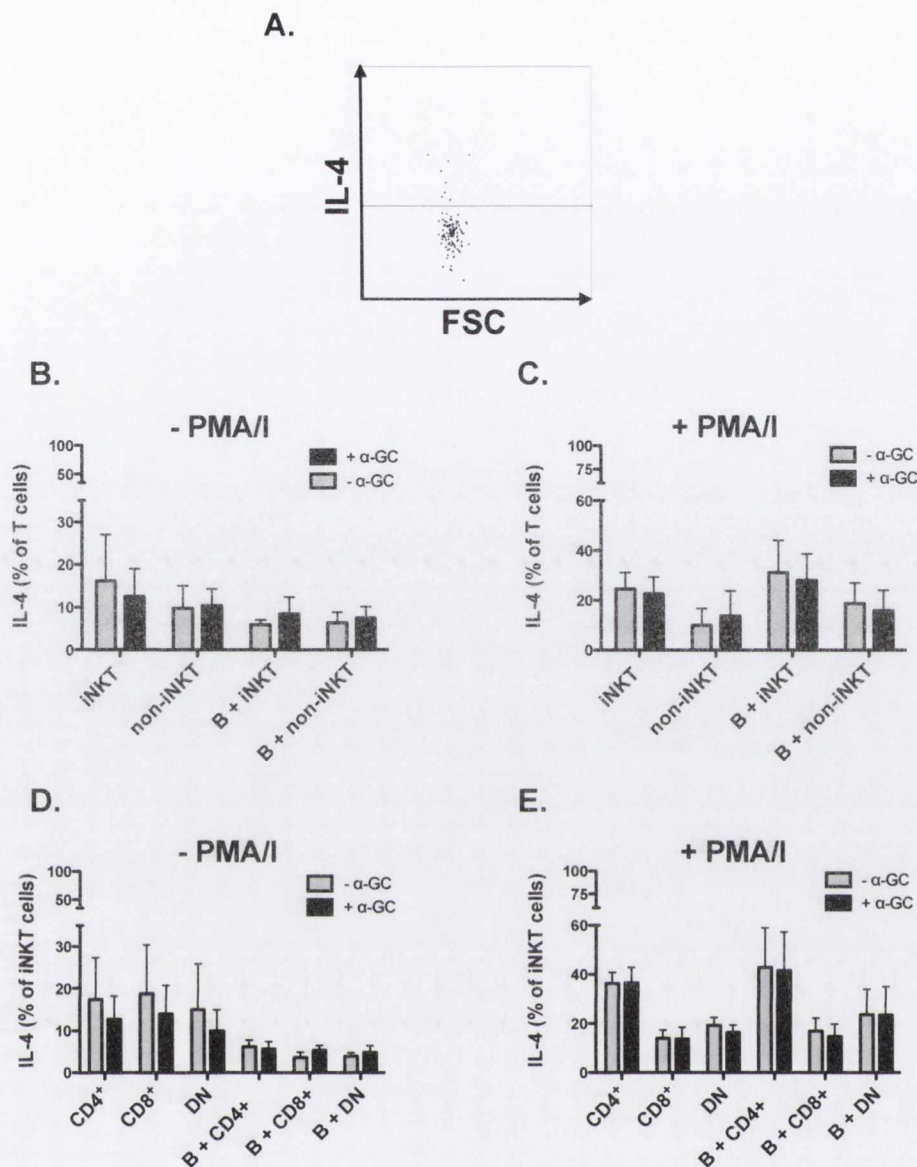


Fig. 4.3. B cells pulsed with 100 ng/ml of α -GalCer do not induce IL-4 production by autologous CD4⁺, CD8⁺, and DN iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from the same 4 donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer over night at 37°C. The autologous iNKT cells were then cultured alone or co-cultured with B cells that that had been cultured in the absence or presence of α -GalCer for 72 hours. After 72 hours brefeldin A was added to the co-cultures and they were re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated for 15 hours. Cells were then stained for intracellular flow cytometry analysis using a mAb against IL-4. **A**, Representative dot plot showing flow cytometric gating on IL-4 producing iNKT cells. **B** and **C**, Frequencies of iNKT cells and non-iNKT cells expressing IL-4 after co-culturing with autologous B cells, in the absence (**B**) or presence (**C**) of PMA/I stimulation ($n = 4$). **D** and **E**, Frequencies of CD4⁺, CD8⁺, and DN iNKT cells expressing IL-4 after co-culturing with autologous B cells, in the absence (**D**) or presence (**E**) of PMA/I stimulation ($n = 4$). Data points are plotted as mean $\pm$ SEM.

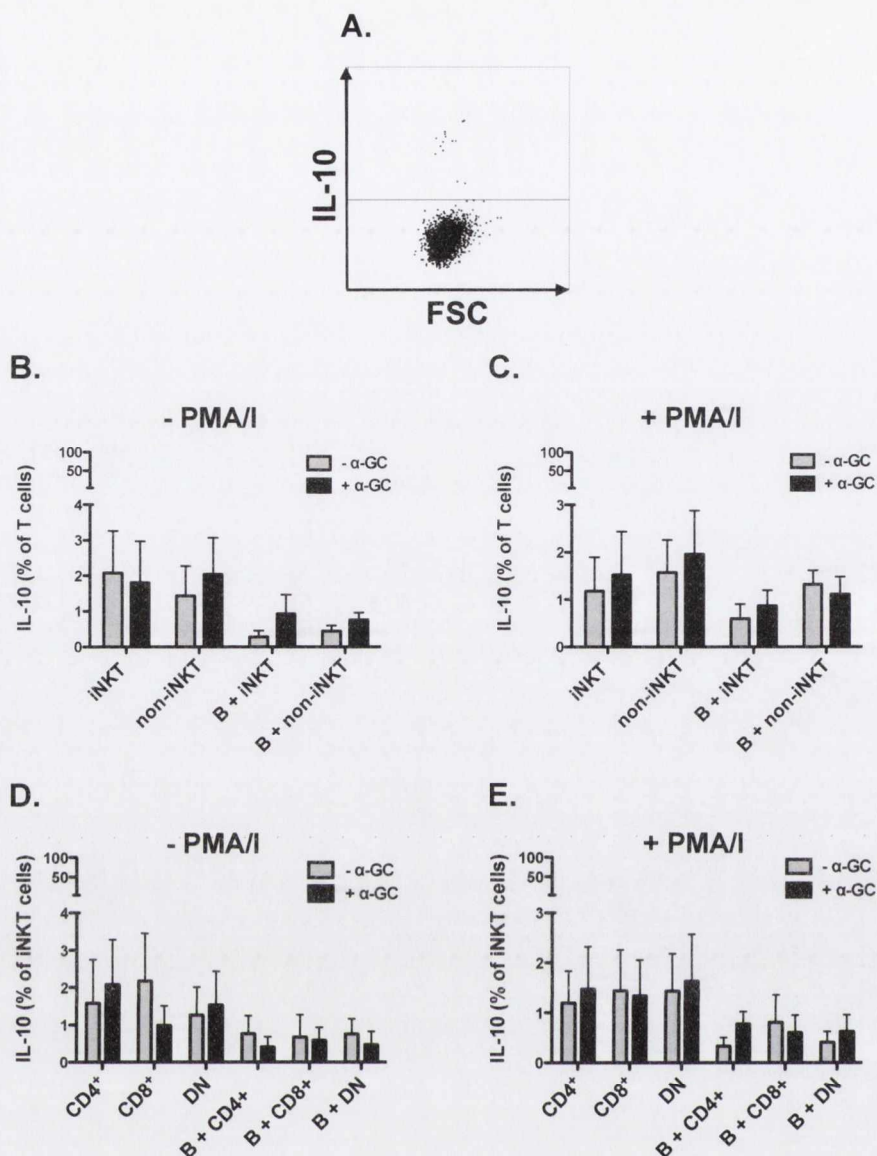


Fig. 4.4. B cells pulsed with 100 ng/ml of α -GalCer do not induce IL-10 production by autologous CD4⁺, CD8⁺, and DN iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from the same 4 donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer overnight at 37°C. The autologous iNKT cells were then cultured alone or co-cultured with B cells that had been cultured in the absence or presence of α -GalCer for 72 hours. After 72 hours brefeldin A was added to the co-cultures and they were re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated for 15 hours. Cells were then stained for intracellular flow cytometry analysis using a mAb against IL-10. **A**, Representative dot plot showing flow cytometric gating on IL-10 producing iNKT cells. **B** and **C**, Frequencies of iNKT cells and non-iNKT cells expressing IL-10 after co-culturing with autologous B cells, in the absence (**B**) or presence (**C**) of PMA/I stimulation ($n = 4$). **D** and **E**, Frequencies of CD4⁺, CD8⁺, and DN iNKT cells expressing IL-10 after co-culturing with autologous B cells, in the absence (**D**) or presence (**E**) of PMA/I stimulation ($n = 4$). Data points are plotted as mean $\pm$ SEM.

4.3.3 Effect of B cells on iNKT_{FH} cell population.

Follicular helper T (T_{FH}) cells possess the ability to home in on B cell rich lymphoid follicles, make cognate interaction with primed B cells, and bring about B cell activation, and ultimately Ab production (Crotty 2011). A similar population is recognised within iNKT cells, and are called iNKT_{FH} cells. Like T_{FH}, they are PD-1^{hi}CXCR5^{hi}, produce IL-21, and have been shown in mice to stimulate lipid-specific Ab production by B cells (Vinuesa & Chang 2013). Although iNKT cells did not show changes in IFN- γ , IL-4, or IL-10 production when assessed after 3 days of co-culture with autologous B cells (see 4.3.5), the possibility of these iNKT cells acquiring an iNKT_{FH} phenotype (PD-1^{hi}CXCR5^{hi}) and producing IL-21 in response to co-culture with B cells was investigated. CD4⁺, CD8⁺, and DN iNKT cells were sorted and expanded in culture for 30 days, from 2 healthy donor PBMC. CD19⁺ B cells were then purified from fresh PBMC of 2 autologous donors and cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. Each T cell lines was cultured alone or co-cultured with the Ag pulsed B cells at a 1:1 ratio for 72 hours. Brefeldin A was then added to co-cultures, which were then split; one half was re-stimulated with PMA/I and the other left unstimulated. Cells re-stimulated with PMA/I were stained for intracellular IL-21, and unstimulated cells were stained for CXCR5 and PD-1, and analysed by flow cytometry (see Fig. 4.5. A). No significant changes in PD-1⁺ CXCR5⁺ iNKT cells or IL-21 producing iNKT cells were observed in the presence of autologous B cells. However, CD8⁺ iNKT cells showed modest increases in both Tfh phenotype and IL-21 production in the presence of B cells, which was not augmented by α -GalCer (Fig. 4.5. B and C).

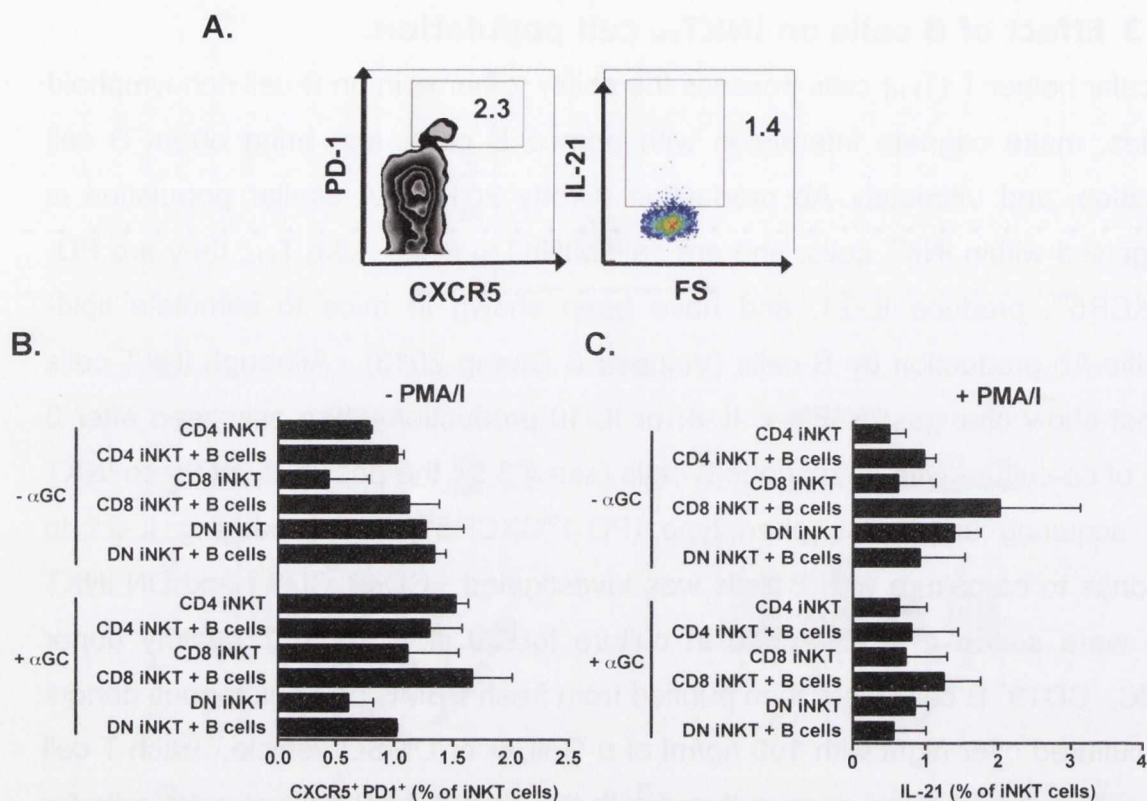


Fig. 4.5. B cells enhance the expression of follicular helper T (Tfh) markers by CD8⁺ iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN) were prepared from 2 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from the same 2 donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α-GalCer (αGC) over night at 37°C. The autologous iNKT cells were then cultured for 72 hours, alone or co-cultured with B cells that that had been cultured in the absence or presence of α-GalCer. After 72 hours brefeldin A was added to the co-cultures and they were re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated for 15 hours. Using flow cytometry, unstimulated cells were analysed for characteristics of Tfh cells, and re-stimulated cells for intracellular IL-21. **A**, Representative flow cytometry zebra plot showing the frequency CXCR5⁺ PD-1⁺ iNKT cells (left), and dot plot showing the frequency of IL-21 expression by iNKT cells (right). **B**, Frequencies of CD4⁺, CD8⁺, and DN iNKT cells expressing both CXCR5 and PD-1, when co-cultured with B cells in the presence and absence of αGC (n = 2). **C**, Frequencies of CD4⁺, CD8⁺, and DN iNKT cells expressing intracellular IL-21, when co-cultured with B cells in the presence and absence of αGC (n = 2). Data points are plotted as mean ± SEM.

4.3.4 CD107a expression by CD4⁺, CD8⁺, DN, and DP iNKT cells in response to B cells pulsed with 4 glycolipids.

CD4⁺, CD8⁺, and DN iNKT cell subsets are known to have different but overlapping functions (O'Reilly et al. 2011). Most studies have only focused on CD4⁺ vs CD4⁻ iNKT cells, without looking specifically at CD8⁺ and DN iNKT cell responses. While most studies looking at iNKT cell function have used PBMC, DC, or CD1d-transfected cell lines (e.g. HeLa and C1r), none have compared these APC to B cells in terms of their abilities to induce iNKT cell degranulation. In this study, the responses of CD4⁺, CD8⁺, DN, and DP iNKT cells to B cells presenting various glycolipids, was investigated. Purified CD19⁺ B cells or CD1d-HeLa cells, that were cultured overnight with 50 ng/ml of α -GalCer, or 5 μ g/ml of either β -GlcCer, OCH, or GSL-1 isolated from *Sphingomonas*, were co-cultured with iNKT cell lines generated from 3 allogeneic donors, for 4 hours in the presence of a mAb against CD107a. CD107a expression was assessed by flow cytometry on total iNKT cells (Fig. 4.6. A) and gated CD4⁺, CD8⁺, DN, and DP iNKT cells. iNKT cells showed minimal CD107a expression when cultured alone, and maximal up-regulation of CD107a in the presence of PMA and ionomycin (PMA/I) (Fig. 4.6. B). There was no significant up-regulation of CD107a on iNKT cells when cultured with the various glycolipids. However, the largest increases in CD107a expression were seen when iNKT cells were co-cultured with CD1d-HeLa pulsed with α -GalCer or β -GlcCer (Fig. 4.6. C). B cells were less capable than CD1d-HeLa cells at inducing CD107a expression by iNKT cells, and the highest CD107a response to B cells was observed in the presence of OCH (Fig. 4.6. D). CD107a responses to B cells pulsed with glycolipids were similar for CD4⁺, CD8⁺, DN, and DP iNKT cells (Fig. 4.6. E). Overall, B cells induce modest CD107a responses by CD4⁺, CD8⁺, DN, and DP iNKT cells when compared to CD1d transfected HeLa cells. Particular glycolipids (e.g. OCH) may be better at inducing iNKT cell responses when presented by B cell in comparison to other APC.

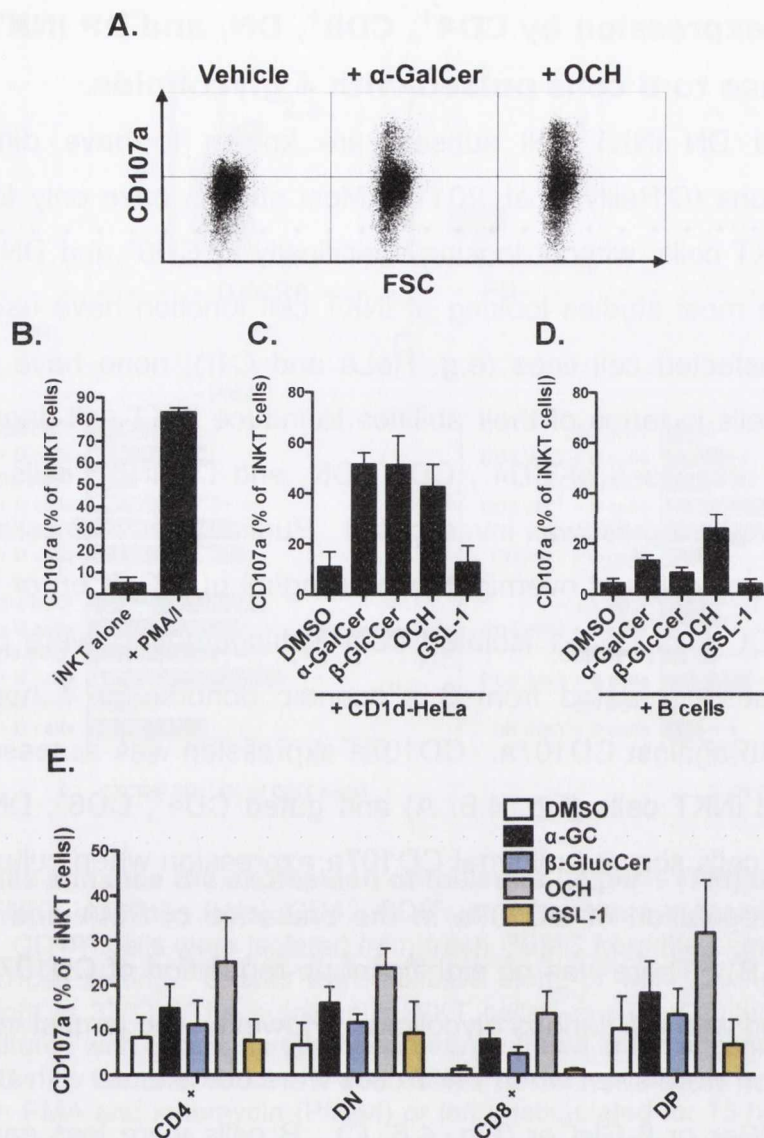


Fig. 4.6. Glycolipid pulsed B cells induce modest cytolytic degranulation by iNKT cells. CD19⁺ cells were isolated from healthy donor PBMC using magnetic bead cell sorting. B cells were cultured with DMSO (vehicle) or pulsed with 50 ng/ml of α -GalCer, 5 μ g/ml of β -GlcCer, 5 μ g/ml of OCH, or 5 μ g/ml of GSL-1 over night at 37°C. B cells were then co-cultured with 3 different allogeneic iNKT cell lines for 4 h in the presence of anti-CD107a mAb. Cells were stained with mAbs against CD4 and CD8 then analysed by flow cytometry. **A**, Representative dot plots of CD107a expression by iNKT cells in the absence or presence of B cells pulsed with α -GalCer or OCH. **B**, CD107a expression by iNKT cells cultured for 4 hours alone, or with PMA and ionomycin (PMA/I) (n = 3). **C**, CD107a expression by iNKT cells co-cultured with glycolipid pulsed CD1d-transfected and mock-transfected HeLa cells (n = 3). **D**, CD107a expression by iNKT cells co-cultured with glycolipid pulsed CD19⁺ cells (n = 3). **E**, CD107a expression by CD4⁺, DN, CD8⁺, and DP iNKT cells co-cultured with glycolipid pulsed CD19⁺ cells (n = 3). Data points are plotted as mean $\pm$ SEM.

4.3.5 Optimisation of iNKT cell stimulation by glycolipids presented by B cells.

The use of α -GalCer pulsed APC to modulate iNKT cell function *in vitro* and *in vivo* is well established. Other glycolipids less potent than α -GalCer have not been as extensively studied, particularly with human iNKT cells. More importantly, there has been no study looking at iNKT cell responses to different glycolipid pulsed PB B cells. iNKT cell responses, measured by assessing cytolytic degranulation (i.e. CD107a expression), were investigated after co-culturing 3 different donor iNKT cell lines with allogeneic B cells that have been pulsed with various concentrations of glycolipids. The glycolipids used were: α -GalCer, the more potent synthetic analogue of α -GalCer (7DW8-5), the endogenous human glycolipid (β -GlcCer), and the synthetic OCH (Fig. 2.1). After 4 hours of co-culture in the presence mAb against CD107a, cells were stained for flow cytometry, and CD107a expression by iNKT cells was measured. iNKT cells showed minimal CD107a expression when cultured alone, and a significant up-regulation of CD107a in the presence of PMA and ionomycin (PMA/I) (Fig. 4.7. A). B cells from all 3 donors were unable to induce an up-regulation of CD107a on iNKT cells in the absence of glycolipid. However, significant increases in CD107a expression on allogeneic iNKT cells were observed when B cells were pulsed with 1000 ng/ml of α -GalCer or 10 ng/ml of 7DW8-5 (Fig. 4.7. A and B). In these experiments, β -GlcCer and OCH failed to significantly up-regulate CD107a responses, but modest increases were observed at 100 ng/ml for β -GlcCer and 10,000 ng/ml for OCH (Fig. 4.7. C and D). These results suggest that 7DW8-5 is the most potent glycolipid for the induction of iNKT cell degranulation when presented by B cells, and B cells require a high concentration of α -GalCer (1000 ng/ml) in order to induce a significant iNKT cell response.

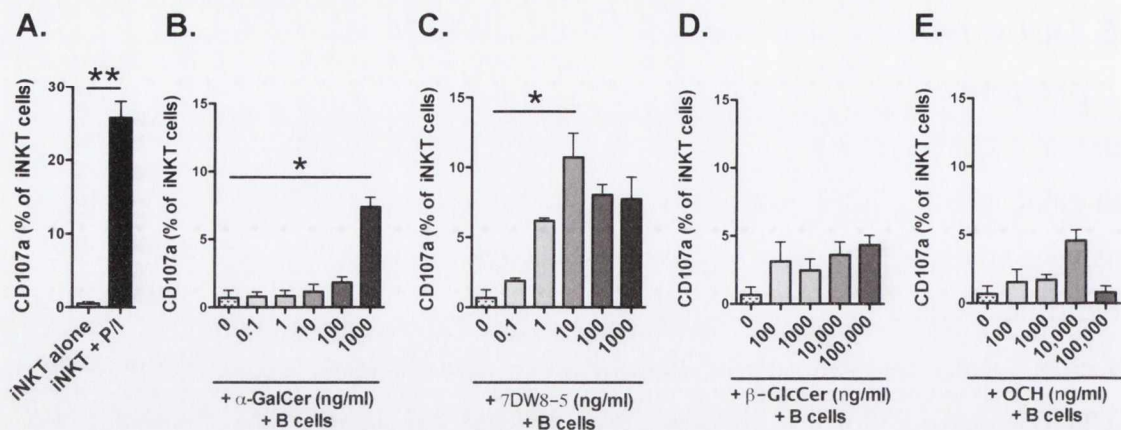


Fig. 4.7. Cytotoxic degranulation of iNKT cells in response to various concentrations of glycolipid. CD19⁺ cells were isolated from healthy donor PBMC using magnetic bead cell sorting. B cells were cultured with DMSO (vehicle) or pulsed with various concentrations of α-GalCer, 7DW8-5, β-GlcCer, and OCH, over night at 37°C. B cells were then co-cultured with 3 different allogeneic iNKT cell lines for 4 h in the presence of anti-CD107a mAb. Cells were analysed by flow cytometry. **A** and **B**, CD107a expression by iNKT cells cultured alone, with PMA and ionomycin (PMA/I) as a positive control (**A**), or with B cells pulsed with 0 – 1000 ng/ml of α-GalCer (**B**). **C**, **D**, and **E**, CD107a expression by iNKT cells co-cultured with B cells pulsed with: 0 – 1000 ng/ml of 7DW8-5 (**C**), with 0 – 100 µg/ml of β-GlcCer (**D**) or OCH (**E**). Data points are plotted as mean ± SEM. Statistical analysis was carried out using the non-parametric Friedman test with post hoc Dunne multiple comparison tests. * P ≤ 0.05, ** P ≤ 0.01.

4.3.6 High glycolipid concentrations facilitate B cell-mediated induction of IFN- γ and IL-4 secretion by iNKT cells.

B cells were found to require higher concentrations of glycolipid than CD1d-HeLa cells to induce significant cytolytic degranulation by iNKT cell (see 4.3.4). The effect of various concentrations of glycolipid on cytokine secretion by iNKT cells in response to B cells was then assessed. Purified CD19⁺ B cells were pulsed overnight with various concentration of α -GalCer, 7DW8-5, β -GlcCer, and OCH, then co-cultured with 3 allogeneic iNKT cell lines overnight. Supernatants were quantitatively analysed by ELISA for the presence of IFN- γ and IL-4. Significant increases in IFN- γ production were only found in co-cultures containing 1000 ng/ml of 7DW8-5 (Fig. 4.8. B). Marginal increases in IFN- γ production were observed in the presence of the highest used concentration of each of the other 3 glycolipids (Fig. 4.8. A, C, and D – left panel). However, significant increases in IL-4 production in co-cultures were induced by 1000 ng/ml of α -GalCer, 100 – 1000 ng/ml of 7DW8-5, 100,000 ng/ml of β -GlcCer, and 10,000 ng/ml of OCH (Fig. 4.8. A, B, C, and D – right panel). These results are in agreement with those obtained from analyzing CD107a expression by iNKT cells (see 4.3.5), and suggesting that 7DW8-5 is the most potent glycolipid to induce significant IFN- γ and IL-4 production from co-cultures of iNKT cells and allogeneic B cells, and that 1000 ng/ml of α -GalCer is the minimum amount required to induce any cytokine response by B cells.

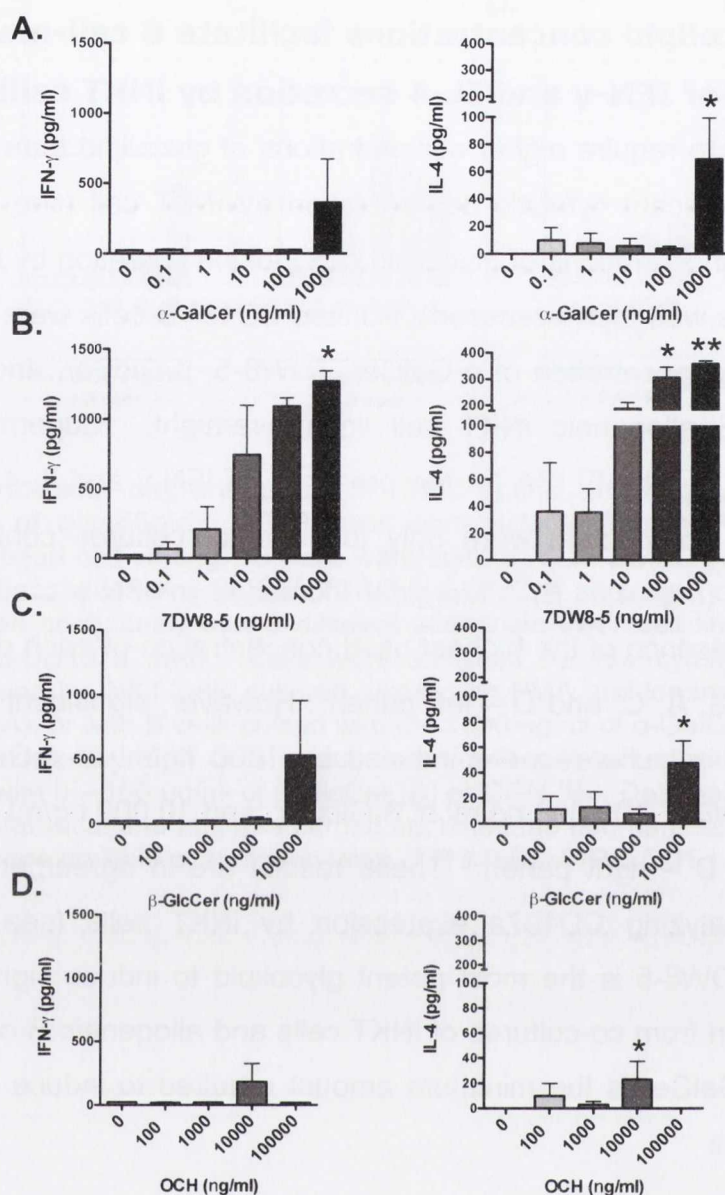


Fig. 4.8. IFN- γ and IL-4 secretion in response to B cells pulsed with various concentrations of glycolipid. CD19⁺ cells were isolated from healthy donor PBMC using magnetic bead cell sorting. B cells were cultured with DMSO (vehicle) or pulsed with various concentrations of α -GalCer, 7DW8-5, β -GlcCer, and OCH, over night at 37°C. B cells were then co-cultured with 3 different allogeneic iNKT cell lines overnight. Supernatants were removed and analysed by ELISA for the presence of IFN- γ (left panel) and IL-4 (right panel). **A, B, C, and D,** Co-cultures of iNKT cells and B cells pulsed with 0 – 1000 ng/ml of α -GalCer-(A), 0 – 1000 ng/ml of 7DW8-5 (B), and with 0 – 100 μ g/ml of β -GlcCer (C) or OCH (D). Data points are plotted as mean $\pm$ SEM. Statistical analysis was carried out using the non-parametric Friedman test with post hoc Dunne multiple comparison tests. * $P \leq 0.05$ compared to no glycolipid.

4.4 Discussion.

B cells are professional APC and are known to express CD1d (Chen & Jensen 2008; Allan et al. 2011). The expression of CD1d by PB B cells was compared to PB monocytes and CD1d-HeLa transfectants. B cells showed relatively low levels of CD1d expression when compared to monocytes and CD1d-HeLa cells. This is important to bear in mind when comparing iNKT cell responses to B cells compared to those in response to monocytes or DC, or the widely used CD1d-HeLa cells.

One of the hallmarks of iNKT cell activation and function is the production of various cytokines, among which are IL-4, IFN- γ , IL-21, and to a lesser extent IL-10. The ability of B cells to present α -GalCer to iNKT cells and induce cytokine production was investigated after 3 days of co-culture. B cells pulsed with 100 ng/ml of α -GalCer did not induce any significant changes in IFN- γ , IL-4, or IL-10 production by total, CD4⁺, CD8⁺, or DN iNKT cells after 3 days co-culture, with or without PMA and ionomycin re-stimulation.

In a preliminary experiment using 2 autologous donor B cells and iNKT cell lines, no significant changes were observed in IL-21 expression by iNKT cells or changes in iNKT_{FH} (PD-1^{hi}CXCR5^{hi}) frequencies. However, in the absence of α -GalCer CD8⁺ iNKT cells showed a noticeable increase in both IL-21 expression and iNKT_{FH} frequencies. The frequencies of PD-1^{hi}CXCR5^{hi} iNKT cells detected were < 2% of total iNKT cells, similar to those observed in mice (Bai et al. 2013). The suggestion that B cells might promote an iNKT_{FH} phenotype within CD8⁺ iNKT cells is contrary to the general understanding that iNKT_{FH} cells are CD4⁺.

CD1d transfected HeLa cells are known to efficiently present α -GalCer and induce the activation of iNKT cells. B cells were compared to CD1d-HeLa cells in terms of their ability to present various glycolipids (α -GalCer, 7DW8-5, β -GlcCer, and OCH) and induce CD107a expression on iNKT cells. OCH presented by B cells gave the best CD107a response by iNKT cells, compared to CD1d-HeLa cells, which gave a better iNKT cell response with α -GalCer. The CD107a responses of CD4⁺, CD8⁺, and DN iNKT cells were similar between the various glycolipids. As expected CD8⁺ iNKT cells gave the highest increase in CD107a in the presence of glycolipid compared to vehicle control. CD8⁺ iNKT cells are known to be the most

cytotoxic of the three iNKT cell subsets, hence they are prime targets to use for cancer immunotherapy. Based on the current findings, CD107a responses by iNKT cell differ between glycolipids depending on the APC. Hence, further work is needed to clarify if certain glycolipids may be favorable for inducing B cell-mediated iNKT cell activation.

The best-established method to study CD1d-dependent activation of iNKT cells is by using the potent glycolipid Ag α -GalCer, which is presented to iNKT cells by APC in the context of the CD1d molecule. The standard dose of α -GalCer used to pulse APC for the purpose of studying iNKT cell responses *in vitro* as well as in *in vivo* clinical trials (Uchida et al. 2008), since the first studies in the mid 1990s, is 100 ng/ml. Furthermore, pulsing irradiated B cells with as little as 50 ng/ml of α -GalCer has been shown to improve iNKT cell expansion *in vitro* (Galli et al. 2003). Purified CD19⁺ B cells were pulsed with various concentrations of α -GalCer, 7DW8-5, β -GlcCer and OCH, then co-cultured with iNKT cell lines and analysed for CD107a expression, as well as secreted IFN- γ , IL-4, and IL-10. Surprisingly, 1000 ng/ml of α -GalCer was required to induce a significant upregulation of CD107a on iNKT cells. Similarly 1000 ng/ml of α -GalCer was required to induce IFN- γ and IL-4 by iNKT cells. In comparison, 7DW8-5 was 10-100 times more potent than α -GalCer, while β -GlcCer and OCH were 10-100 times less potent than α -GalCer at inducing a significant CD107a and cytokine response, when presented by B cells to allogeneic iNKT cells. Although the study by Galli and colleagues showed that 50 ng/ml was enough to induce iNKT cell proliferation, they used irradiated B cells, which may influence the ability of B cells to present α -GalCer. In addition, another study demonstrated using peripheral blood B cells that a concentration higher than 100 ng/ml is required to induce significant increases in IFN- γ production by iNKT cells (Allan et al. 2011). These findings suggest that B cells are less efficient at presenting α -GalCer to iNKT cells, and hence future studies should consider increasing the concentration of α -GalCer in order to achieve optimal iNKT cell responses.

These findings suggest that B cells are capable of inducing both cytolytic degranulation, as indicated by CD107a upregulation, and cytokine expression by iNKT cells. However, B cells require a higher concentration of glycolipid than other APC to induce significant iNKT cell responses. Furthermore, different

glycolipids may be considered as better stimulators of iNKT cell activity when presented by B cells. And lastly but not least, is the importance of considering all iNKT cell subsets when investigating iNKT_{FH} phenotype and function, as our preliminary findings suggested a relatively higher induction of iNKT_{FH} by CD8⁺ iNKT cells.

**Chapter 5 Influence of iNKT cells on B cell
phenotype and function.**

5.1 Introduction.

B cells are a subset of lymphocytes that are clonally diverse; they possess Ag specific surface immunoglobulin receptors or BCR, which infer clonality and diversity. In an adult human, B cells are produced in the bone marrow, where they complete their maturation, before being released as naïve B cells into peripheral circulation. In the periphery, naïve B cells migrate to secondary lymphoid tissue (e.g. lymph nodes), where they encounter foreign Ag and consequently undergo various stages of functional maturation, ultimately leading to the development of Ab-producing plasma cells, which are the hallmark of humoral or Ab mediated immunity (LeBien & Tedder 2008).

The journey of B cell differentiation in the periphery starts with the functionally immature population of transitional B cells that are phenotypically characterized as $CD24^{hi}CD38^{hi}IgD^{+}CD27^{-}$ B cells. Transitional B cells gradually differentiate into mature naïve B cells, going from transitional 1 (T1) $\rightarrow$ T2 $\rightarrow$ T3 $\rightarrow$ naïve B cells. Naïve B cells are fully mature B cells that have not yet encountered Ag, and are phenotypically $CD24^{int}CD38^{int}IgD^{+}CD27^{-}$ B cells. Naïve B cells encounter their specific Ags in secondary lymphoid tissue, and clonally expand and differentiate into short-lived IgM producing plasma cells, or enter a germinal center (GC) reaction, undergo class switching, and mature into memory cells or plasmablasts. Plasmablasts then become a short-lived or long-lived plasma cell depending on the environmental cues they receive. B cells lose their expression of IgD as they undergo class switching, and gain CD27 expression as they differentiate into memory B cells (Bemark et al. 2012; Radbruch et al. 2006; Palanichamy et al. 2009).

A relatively small proportion of T2 B cells differentiate in the spleen into marginal zone B (MZB) cells. MZB cells are thought to be naïve B cells with phenotypic and functional properties of memory B cells (Radbruch et al. 2006; Pillai & Cariappa 2009). Circulating MZB cells are identified in humans as $IgM^{+}IgD^{+}CD27^{+}$ B cells, they are situated in the marginal zone of the spleen, and one of the first responders to blood born Ags (e.g. encapsulated bacteria) (Weller et al. 2013; Vinuesa & Chang 2013). They are known in both mice and humans to constitutively express high levels of CD1d (Allan et al. 2011).

Peptide Ags that B cells encounter fall into one of two categories: T cell-independent Ags, that are recognized by the BCR and elicit a B cell response without any cognate T cell interaction, and T cell dependent Ags, which require Ag presentation on MHC class II and cognate interaction with Ag-specific T cells, which provides necessary help for B cell activation. B cell responses that are T cell-dependent are well characterized and involve cognate interaction with CD4⁺ T helper cells. CD4⁺ T cells present in lymphoid tissues that express the chemokine receptor CXCR5 are termed T_{FH} cells. T_{FH} cells are major sources of IL-21, which in turn is the most potent inducer of B cells differentiation and Ab production (Bryant et al. 2007).

iNKT cells as described in previous chapters, are innate like lymphocytes that are capable of recognizing glycolipid antigens presented to them in the context of a CD1d molecule by APCs (Godfrey et al. 2004). They are present in a pre-activated state, ready to rapidly and effectively respond to stimulation. An *in vitro* study on PB and tonsillar B cells first reported that iNKT cells provide help to B cells for Ab production and proliferation (Galli et al. 2003). Many studies in murine models have since shown that iNKT cells and CD1d are required for the production of pathogen specific IgM (Belperron et al. 2005), IgG antibodies (Schofield, 1999; Lee et al. 2005), as well as IgE production in experimental asthma models (Lisbonne et al. 2003). Studies on mice have also demonstrated that α -GalCer can be used as an adjuvant to enhance humoral antibody responses (Ko, Ko, & Chang, 2005; Galli et al., 2007; Lang et al., 2006), in a CD1d dependent manner (Lang et al. 2008). iNKT cells were not only found to enhance but sustain antibody levels in immunized mice, and enhance recall responses after re-challenge (Galli et al. 2007). They also appear to be essential for optimal plasma cell generation (Devera et al. 2008).

iNKT cells are potent producers of IL-21 in mice (Coquet et al. 2007), and can provide cognate help for lipid antigen-specific B cells in mice (Leadbetter et al. 2008). Based on studies on mice, a new model of T cell-dependent B cell activation (T cell-dependent type 2) was proposed. In this model, lipid Ag presented by B cells in the context of a CD1d molecule, allows for IL-21-dependent cognate help by iNKT cells, resulting in the formation of extra-follicular plasmablasts, germinal center formation, and robust primary IgG response, but do

not enhance memory response to such lipid antigens (King et al. 2011). MZB cells are suggested to be the main target for iNKT cell help. In mice MZB cells have been shown to induce better lipid Ag-driven proliferation of iNKT cells when compared to follicular B cells (Leadbetter et al. 2008; Barral et al. 2008), and were also suggested to preferentially interact with iNKT cells in mouse spleen (Muppidi et al. 2011). However, there has been no research carried in humans to suggest that iNKT cells play a role in B cell differentiation.

In addition to their primary effector role in Ab mediated immunity, B cells are known to hold Ab-independent immune regulatory functions. B cells produce a vast array of cytokines including IFN- γ , IL-4, IL-12, and IL-10, through which they can elicit immune stimulatory or immune regulatory effects (Pistoia 1997; Harris et al. 2000). Studies on mice over the past 3 decades have reported that B cells can prevent and control autoimmunity and inflammation (Mauri & Bosma 2012). In mice, the main mechanism by which B cells regulate immune function is through IL-10 production, and these B cells have been termed B10 cells (Kalampokis et al. 2013). B10 cells are contained within a number of B cell subsets. CD5⁺CD1d^{hi} B cells are one particular subset that are enriched for B10 cells in both mouse (Yanaba et al. 2008) and humans (Blair et al. 2010). In humans, B10 cells and CD5⁺CD1d^{hi} B cells were both enriched in the CD24^{hi}CD38^{hi} transitional (T1) B cell subset, which were found to suppress Th1 activity by CD4⁺ T cells. However, the suppressive activity of these T1 cells was not fully blocked by IL-10 neutralisation, which suggests that other suppressive molecules are at play in the function of human regulatory B cell activity (Blair et al. 2010).

The possible regulatory role for IL-10 producing B cells in humans was highlighted by various studies. In one study, patients with MS had impaired IL-10 production by B cells (Duddy et al. 2007). Another found that a population of IL-10-producing CD1d^{hi} B cells helped ameliorate the disease in MS patients (Correale et al. 2008).

Moreover, CD24^{hi}CD38^{hi} B cells improved the outcome of patients with renal transplants (Newell et al. 2010). In an autoimmune mouse model, the *in vivo* presence of iNKT cells results in the selective reduction of IL-10 producing autoreactive B cell frequencies, while non-autoreactive B cells are markedly activated (Yang et al. 2011). This suggests that iNKT cells can differentially

regulate B cell function. However, there has been no research to date looking at the effect of iNKT cells on regulatory B cell function or phenotype in humans.

5.2 Objectives.

The aim of this part of the study is to investigate whether CD4⁺, CD8⁺, or DN iNKT cell subsets can differentially affect B cell activation, differentiation, and antibody production *in vitro*. Identifying the relationship between the various iNKT cells subsets and B cell phenotype and function, may help tailor therapies for various B cell-related diseases, where iNKT cells can be either used as a tool or target for immunotherapy to help correct B cell dysfunction.

- To optimise the detection of intracellular IL-10 from peripheral blood B cells.
- To determine whether CD4⁺, CD8⁺, or DN iNKT cells affect B cell differentiation after *in vitro* co-culture.
- To assess the ability of CD4⁺, CD8⁺, or DN iNKT cells to induce the differentiation of MZB cells after *in vitro* co-culture with peripheral blood B cells.
- To compare CD4⁺, CD8⁺, or DN iNKT cells in terms of their ability to induce the secretion of IgM, IgA, and total IgG by peripheral blood B cells after *in vitro* co-culture.
- To assess the role of CD1d, IL-4, and IL-13 in the effects elicited by iNKT cells on B cell activation and antibody production.
- To determine whether CD4⁺, CD8⁺, or DN iNKT cells can induce the differentiation of CD5⁺CD1d^{hi} and CD24^{hi}CD38^{hi} regulatory B cell subsets *in vitro*.
- To look at IL-10, IL-4, and IFN- γ production by B cells after co-culture with CD4⁺, CD8⁺, or DN iNKT cells.
- To investigate changes in surface expression of markers of activation, co-stimulation, and antigen presentation on B cells after *in vitro* co-culture with CD4⁺, CD8⁺, or DN iNKT cells.
- To investigate the difference in the above parameters between autologous and allogeneic co-cultures of B cells and iNKT cells.

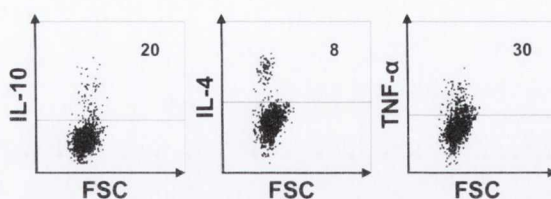
5.3 Results.

5.3.1 Optimisation of B cell stimulation for TNF- α , IL-4, and IL-10 expression.

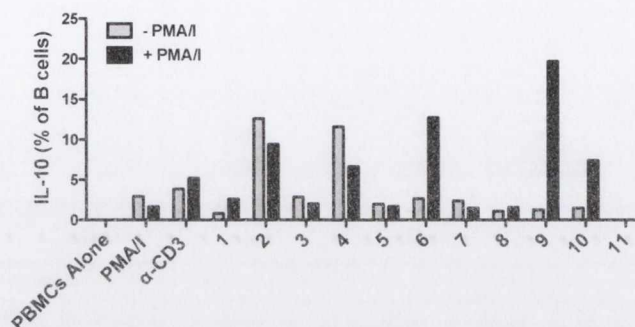
Naïve and memory B cells were previously shown to produce IL-10, TNF- α , and IL-6 in response to the TLR-9 agonist CpG (Wagner et al. 2004). IL-10 production by human B cells has also been shown to occur upon CD40 ligation in the absence of Ag-specific TCR engagement (Duddy et al. 2004). There has been great interest in the ability of B cells to produce IL-10, with a focus on targeting these cells for immunotherapy; these IL-10 producing B cells have been given the name regulatory B cells (Breg). One part of this study is to look at the ability of iNKT cell to induce the differentiation of Breg cells, and IL-10 production by B cells. For the purpose of optimizing the stimulation of B cells to allow for positive detection of IL-10 in particular, and other cytokines of interest such as IL-4 and TNF- α , a number of stimulations were tested on a freshly purified PBMC in culture. Particular stimulations were chosen based on previously published studies looking at B cell activation and differentiation. Some of these studies findings include: IL-10 production by human B cells after stimulating PBMC for 4 days in culture with 1 μ M CpG (10 μ g/ml), and PMA/I for the last 4 hours (Das et al. 2012). Significant proliferation of both human CD27⁻ naïve and CD27⁺ memory B cells when culturing each population in round bottom wells, at 1×10^5 per well, and in the presence of CpG (10 μ g/ml), IL-2 (20 IU/ml), and IL-15 (10 ng/ml) for 4 days (Huggins et al. 2007). Significant up-regulation of IL-10 and TNF- α on human CD19⁺ B cells after 3 days of stimulation in the presence of CpG (3 μ g/ml) and CD40L transfected BHK cells (Wagner et al. 2004); CD40L transfected cells were replaced by soluble CD40L (sCD40L) in this study. The APC markers CD80, CD86, and MHC II were up-regulated on CD19⁺ PBMC when cultured long-term in the presence of sCD40L (2 μ g/ml) and IL-4 (2 ng/ml) (Wu et al. 2010). Significant increases in CpG specific B cell proliferation in the presence of BAFF (10 ng/ml) and CpG (10 μ g/ml), using bovine cells (Buchanan et al. 2011). Based on the above, various combinations of CpG, BAFF, sCD40L, IL-4, and IL-15 were used to investigate the optimal stimulation for cytokine expression by CD19⁺ PBMC after 3 days of stimulation at 2×10^5 PBMC/per round bottom well. For comparison, other common T cell stimulations were used, such as mAb against CD3 and PMA/I. Brefeldin A was added for a further 15 hours in the absence or presence of PMA/I,

then cells were stained and analysed by flow cytometry for IL-10, IL-4, and TNF- α production. Based on the results obtained from one donor PBMC, the stimulation which induced the highest frequencies of IL-10 and IL-4 after re-stimulation with PMA/I, was the combination of BAFF (10 ng/ml) and CpG (10 μ g/ml) (Fig. 5.1. A and B). TNF- α was up-regulated when PBMC were re-stimulated with PMA/I regardless of previous stimulations (Fig. 5.1. C). In the absence of re-stimulation with PMA/I, an initial stimulation with CpG (3 μ g/ml) alone or in combination with PMA (50 ng/ml) and ionomycin (1 μ g/ml) was optimal for IL-10 and TNF- α production (Fig. 5.1. A and C). Overall, culturing PBMC for 3 days with BAFF (10 ng/ml) and CpG (10 μ g/ml), followed by a PMA/I re-stimulation allowed for optimal induction of intracellular IL-10, IL-4, and TNF- α expression.

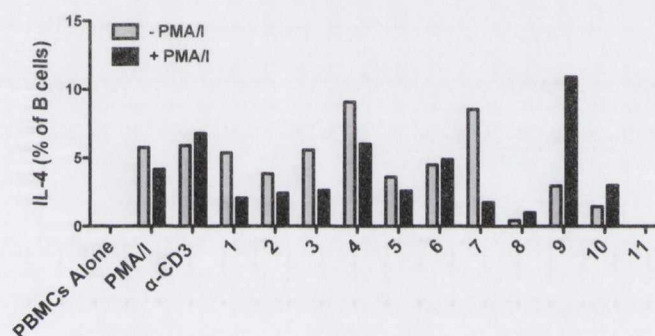
A.



B.



C.



D.

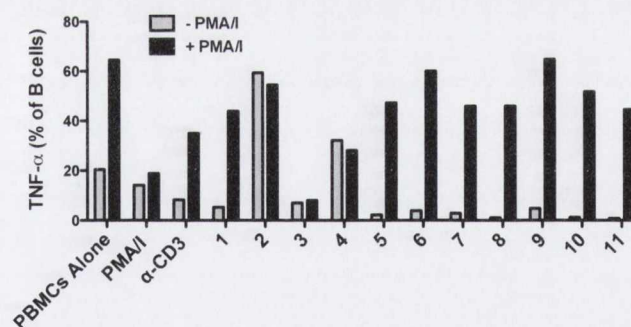


Fig. 5.1. IL-10, IL-4, and TNF- α production by B cells stimulated with various B cell agonists. PBMC from one healthy donor were cultured alone, with PMA (50 ng/ml) and ionomycin (1 μ g/ml) (PMA/I), anti-hCD3 mAb (α -CD3) (1 μ g/ml), or with one of 11 different B cell stimulations: 1 (0.1 μ g/ml CpG), 2 (3 μ g/ml CpG), 3 (10 μ g/ml CpG) (Das et al. 2012), 4 (3 μ g/ml CpG + PMA/I), 5 (10 μ g/ml CpG + 20 IU/ml IL-2 + 10 ng/ml IL-15) (Huggins et al. 2007), 6 (3 μ g/ml CpG + 2 μ g/ml sCD40L) (Wagner et al. 2004; Wu et al. 2010), 7 (0.4 μ g/ml sCD40L + 100 ng/ml IL-4), 8 (2 μ g/ml sCD40L + 2 ng/ml IL-4) (Wu et al. 2010), 9 (10 ng/ml BAFF + 10 μ g/ml CpG) (Buchanan et al. 2011), 10 (10 ng/ml BAFF + 1 μ g/ml CpG), 11 (10 ng/ml BAFF + 2 μ g/ml sCD40L), for 72. After 72 hours brefeldin A was added to the co-cultures and they were re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated for 15 hours. Cells were then stained for intracellular flow cytometry analysis using mAb against IL-10, IL-4, and TNF- α . **A**, Representative dot plots of CD19⁺ PBMC showing frequencies of IL-10, IL-4, and TNF- α expression. **B**, **C**, and **D**, Frequencies of CD19⁺ PBMC expressing intracellular IL-10 (**B**), IL-4 (**C**), and TNF- α (**D**).

5.3.2 Optimisation of stimulation times for TNF- α , IL-4, and IL-10 expression by B cells.

After testing 11 different B cell stimuli, the optimal stimulation for the induction of intracellular IL-10, IL-4 and TNF- α by CD19⁺ PBMC after PMA/I re-stimulation was BAFF (10 ng/ml) and CpG (10 μ g/ml), while CpG (3 μ g/ml) was the best option for cytokine induction in the absence of PMA/I re-stimulation (see 5.3.1). Many studies looking at B cell activation, proliferation, and IL-10 production have suggested incubation times from 3 – 4 days (Wagner et al. 2004; Das et al. 2012). The culture incubation time required for optimal IL-10, IL-4 and TNF- α expression by B cells was investigated. Freshly isolated PBMC were cultured for 3 or 5 days in the presence of BAFF (10 ng/ml) and CpG (10 μ g/ml), or CpG (10 μ g/ml) alone, or CpG (3 μ g/ml). Brefeldin A was added for a further 15 hours in the absence or presence of PMA/I, then cells were stained and analysed by flow cytometry for IL-10, IL-4, and TNF- α production. Based on the results obtained from one donor PBMC, in the absence of PMA/I re-stimulation, there was no noticeable difference between 3 days and 5 days of stimulation (Fig. 5.2. A, C, and E). On the other hand, when PBMC were re-stimulated with PMA/I, a noticeable increase was observed in both intracellular IL-10 and TNF- α expression at day 3 when compared to day 5 was observed (Fig. 5.2. B and F). However, in this experiment there was no difference in cytokine production between the different stimulation. These results suggest that 3 days is enough to stimulate optimal IL-10, IL-4, and TNF- α production by CD19⁺ PBMC.

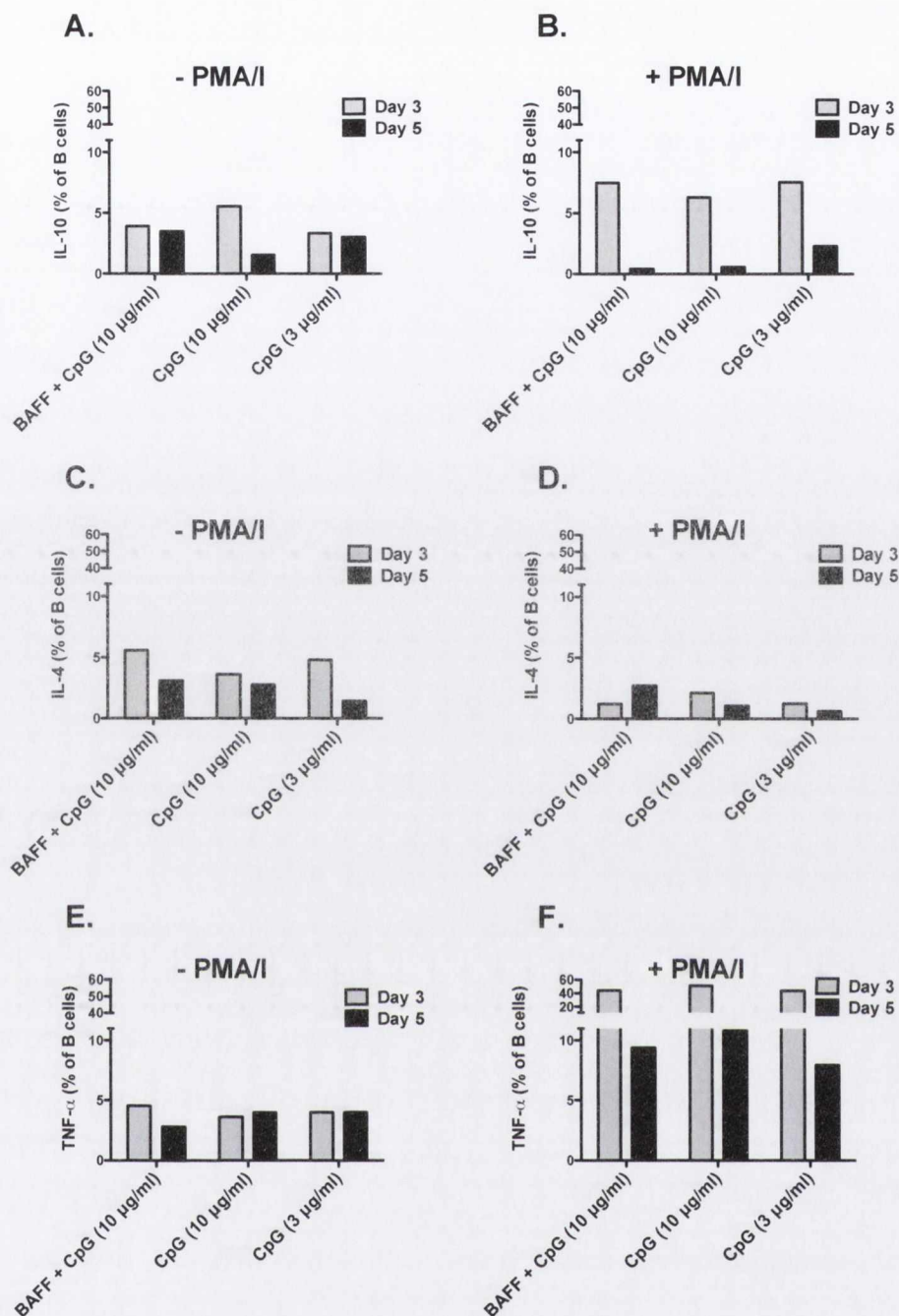


Fig. 5.2. Optimisation of B cell stimulation times for IL-10, IL-4, and TNF- α production. PBMC from one healthy donor were stimulated with: 10 ng/ml of BAFF with 10 μ g/ml of CpG, or 10 μ g/ml of CpG alone, or 3 μ g/ml of CpG, and cultured for 3 days or 5 days. PBMC were then either re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated, all in the presence of brefeldin A for a further 5 hours. Cells were stained for intracellular IL-10, IL-4, and TNF- α , and analysed by flow cytometry. **A** and **B**, Frequencies of B cells producing IL-10, with (**A**) or without (**B**) re-stimulation. **C** and **D**, Frequencies of B cells producing IL-4, with (**C**) or without (**D**) re-stimulation. **E** and **F**, Frequencies of B cells producing TNF- α , with (**E**) or without (**F**) re-stimulation.

5.3.3 Optimisation of PMA/I re-stimulation times for the detection of TNF- α , IL-4, and IL-10 expression by B cells.

In order to maximize the detection of cytokine production by B cells, the duration of re-stimulation with PMA/I was compared at 5 hours or over night (~ 15 hours). Freshly isolated PBMC were cultured for 3 days in the presence of BAFF (10 ng/ml) and CpG (10 μ g/ml), or CpG (10 μ g/ml) alone, or CpG (3 μ g/ml). Brefeldin A was added for a further 5 or 15 hours in the presence of PMA/I, then cells were stained and analysed by flow cytometry for IL-10, IL-4, and TNF- α production (Fig. 5.3.). Based on the results obtained from one donor PBMC, over night re-stimulation with PMA/I resulted in higher frequencies of B cells producing IL-4 and IL-10 detected (Fig. 5.3. A and B), while higher frequencies of B cells producing TNF- α were detected when cells were re-stimulated for only 5 hours (Fig. 5.3. C).

5.3.4 Optimisation of TNF- α , IL-4, and IL-10 expression by B cells, in the presence or absence of PBMC.

Previous optimisations for B cell stimulations were carried out using PBMC, which contain other cells that may influence the stimulation of B cells, such as T cells and monocytes. Whether purified B cells can be optimally stimulated using BAFF and CpG, or if they required the presence of PBMC was investigated. CD19⁺ B cells were isolated from PBMC using magnetic bead cell separation. Purified B cells or PBMC were then cultured for 3 days in the presence of BAFF (10 ng/ml) and CpG (10 μ g/ml), or CpG (10 μ g/ml) alone, or CpG (3 μ g/ml). Brefeldin A was added for a further 5 hours in the presence of PMA/I, then cells were stained and analysed by flow cytometry for IL-10, IL-4, and TNF- α production. Based on the results obtained from one donor PBMC, there was no major difference in cytokine production by B cells in the presence or absence of PBMC (Fig. 5.4). However, slight increases in IL-10 production were observed in purified B cells compared to PBMC (Fig. 5.4. A).

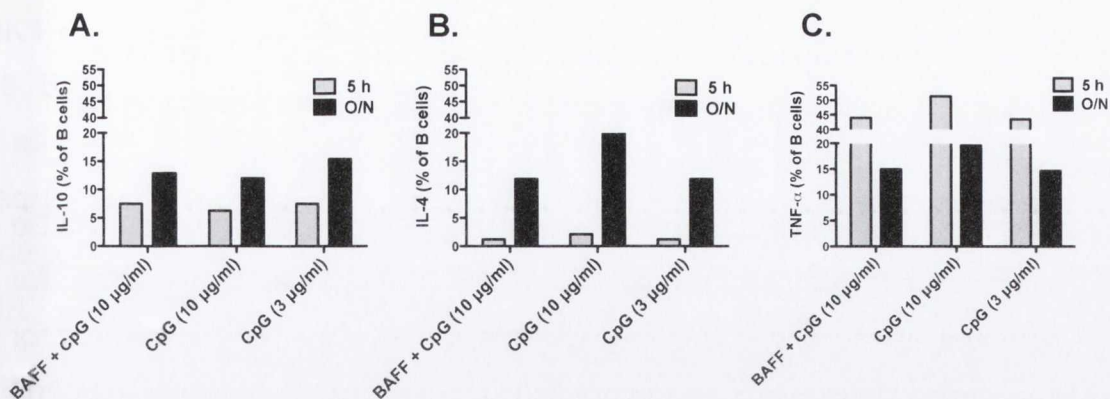


Fig. 5.3. Optimisation of B cell re-stimulation times for IL-10, IL-4, and TNF- α production. PBMC from one healthy donor were stimulated with: 10 ng/ml of BAFF with 10 µg/ml of CpG, or 10 µg/ml of CpG alone, or 3 µg/ml of CpG, and cultured for 3 days. PBMC were then re-stimulated with PMA and ionomycin (PMA/I) for 5 hours or overnight in the presence of brefeldin A. Cells were stained for intracellular IL-10, IL-4, and TNF- α , and analysed by flow cytometry. **A**, **B**, and **C**, Frequencies of B cells producing IL-10 (**A**), IL-4 (**B**), and TNF- α (**C**) after 5 hour or overnight re-stimulation with PMA/I.

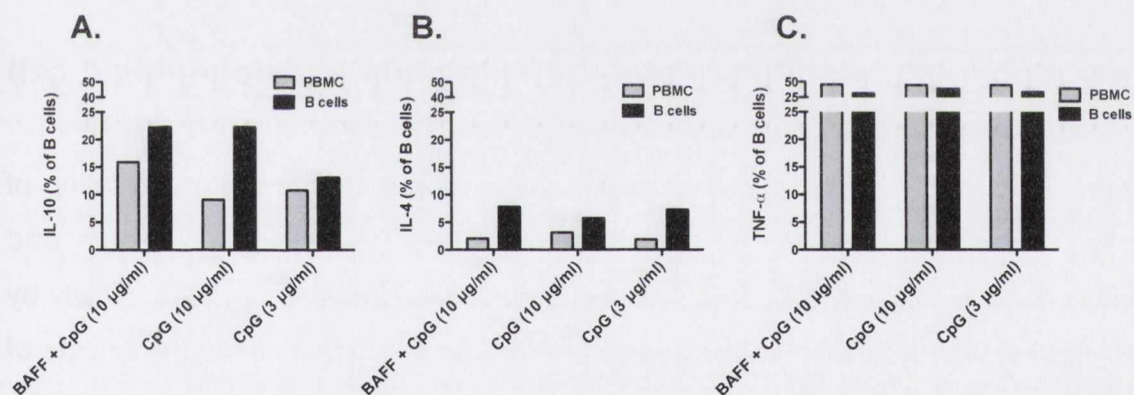


Fig. 5.4. PBMC are not required for IL-10, IL-4, and TNF- α production by BAFF and CpG stimulated B cells. CD19⁺ cells were isolated from one healthy donor PBMC using magnetic bead cell sorting. PBMC or purified CD19⁺ B cells from one healthy donor were stimulated with: 10 ng/ml of BAFF with 10 µg/ml of CpG, or 10 µg/ml of CpG alone, or 3 µg/ml of CpG, and cultured for 3 days. PBMC were then re-stimulated with PMA and ionomycin (PMA/I) for 5 hours in the presence of brefeldin A. Cells were stained for intracellular IL-10, IL-4, and TNF- α , and analysed by flow cytometry. **A**, **B**, and **C**, Frequencies of IL-10 (**A**), IL-4 (**B**), and TNF- α (**C**) producing-B cells from PBMC or purified CD19⁺ cells.

5.3.5 Comparing staining at room temperature or 4°C for the detection of intracellular TNF- α , IL-4, and IL-10 in CD19⁺ PBMC.

The staining method carried out for the detection of intracellular cytokines can be carried out at room temperature or at 4°C. The optimal temperature for intracellular cytokine staining in B cells was investigated. PBMC were cultured for 3 days in the presence of BAFF (10 ng/ml) and CpG (10 μ g/ml), or CpG (10 μ g/ml) alone, or CpG (3 μ g/ml). Brefeldin A was added for a further 5 hours in the presence of PMA/I, then cells were stained and analysed by flow cytometry for IL-10, IL-4, and TNF- α production. Based on the results obtained from one donor PBMC, staining at 4°C resulted in better detection of intracellular IL-10 and TNF- α in cells that were not re-stimulated with PMA/I (Fig. 5.5. A, C, and E). However, in cells that were re-stimulated, there was no noticeable difference between staining methods in the detection of any of the three cytokines (Fig. 5.5. B, D, and F).

5.3.6 Effect of CD4⁺, CD8⁺, and DN iNKT cells on allogeneic B cell differentiation.

Human CD4⁺ and DN iNKT cells have been reported to induce the proliferation of both naïve and memory B cell subsets *in vitro* (Galli et al. 2003). Naïve and memory B cells, both un-switched and switched can be identified phenotypically by their expression of CD27 and IgD (Bemark et al. 2012). In this study, the effects of CD4⁺, CD8⁺, and DN iNKT cells on B cell differentiation was investigated. CD4⁺, CD8⁺, and DN iNKT cells were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells from 4 donors were then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone or co-cultured with CD4⁺, CD8⁺, and DN iNKT cells at a 1:1 ratio for 7 days. Cells were stained with mAb against CD27 and IgD, and analysed by flow cytometry for changes in frequencies of naïve/transitional (IgD⁺CD27⁻), un-switched memory (IgD⁺CD27⁺), switched memory (IgD⁻CD27⁺), and CD27⁻ memory (IgD⁻CD27⁻). There was no change in the frequencies of naïve/transitions B cells after 7 days co-culture with allogeneic iNKT cells (Fig. 5.6. A). The frequencies of un-switched memory B cells were significantly increased in the presence of allogeneic CD4⁺

iNKT cells, and visibly increased in the presence of CD8⁺ and DN iNKT cells (Fig. 5.6. B). While switched memory B cells were significantly reduced in the presence of all three iNKT cell subsets (Fig. 5.6. C), no significant changes occurred in the frequencies of CD27⁺ memory B cell (Fig. 5.6. D). All changes occurred independent of α -GalCer stimulation. These results suggest a possible role for iNKT cells in memory B cell differentiation.

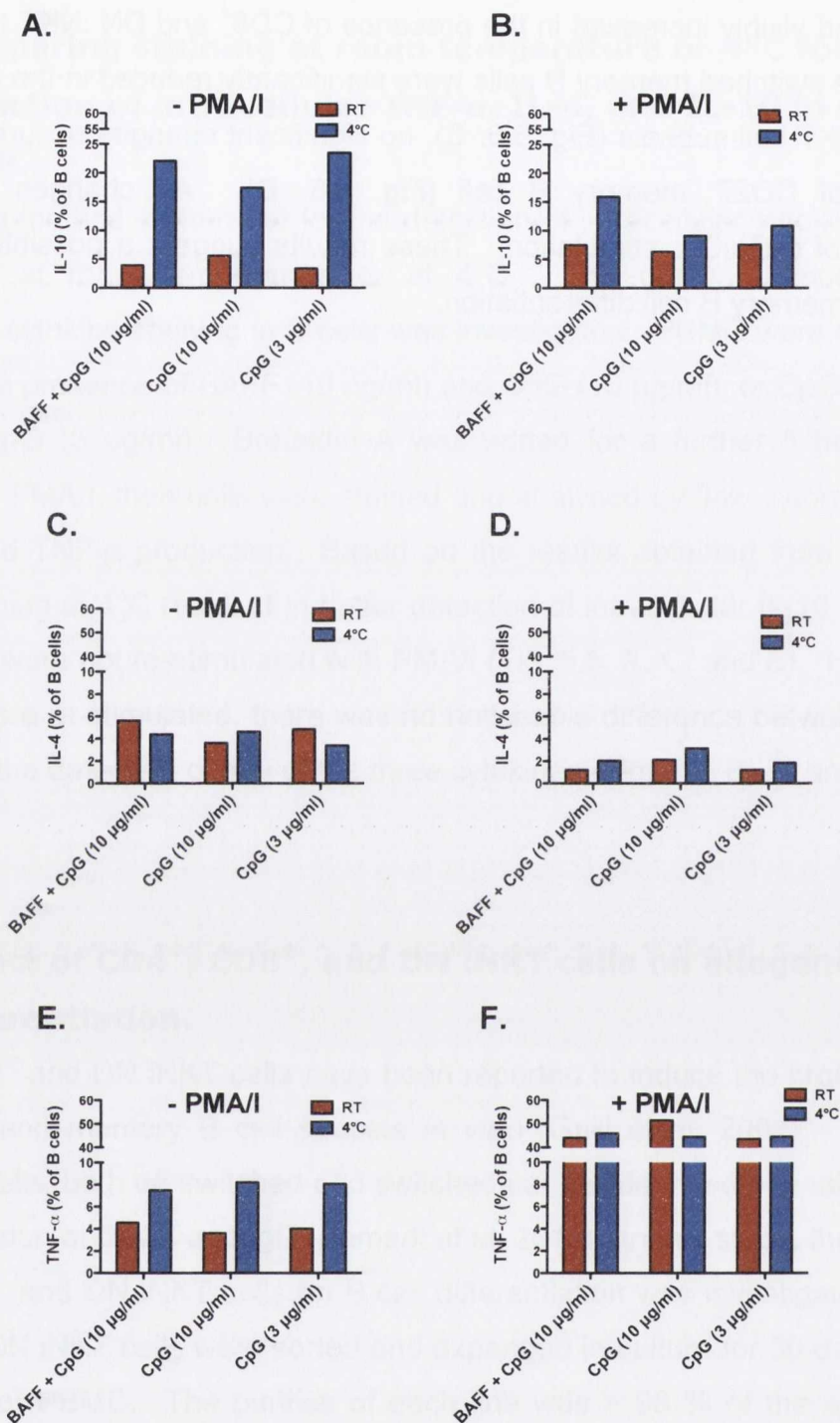


Fig. 5.5. Comparison of mAb staining at room temperature and 4°C for optimal intracellular IL-10, IL-4, and TNF- α detection in B cells. PBMC from one healthy donor were stimulated with: 10 ng/ml of BAFF with 10 µg/ml of CpG, or 10 µg/ml of CpG alone, or 3 µg/ml of CpG, and cultured for 3 days. PBMC were then either re-stimulated with PMA and ionomycin (PMA/I) or left un-stimulated, all in the presence of brefeldin A for a further 5 hours. Cells were stained on ice (4°C) or at room temperature for intracellular IL-10, IL-4, and TNF- α , and analysed by flow cytometry. **A** and **B**, Frequencies of B cells producing IL-10, without (**A**) or with (**B**) re-stimulation. **C** and **D**, Frequencies of B cells producing IL-4, without (**C**) or with (**D**) re-stimulation. **E** and **F**, Frequencies of B cells producing TNF- α , without (**E**) or with (**F**) re-stimulation.

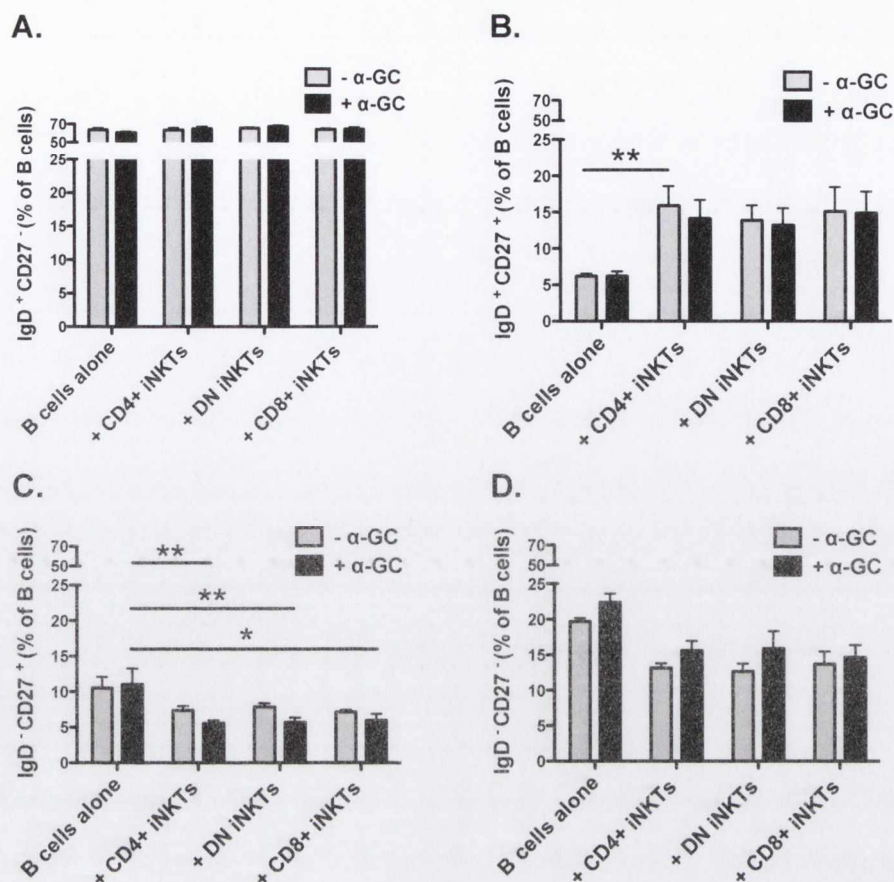


Fig. 5.6. iNKT cell influence the differentiation of allogeneic B cells. iNKT cell lines were prepared from healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from allogeneic donors, using magnetic bead cell sorting. B cells were cultured alone (-α-GC) or with 100 ng/ml of α-GalCer (+α-GC) over night at 37°C. B cells were then cultured alone or co-cultured with CD4⁺, CD8⁺, and DN iNKT cells for 7 days. B cells were then stained using mAb against IgD and CD27, and analysed by flow cytometry. Displayed are frequencies of IgD⁺CD27⁻ naïve/transitional B cells (A), IgD⁺CD27⁺ unswitched memory B cells (B), IgD⁻CD27⁺ switched memory B cells (C), and IgD⁻CD27⁻ CD27⁻ memory B cells (D), after co-culture with allogeneic iNKT cells in the absence and presence of α-GalCer (n = 4). Data points are plotted as mean ± SEM. Statistical analysis was carried out using Friedman test with post hoc Dunne multiple comparison tests. * P ≤ 0.05, ** P ≤ 0.01.

5.3.7 iNKT cells induce marginal zone B (MZB) cells.

Marginal zone B cells are found in the marginal zone of the spleen, and they represent a subset of B cells that seem innate in function, but express memory phenotype and functional attributes. They can be identified as having high levels of CD1d expression and are IgM⁺IgD⁺CD27⁺ (Radbruch et al. 2006; Pillai & Cariappa 2009). iNKT cells have been suggested to interact with MZB cells, due to their high levels of surface CD1d (Allan et al. 2011; Muppidi et al. 2011). The effect of co-culturing iNKT cells and peripheral blood B cells on the frequencies of MZB cells was investigated. CD4⁺, CD8⁺, and DN iNKT cells were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells from 4 allogeneic donors were then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone or co-cultured with CD4⁺, CD8⁺, and DN iNKT cells at a 1:1 ratio for 7 days. Cells were stained with mAb against CD27, IgD, and IgM, and analysed by flow cytometry for changes in frequencies of MZB cells (IgD⁺CD27⁺IgM⁺) (Fig. 5.7. A and B). Frequencies of MZB cells were significantly increased in the presence of allogeneic CD4⁺ and CD8⁺ iNKT cells, and visibly increased in the presence of DN iNKT cells (Fig. 5.7. C). These results suggest that iNKT cells may promote the differentiation of MZB cells without the need for α -GalCer.

5.3.8 iNKT cells induce antibody secretion by autologous B cells.

A previous *in vitro* study on human peripheral blood B cells has shown using expanded CD4⁺ and DN iNKT cell clones that iNKT cells induce IgM and IgG production by B cells (Galli et al. 2003). The differential effect of CD4⁺, CD8⁺, and DN polyclonal iNKT cells on IgM, IgA, and total IgG production by B cells was investigated in this study. CD4⁺, CD8⁺, and DN iNKT cells, as well as CD3⁺ non-iNKT cells, were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells from 4 autologous donors were then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone or co-cultured with CD4⁺, CD8⁺, and DN iNKT cells at a 1:1 ratio for 10 days. Levels of secreted total IgG, IgM, and IgA in the culture supernatants were tested using a

cytometric bead array (CBA) assay, and analysed by flow cytometry. Data obtained from this experiment was amalgamated with others obtained by Dr. Grace Zeng (n = 3), who independently carried out the same experiment. iNKT cells were capable of inducing significant increases in IgM, IgG, and IgA secretion by B cells (Fig. 5.8. A, B, and C). A more significant increase in antibody production by B cells was observed in the presence of CD4⁺ iNKT cells when compared to CD8⁺ and DN iNKT cells, and in the absence of α -GalCer (Fig. 5.8). This suggests a role for iNKT cells in antibody mediated B cell responses *in vivo*, as was previously demonstrated in mouse models (Galli et al. 2003; Belperron et al. 2005; Lee et al. 2005; Lisbonne et al. 2003).

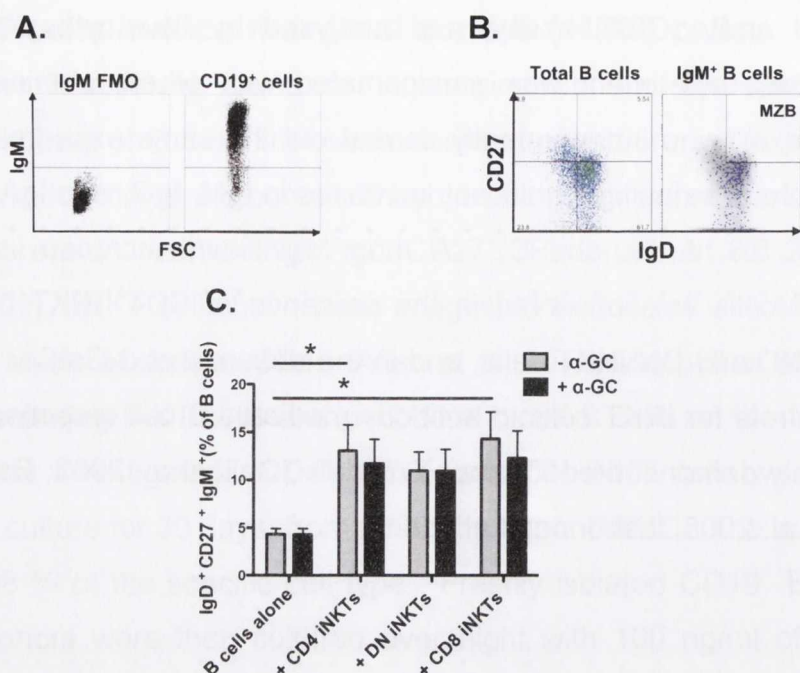


Fig. 5.7. Frequencies of IgD⁺CD27⁺IgM⁺ B cells are increased when co-cultured with allogeneic iNKT cells. iNKT cell lines were prepared from healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from allogeneic donors, using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer over night at 37°C. B cells were then cultured alone or co-cultured with CD4⁺, CD8⁺, and DN iNKT cells for 7 days. B cells were then stained using mAb against IgD, IgM, and CD27, and analysed by flow cytometry. **A**, Representative dot plots showing the gating on IgM⁺ B cells (CD19⁺ cells), using an IgM FMO. **B**, Representative polychromatic dot plot (left) showing CD27 and IgD expression by B cells, and (right) density plot highlighting in blue the IgM⁺ cells, and the gating on marginal zone-like B cells (MZB). **C**, Frequencies of IgD⁺CD27⁺IgM⁺ B cells (MZB) after co-culture with allogeneic iNKT cells (n = 4). Data points are plotted as mean $\pm$ SEM. Statistical analysis was carried out using Friedman test with post hoc Dunne multiple comparison tests. * P $\leq$ 0.05.

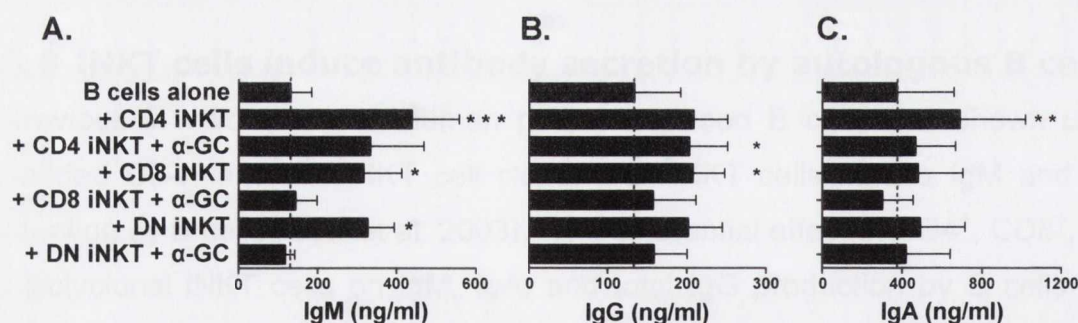


Fig. 5.8. Increase in IgG, IgM, and IgA secretion by B cells in response to autologous iNKT cells. iNKT cell lines were prepared from healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from allogeneic donors, using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer over night at 37°C. B cells were then cultured alone or co-cultured with iNKT cells for 10 days. Supernatants were analysed using a cytometric bead assay for the detection of secreted total IgG, IgM, and IgA by flow cytometry. **A**, **B**, and **C**, Concentrations of total IgM (**A**), IgG (**B**), and IgA (**C**) in co-culture supernatants of B cells and autologous iNKT cells (n = 7). Data points are obtained from two independent experiments, and plotted as mean $\pm$ SEM. Statistical analysis was carried out using Friedman test with post hoc Dunne multiple comparison tests. * P $\leq$ 0.05, ** P $\leq$ 0.01, *** P $\leq$ 0.001.

5.3.9 iNKT cell-mediated antibody secretion is blocked by antibodies against CD1d but not IL-4 or IL-13.

An *in vivo* study in mice has demonstrated that iNKT cell help to B cells for the production of lipid specific Abs was CD1d dependent and did not require IL-4 (Leadbetter et al. 2008). IL-4 and IL-13 are Th2 cytokines produced by T_{FH} cells and play a role in Ab-mediated immunity (Vinuesa et al. 2005). The requirement of CD1d, IL-4, or IL-13 by B cells to produce Ab in response to iNKT cell stimulation was investigated. iNKT cells were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells were cultured alone, or co-cultured with iNKT cells at 1:1 ratios for 10 days in the absence and presence of blocking mAb against CD1d, IL-4, or IL-13. Levels of secreted total IgG, IgM, and IgA in the culture supernatants were tested using a cytometric bead array (CBA) assay, and analysed by flow cytometry. Total allogeneic iNKT cells did not significantly influence Ab secretion by B cells (Fig. 5.9). However, there was a modest increase in total IgG when B cells were co-cultured with iNKT cells, and this increase was abolished in the presence of CD1d blocking mAb (Fig. 5.9. A). Although iNKT cells did not enhance either IgM or IgA secretion by B cells, blocking CD1d resulted in a noticeable decrease in the levels of both IgM and IgA (Fig. 5.9. B and C). These results suggest that iNKT cells modulation of Ab secretion by B cells is CD1d dependent.

5.3.10 iNKT cells induce CD69 and IL-21R expression by autologous and allogeneic B cells.

iNKT cells in humans were found to activate B cells, causing an upregulation of CD69. This activation was mainly caused by CD4⁺ iNKT (Lin, Nieda, Rozenkov, et al. 2006). IL-21 receptor (IL-21R) is expressed on both peripheral blood T cells and B cells, and is upregulated upon activation (Habib et al. 2003). The effect of iNKT cells on the expression of both CD69 and IL-21R on B cells was investigated. CD4⁺, CD8⁺, and DN iNKT cells, as well as CD3⁺ non-iNKT cells, were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells from 4 autologous donors were then cultured overnight with 100

ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone, with 10 ng/ml of BAFF and 10 μ g/ml of CpG, or co-cultured with autologous CD4⁺, CD8⁺, and DN iNKT cells or non-iNKT cells at a 1:1 ratio for 3 days. B cells were also co-cultured with allogeneic CD4⁺, CD8⁺, and DN iNKT cells for 7 days. Cells were stained using mAb against CD69 and IL-21R and analysed by flow cytometry (Fig. 5.10. A). Frequencies of IL-21R⁺ B cells were significantly increased in the presence of α -GalCer and autologous CD4⁺ iNKT cells, which were comparable to frequencies in the presence of non-iNKT cells (Fig. 5.10. B). Marginal increases in the frequencies of IL-21R⁺ B cells were also observed in the presence of allogeneic CD4⁺ iNKT cells, but in the absence of α -GalCer (Fig. 5.10. C). CD69 expression on B cells were also significantly increased in the presence of autologous CD4⁺ iNKT cells (Fig. 5.10. D), as well as allogeneic CD4⁺ iNKT cells (Fig. 5.10. E). These results suggest that CD4⁺ iNKT cells induce an up-regulation in both CD69 and IL-21R expression on autologous and allogeneic B cells, independent of α -GalCer stimulation.

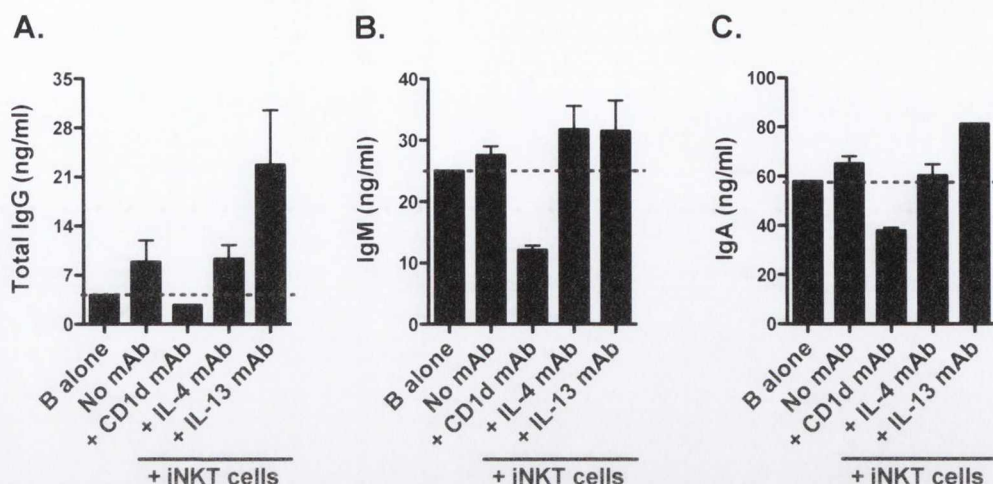


Fig. 5.9. iNKT cell-mediated antibody secretion is blocked by antibodies against CD1d but not IL-4 or IL-13. iNKT cell lines were prepared from healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from allogeneic donors, using magnetic bead cell sorting. B cells were then co-cultured with iNKT cells in the absence or presence of 10 μ g/ml blocking mAb against CD1d, IL-4, and IL-13, for 10 days. Supernatants were analysed using a cytometric bead assay for the detected of secreted total IgG, IgM, and IgA by flow cytometry. **A**, **B** and **C**, Concentrations of total IgG (**A**), IgM (**B**), and IgA (**C**) in co-culture supernatants of B cells and allogeneic iNKT cells in the presence and absence of blocking antibodies ($n = 4$). Data points are plotted as mean $\pm$ SEM.

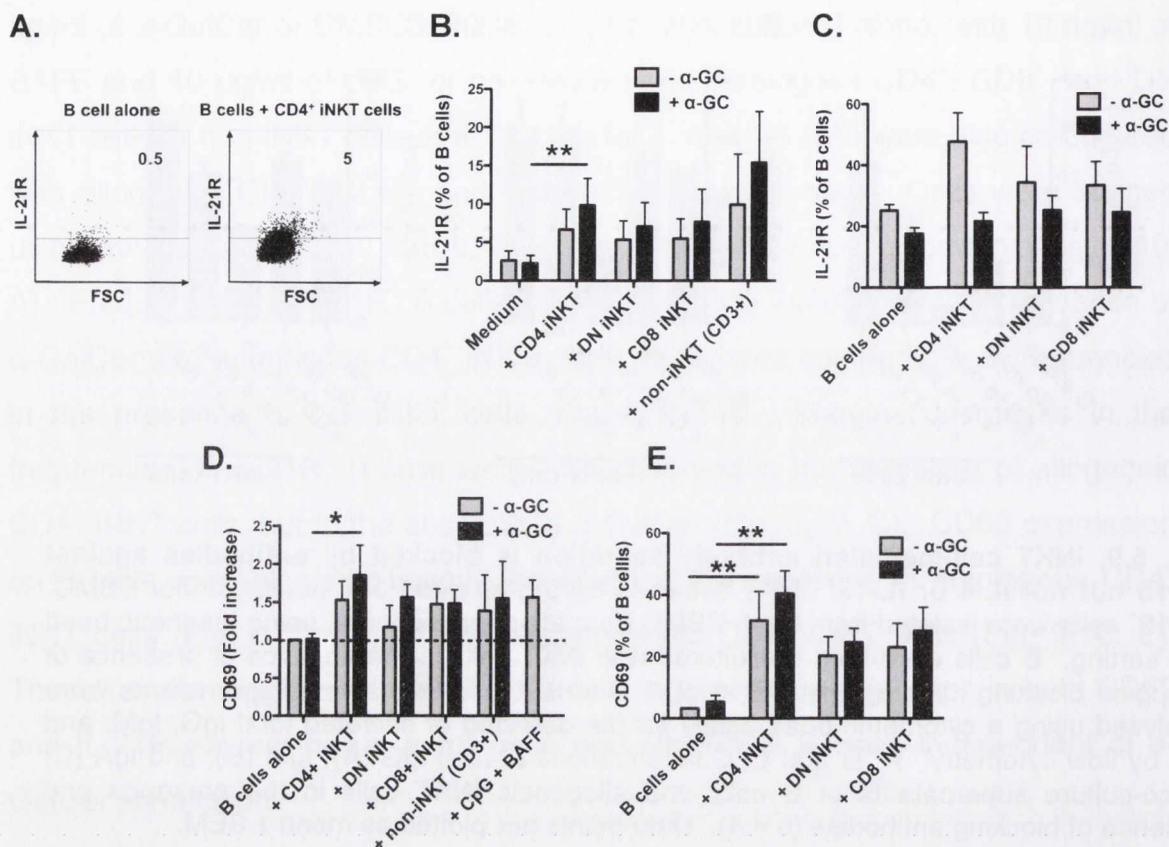


Fig. 5.10. IL-21R and CD69 expression on B cells is increased by autologous and allogeneic CD4⁺ iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from autologous and allogeneic donors, using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α-GalCer over night at 37°C. B cells were then cultured alone, with BAFF (10 ng/ml) and CpG (10 μg/ml) as a positive control, or co-cultured with autologous or allogeneic iNKT cells, or CD3⁺ non-iNKT cells for 3 or 7 days. B cells were then stained using mAb against IL-21R and CD69, and analysed by flow cytometry. **A** and **B**, Frequencies of B cells expressing IL-21R after 3 days co-culture with autologous (**A**) or 7 days with allogeneic (**B**) iNKT cells (n = 4). **C**, Frequencies of B cells expressing CD69, expressed as a fold increase from B cell alone, after 3 days co-culture with autologous iNKT cells (n = 4). **D**, Frequencies of B cells expressing CD69 after 7 days co-culture with allogeneic iNKT cells (n = 4). Data points are plotted as mean ± SEM. Statistical analysis was carried out using Friedman test with post hoc Dunne multiple comparison tests. * P ≤ 0.05, ** P ≤ 0.01.

5.3.11 iNKT cells induce autologous and allogeneic

CD1d^{hi}CD5⁺ B cells.

There is a direct relationship between CD5 expression and IL-10 production by human B cells (Gary-Gouy et al. 2002). A subset of B cells that are CD5⁺CD1d^{hi} have been shown in both mice and humans to be enriched for IL-10 producing regulatory B cells (Blair et al. 2010). CD4⁺, CD8⁺, and DN iNKT cells, as well as CD3⁺ non-iNKT cells, were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells from 4 autologous donors were then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone, with 10 ng/ml of BAFF and 10 μ g/ml of CpG, or co-cultured with autologous CD4⁺, CD8⁺, and DN iNKT cells or non-iNKT cells at a 1:1 ratio for 3 days. B cells were also co-cultured with allogeneic CD4⁺, CD8⁺, and DN iNKT cells for 3 and 8 days. Cells were stained using mAb against CD1d and CD5 and analysed by flow cytometry (Fig. 5.11. A). Significant and modest increases in CD5⁺CD1d^{hi} B cells, in the absence of α -GalCer, were observed in the presence of autologous DN and CD4⁺ iNKT cells respectively (Fig. 5.11. B). Allogeneic B cells also showed significant increase in the frequencies of CD5⁺CD1d^{hi} B cells when co-cultured with CD4⁺ iNKT cells. These increases were independent of α -GalCer, and required a culture time of 8 days (Fig. 5.11. C and D). These results suggest that CD4⁺ and DN iNKT cells can induce the differentiation of CD5⁺CD1d^{hi} B cells, which in turn may function as regulatory B cells.

5.3.12 CD4⁺ iNKT cells induce autologous and allogeneic

CD24^{hi}CD38^{hi} B cells.

CD24^{hi}CD38^{hi} B cells are a subset of transitional B cells that have been shown in humans to possess immunosuppressive properties (Blair et al. 2010). CD4⁺, CD8⁺, and DN iNKT cells, as well as CD3⁺ non-iNKT cells, were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells from 4 autologous donors were then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone, with 10 ng/ml of BAFF and 10 μ g/ml of CpG, or co-cultured with autologous CD4⁺, CD8⁺, and DN iNKT cells or non-

iNKT cells at a 1:1 ratio for 3 days. B cells were also co-cultured with allogeneic CD4⁺, CD8⁺, and DN iNKT cells for 7 days. Cells were stained using mAb against CD24 and CD38 and analysed by flow cytometry (Fig. 5.12. A). The frequencies of CD24^{hi}CD38^{hi} B cells were unchanged in the presence of autologous iNKT cells (Fig. 5.12. B). However, allogeneic CD4⁺ iNKT cells resulted in a significant increase in the frequencies of CD24^{hi}CD38^{hi} B cells, independent of α -GalCer, after 7 days in culture (Fig. 5.12. C). These results suggest that CD4⁺ iNKT cells can induce the differentiation of CD24^{hi}CD38^{hi} B cells.

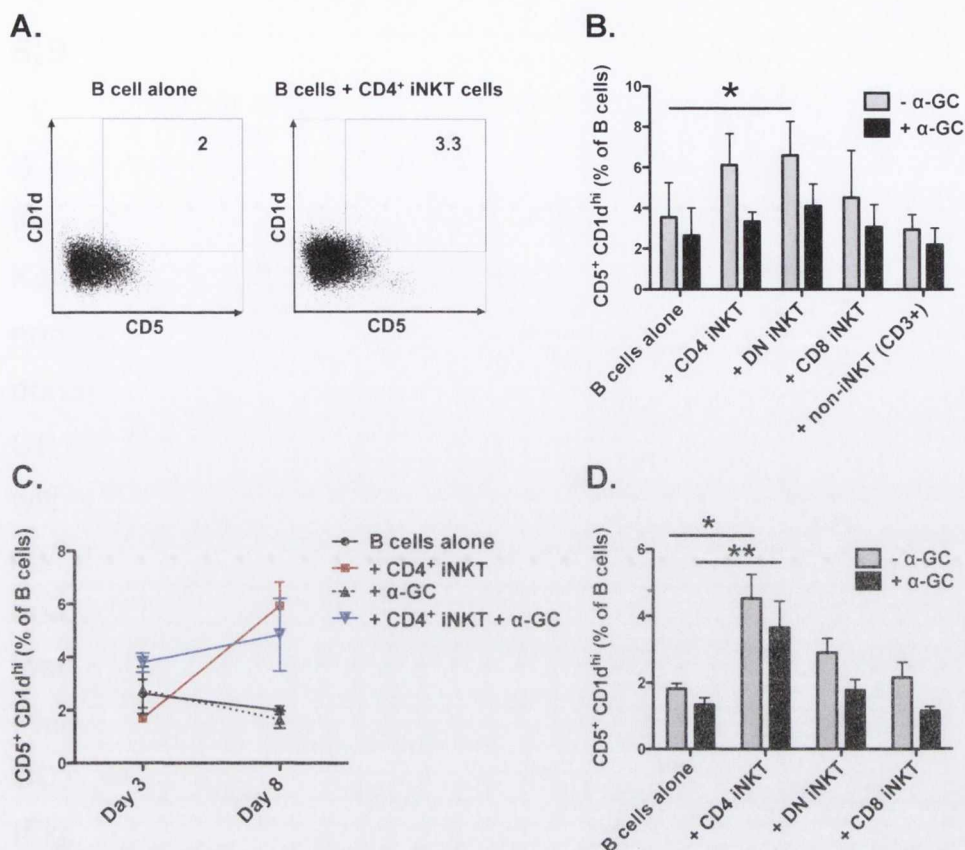


Fig. 5.11. Frequencies of CD1d^{hi}CD5⁺ B cells are increased by culturing B cells with CD4⁺ or DN iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from 4 allogeneic or 4 autologous donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer over night at 37°C. B cells were then cultured alone, or co-cultured with autologous or allogeneic iNKT cells, or CD3⁺ non-iNKT cells. B cells were then stained using mAb against CD1d and CD5, and analysed by flow cytometry. **A**, Representative dot plots of CD19⁺ B cells, showing the gating on CD1d^{hi}CD5⁺ B cells. **B**, Frequencies of CD1d^{hi}CD5⁺ B cells after 3 days of co-culture with autologous iNKT cells. **C**, Frequencies of CD1d^{hi}CD5⁺ B cells after 3 or 7 days co-culture with allogeneic iNKT cells. **D**, Frequencies of CD1d^{hi}CD5⁺ B cells after 7 days co-culture with allogeneic iNKT cells. Data points are plotted as mean $\pm$ SEM. Statistical analysis was carried out using the non-parametric Friedman test with post hoc Dunne multiple comparison tests. * $P \leq 0.05$, ** $P \leq 0.01$.

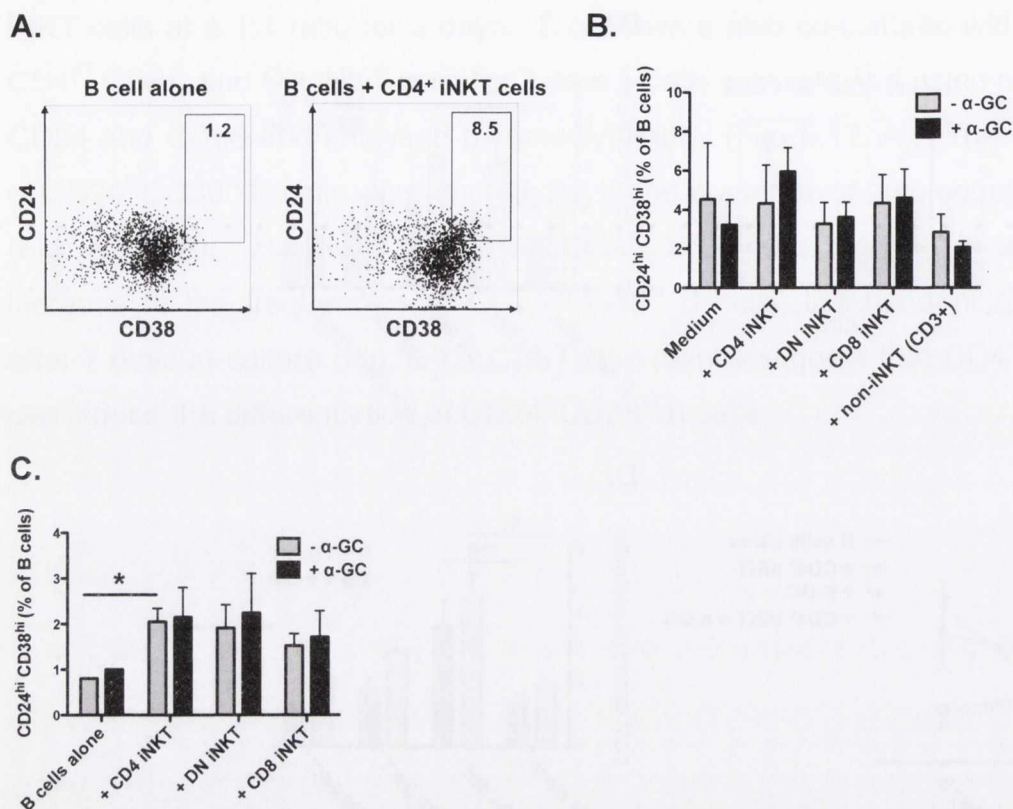


Fig. 5.12. Frequencies of CD24^{hi}CD38^{hi} B cells are increased by culturing B cells with allogeneic iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from 4 allogeneic or 4 autologous donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α-GalCer over night at 37°C. B cells were then cultured alone, or co-cultured with autologous or allogeneic iNKT cells, or CD3⁺ non-iNKT cells. B cells were then stained using mAb against CD24 and CD38, and analysed by flow cytometry. **A**, Representative dot plots of CD19⁺ B cells, showing the gating on CD24^{hi}CD38^{hi} B cells. **B**, Frequencies of CD24^{hi}CD38^{hi} B cells after 3 days co-culture with autologous iNKT cells. **C**, Frequencies of CD24^{hi}CD38^{hi} B cells after 8 days co-culture with allogeneic iNKT cells. Data points are plotted as mean ± SEM. Statistical analysis was carried out using the non-parametric Friedman test with post hoc Dunne multiple comparison tests. * P ≤ 0.05.

5.3.13 iNKT cells do not induce IFN- γ , IL-4, or IL-10 expression by autologous B cells.

B cells are capable of producing many cytokines including IL-10, through which they can elicit immune stimulatory or immune regulatory effects (Pistoia 1997; Kalampokis et al. 2013). CD4⁺, CD8⁺, DN, as well as total iNKT cells, and CD3⁺ non-iNKT cells were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. CD19⁺ B cells were purified from 4 donors fresh PBMC, then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone, with 10 ng/ml of BAFF and 10 μ g/ml of CpG, or co-cultured with autologous CD4⁺, CD8⁺, and DN iNKT cells or non-iNKT cells at a 1:1 ratio for 3 days. Brefeldin A was then added to co-cultures, which were then split; one half was re-stimulated with PMA/I and the other left un-stimulated. Cells were finally stained with mAb against CD3, CD19, IFN- γ , IL-4, and IL-10, and analysed by flow cytometry (Fig. 5.13 A), (Fig. 5.14 A), and (Fig. 5.15. A). Autologous iNKT cells did not significantly influence IFN- γ , IL-4, or IL-10 expression by B cells (Fig. 5.13 – 5.15).

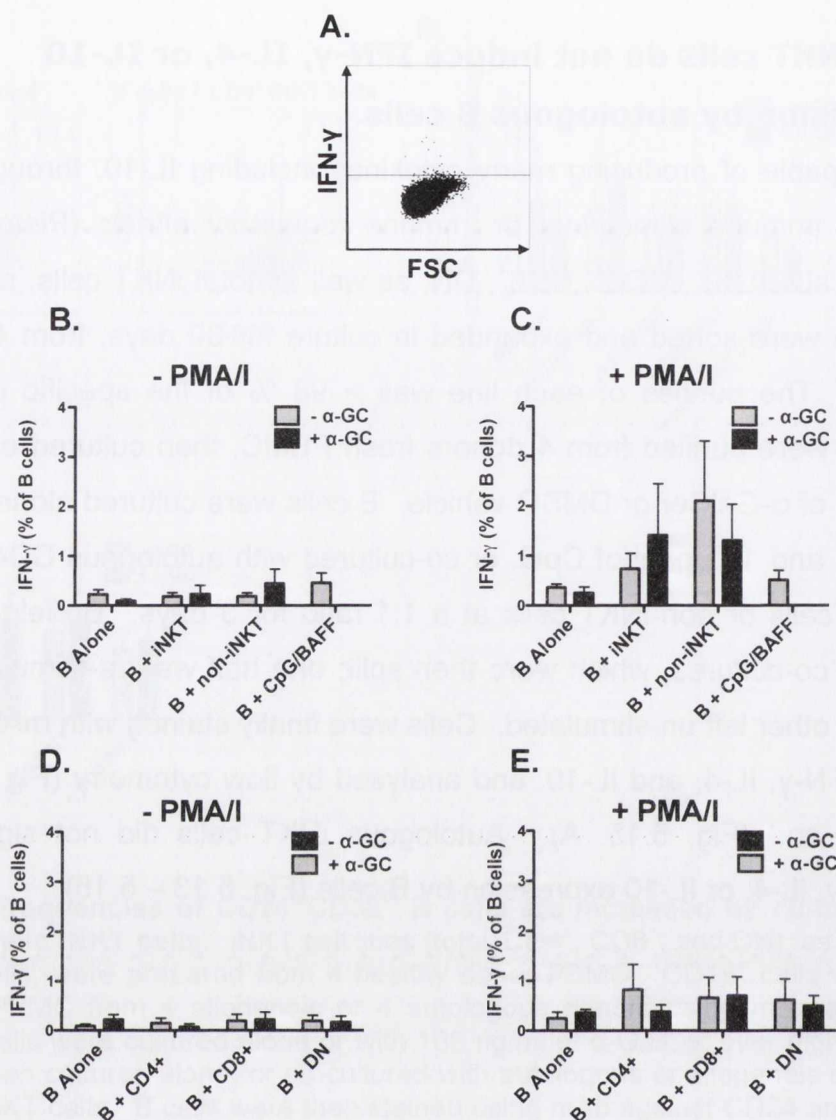


Fig. 5.13. iNKT cells do not induce IFN- γ production by B cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from the same 4 donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer over night at 37°C. B cells were then cultured alone, with BAFF (10 ng/ml) and CpG (10 μ g/ml), or co-cultured with autologous iNKT cells for 72 hours. After 72 hours brefeldin A was added to the co-cultures and they were re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated for 15 hours. Cells were then stained for intracellular flow cytometry analysis using a mAb against IFN- γ . **A**, Representative dot plot of IFN- γ producing B cells. **B** and **C**, Frequencies of B cells expressing intracellular IFN- γ after co-culturing with autologous total iNKT cells or non-iNKT cells, in the absence (**B**) or presence (**C**) of PMA/I re-stimulation. **D** and **E**, Frequencies of B cells expressing intracellular IFN- γ in response to autologous CD4⁺, CD8⁺, and DN iNKT cells, in the absence (**D**) or presence (**E**) of PMA/I re-stimulation. Data points are plotted as mean $\pm$ SEM.

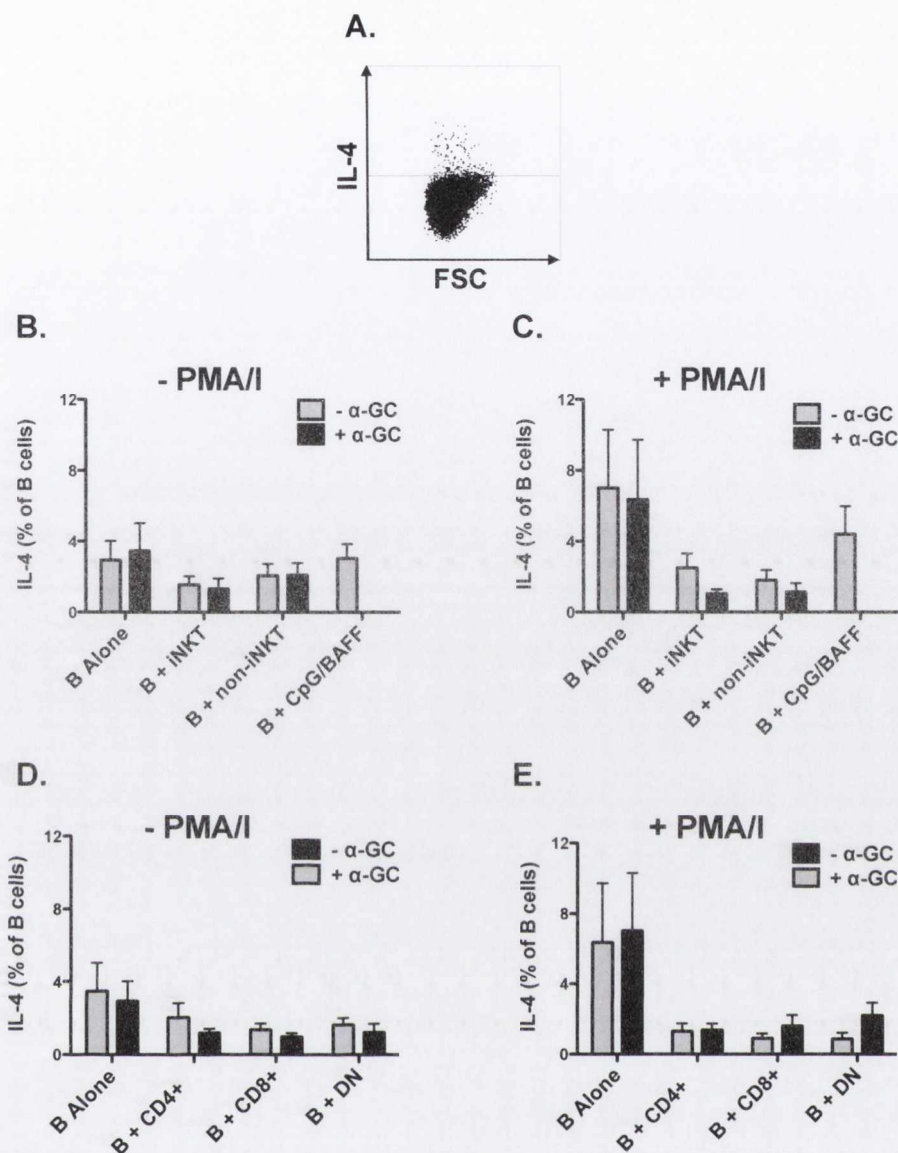


Fig. 5.14. iNKT cells do not induce IL-4 production by B cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from the same 4 donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer over night at 37°C. B cells were then cultured alone, with BAFF (10 ng/ml) and CpG (10 μ g/ml), or co-cultured with autologous iNKT cells for 72 hours. After 72 hours brefeldin A was added to the co-cultures and they were re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated for 15 hours. Cells were then stained for intracellular flow cytometry analysis using a mAb against IL-4. **A**, Representative dot plot of IL-4 producing B cells. **B** and **C**, Frequencies of B cells expressing intracellular IL-4 after co-culturing with autologous total iNKT cells or non-iNKT cells, in the absence (**B**) or presence (**C**) of PMA/I re-stimulation. **D** and **E**, Frequencies of B cells expressing intracellular IL-4 in response to autologous CD4⁺, CD8⁺, and DN iNKT cells, in the absence (**D**) or presence (**E**) of PMA/I re-stimulation. Data points are plotted as mean $\pm$ SEM.

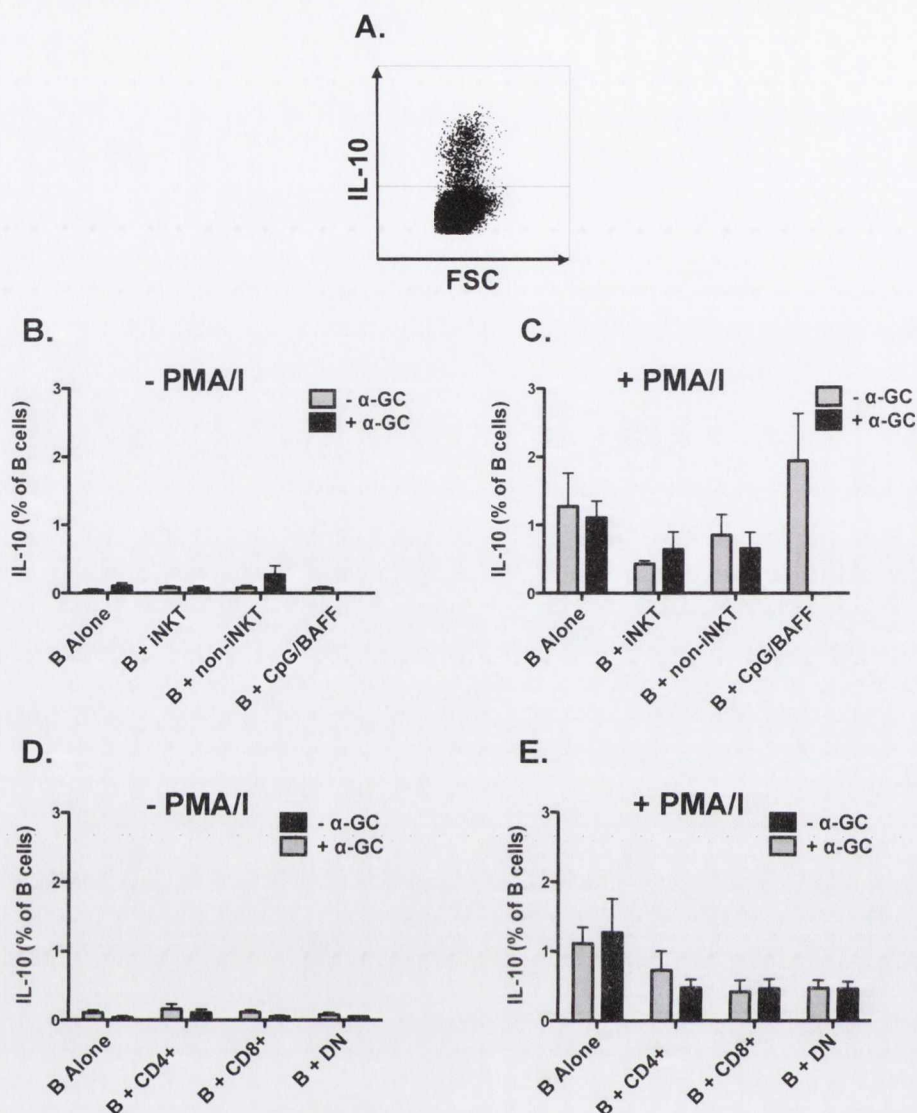


Fig. 5.15. iNKT cells do not induce IL-10 production by B cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from the same 4 donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α -GalCer over night at 37°C. B cells were then cultured alone, with BAFF (10 ng/ml) and CpG (10 μ g/ml), or co-cultured with autologous iNKT cells for 72 hours. After 72 hours brefeldin A was added to the co-cultures and they were re-stimulated with PMA and ionomycin (PMA/I) or left unstimulated for 15 hours. Cells were then stained for intracellular flow cytometry analysis using a mAb against IL-10. **A**, Dot plot showing flow cytometric gating on IL-10 producing B cells when stimulated with CpG and BAFF. **B** and **C**, Frequencies of B cells expressing intracellular IL-10 after co-culturing with autologous total iNKT cells or non-iNKT cells, in the absence (**B**) or presence (**C**) of PMA/I re-stimulation. **D** and **E**, Frequencies of B cells expressing intracellular IL-10 in response to autologous CD4⁺, CD8⁺, and DN iNKT cells, in the absence (**D**) or presence (**E**) of PMA/I re-stimulation. Data points are plotted as mean $\pm$ SEM.

5.3.14 CD4⁺ and DN iNKT cells increase CD1d expression on autologous B cells.

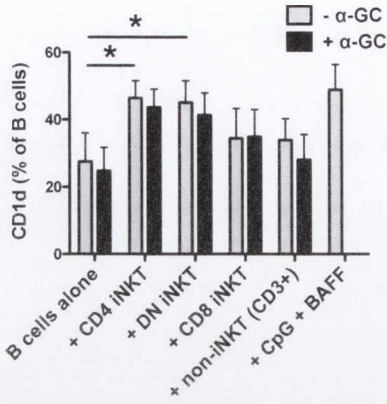
B cells are APC capable of presenting both protein and lipid Ags through class II MHC (e.g. HLA-DR) and CD1d respectively (Chen & Jensen 2008). The ability of iNKT cells to alter the surface expression of either molecule was investigated. CD4⁺, CD8⁺, and DN iNKT cells, as well as CD3⁺ non-iNKT cells, were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells from 4 autologous donors were then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone, with 10 ng/ml of BAFF and 10 μ g/ml of CpG, or co-cultured with autologous CD4⁺, CD8⁺, and DN iNKT cells or non-iNKT cells at a 1:1 ratio for 3 days. Cells were finally stained with mAb against CD1d and HLA-DR, and analysed by flow cytometry. There was a significant increase in the frequencies of CD1d⁺ B cells in the presence of both CD4⁺ and DN iNKT cells (Fig. 5.16. A). While, no significant change in HLA-DR expression on B cells when co-cultured with autologous iNKT cells (Fig. 5.16. B). These results suggest that CD4⁺ and DN iNKT cells induce an up-regulation of CD1d on autologous B cells, in an α -GalCer independent manner.

5.3.15 The effect of iNKT cells on CD40 and CD86 expression by autologous and allogeneic B cells.

CD40 and CD86 are important co-stimulatory molecules found on APC (e.g. B cells) that engage their ligands on the T cell (e.g. iNKT cell) surface (Cells et al. 1997). Upregulation of such molecules results in enhanced co-stimulation, and consequently immune activation. CD40/CD40L interaction has been identified as vital for IL-10 production and immune suppression by B cells (Duddy et al. 2004). CD4⁺, CD8⁺, and DN iNKT cells, as well as CD3⁺ non-iNKT cells, were sorted and expanded in culture for 30 days, from 4 healthy donor PBMC. The purities of each line was > 98 % of the specific cell type. Freshly isolated CD19⁺ B cells from 4 autologous donors were then cultured over night with 100 ng/ml of α -GalCer or DMSO vehicle. B cells were cultured alone, with 10 ng/ml of BAFF and 10 μ g/ml of CpG, or co-cultured with autologous CD4⁺, CD8⁺, and DN iNKT cells or non-iNKT cells at a 1:1 ratio for 3 days. Cells were finally stained with mAb against

CD40 and CD86, and analysed by flow cytometry. CD40 expression was increased on B cells in the presence of allogeneic, but not autologous iNKT cells (Fig. 5.17. A and B). These increases in CD40 occurred in the presence of all iNKT cell subsets, but were only statistically significant in the presence of CD4⁺ and CD8⁺ iNKT cells (Fig. 5.17. A). Significant up-regulation of CD40 expression induced by allogeneic iNKT cells was CD1d dependent, and may be influenced by IL-4 and IL-13 (Fig. 5.17. C). However, CD86 expression on B cells was not affected by co-culturing with autologous iNKT cells, when compared to non-iNKT cells (Fig. 5.18. A and B). These results suggest that B cells up-regulate CD40 expression in response to allogeneic iNKT cells, in a CD1d dependent manner.

A.



B.

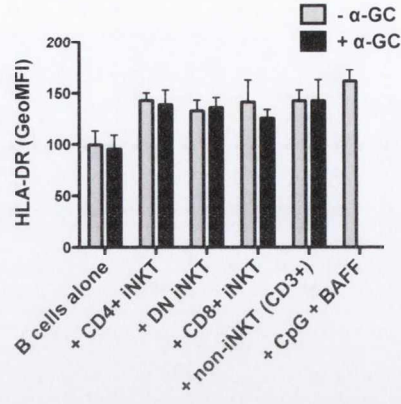


Fig. 5.16. CD1d and HLA-DR expression on B cells in response to culturing them with autologous iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from 4 healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from the same 4 donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α-GalCer over night at 37°C. B cells were then cultured alone, with BAFF (10 ng/ml) and CpG (10 μg/ml) as a positive control, or co-cultured with autologous CD4⁺, CD8⁺, and DN iNKT cells, as well as CD3⁺ non-iNKT cells, for 72 hours. B cells were then stained using mAb against CD1d and HLA-DR, and analysed by flow cytometry. **A**, Frequencies of B cells expressing CD1d after 3 days co-culture with autologous iNKT cells. **B**, Levels of expression of HLA-DR on B cells after 3 days co-culture with autologous iNKT cells. Data points are plotted as mean ± SEM. Statistical analysis was carried out using the non-parametric Friedman test with post hoc Dunne multiple comparison tests. * P ≤ 0.05.

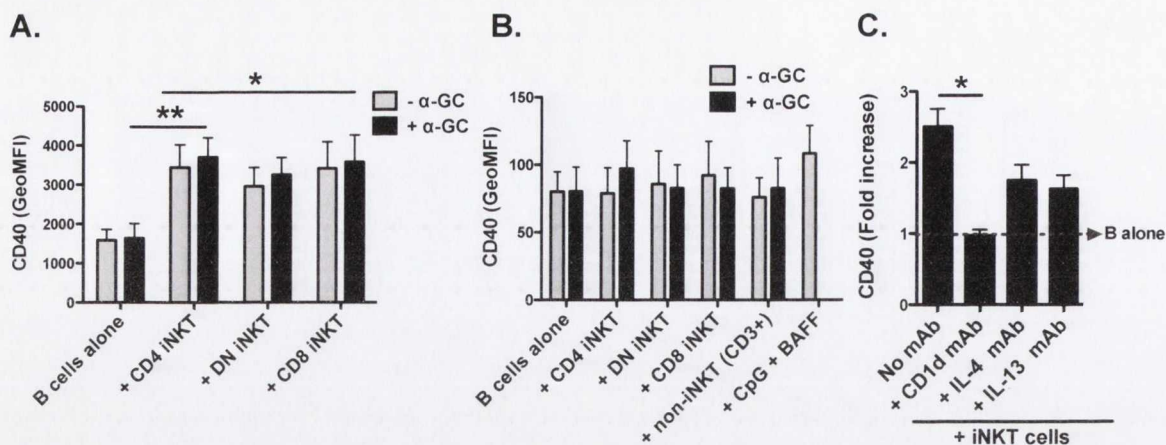


Fig. 5.17. CD40 expression is increased in a CD1d-depent manner, by B cells in response to co-culture with allogeneic iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from healthy donor PBMC. CD19⁺ cells were isolated from the same donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α-GalCer over night at 37°C. B cells were then cultured alone, with BAFF (10 ng/ml) and CpG (10 μg/ml), or co-cultured with iNKT cells, as well as CD3⁺ non-iNKT cells for 3 or 7 days, in the absence or presence of 10 μg/ml of blocking mAb against CD1d, IL-4, or IL-13. B cells were then stained using mAb against CD40, and analysed by flow cytometry. **A**, Levels of expression of CD40 on B cells after 7 days co-culture with allogeneic iNKT cells (n = 4). **B**, Levels of expression of CD40 on B cells after 3 days co-culture with autologous iNKT cells (n = 4). **C**, Levels of expression of CD40 on B cells after 3 days co-culture with allogeneic iNKT cells, presented as fold increase compared to B cells alone, in the presence of blocking mAb against CD1d, IL-4, and IL-13 (n = 4). Data points are plotted as mean ± SEM. Statistical analysis was carried out using the non-parametric Friedman test with post hoc Dunne multiple comparison tests. * P ≤ 0.05, ** P ≤ 0.01.

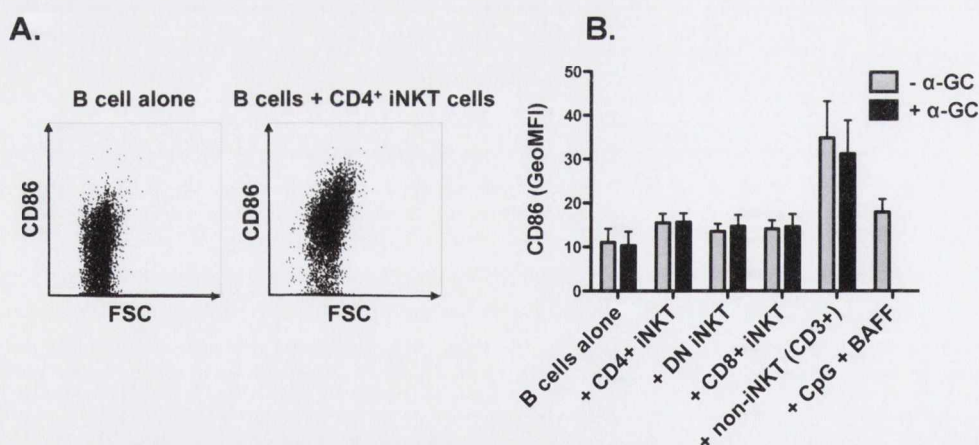


Fig. 5.18. CD86 expression by B cells in response to co-culture with autologous iNKT cells. iNKT cell lines (total, CD4⁺, CD8⁺, and DN), as well as CD3⁺ non-iNKT cells, were prepared from healthy donor PBMC. CD19⁺ cells were isolated from the same donors using magnetic bead cell sorting. B cells were cultured alone or with 100 ng/ml of α-GalCer over night at 37°C. B cells were then cultured alone, with BAFF (10 ng/ml) and CpG (10 μg/ml), or co-cultured with autologous iNKT cells, as well as CD3⁺ non-iNKT cells for 3 days. B cells were then stained using mAb against CD86, and analysed by flow cytometry. **A**, Representative dot plots showing CD86 expression on gated CD19⁺ B cells. **B**, Levels of expression of CD86 on B cells after 3 days co-culture with autologous iNKT cells (n = 4). Data points are plotted as mean ± SEM.

5.4 Discussion.

B lymphocytes possess a number of immune functions; they are the only cell to produce Abs, they secrete various cytokine, and differentiate into memory cells, as well as being professional APC. They express both MHC class II for protein Ag presentation, and CD1d for lipid Ag presentation. A particular subset of B cells called MZB cells have particularly high levels of surface CD1d expression, and have been found in humans to recirculate from the spleen and possess an $\text{IgM}^+\text{IgG}^+\text{CD27}^+$ phenotype. Various studies in mice have shown that the CD1d-restricted iNKT cell can induce lipid specific Ab production by B cells; such B cells are likely to be MZB cells, and the particular iNKT cells involved produce IL-21 and are dubbed T_{FH} iNKT cells (Leadbetter et al. 2008; Barral et al. 2008).

So far there has been limited research in humans to confirm these findings. Also, any studies that have been carried out looking at the role of iNKT cells in B cell activation and proliferation have not analysed the differences between CD4^+ , CD8^+ , and DN iNKT cells. In this study, the aim was to look at peripheral blood B cell phenotype and function and how they might be altered in the presence of autologous or allogeneic CD4^+ , CD8^+ , and DN polyclonal iNKT cell lines. In addition, all of the experimental outputs were assessed in the presence and absence of the glycolipid α -GalCer (100 ng/ml), as many studies have demonstrated its requirement for iNKT cell function.

Worthy of noting, is that all B cells as well as iNKT cell lines, used in the co-culture experiments, were > 98 % pure. iNKT cells are not MHC restricted, hence do not react to foreign MHC on allogeneic B cells. In theory, the only differences that should arise when comparing allogeneic vs autologous cells should be related to donor variability, and not due to alloreactivity.

One of the main objectives of this chapter was to investigate the role of iNKT cells in the induction of a regulatory phenotype and function on B cells. Immunosuppressive functions by B cells are totally (i.e. in mice) or partially (i.e. in humans) attributed to IL-10 production by B cells. The detection of IL-10 from B cells has proven challenging in the past, hence the need to optimise a method for IL-10 detection by B cells. The optimal conditions required for IL-10 detection may vary from those needed for the detection of other cytokines of interest, such as IL-

4. For this reason, IL-4 and TNF- α were chosen for comparative analysis through all the optimisation experiments.

The optimal control stimulation for IL-10 production, as well as for IL-4 and TNF- α was determined. A number of B cell stimulatory ligands were chosen based on previous publications demonstrating their effect on B cell differentiation, proliferation, or cytokine production. In agreement with previous studies, CpG-ODNs were the best stimulator of IL-10 production (Blair et al. 2010; Barr et al. 2007; Gantner et al. 2003). However, we found combining CpG (10 μ g/ml) with BAFF (10 ng/ml) induced the most IL-10 and IL-4 production after re-stimulation with PMA and ionomycin. Based on previous studies, the time of stimulation that allowed for IL-10 detection was 3 days or longer. For this reason 3 and 5 days were chosen, with the former giving higher levels of IL-10 and TNF- α . 15 hours or overnight re-stimulation with PMA and ionomycin resulted in more IL-10 and IL-4 production, as well as being practically more convenient than short stimulations times, as it allowed for next-day staining for flow cytometry. All previous optimisations were carried out using PBMC, hence the presence of other cells like T cells may affect the activation and cytokine production by B cell. However, when purified B cells were compared to PBMC, there was no compromise in cytokine production by B cells when they were stimulated in the absence of PBMC. One last aspect to consider for intracellular cytokine detection is the temperature at which staining is carried out. Although most staining protocols specify room temperature, some recommend (e.g. when staining using Miltenyi Biotech Abs) staining at 4°C. Based on this one experiment, the levels of IL-10 in particular, and to a lesser extent IL-4, that were detected at 4°C were much higher than those stained at room temperature.

Human CD4⁺ iNKT cells have previously been shown to induce the proliferation of naïve and memory B cell subsets (Galli et al. 2003). Whether iNKT cells can influence the differentiation of these various B cell subsets is unclear. To shed some light on this, naïve and transitional (IgD⁺CD27⁻), un-switched memory (IgD⁺CD27⁺), switched memory (IgD⁻CD27⁺), CD27⁻ memory (IgD⁻CD27⁻), and MZB (IgM⁺IgD⁺CD27⁺) B cells were first co-culture with total iNKT cells (Appendix Fig. 8.2), then with CD4⁺, CD8⁺, and DN iNKT cells. Based on percentage of total B cells, unswitched memory and MZ B cells were increased, and switched

memory was decreased significantly in the presence of all three iNKT cell subsets. However, absolute numbers of B cells were not measured, hence it is unclear whether iNKT cells induce the differentiation or proliferation of un-switched memory B and MZB cells, or if they merely promote their survival in culture, while other B cells undergo apoptosis. Based on these findings, it can be speculated that CD4⁺, CD8⁺, and DN iNKT cells promote a more MZB and un-switched memory B cell phenotypes in peripheral blood B cells.

One of the ultimate and exclusive B cell functions is the ability to produce and secrete Abs. There are two main types of Abs produced by B cells: 1) Natural Abs targeted against highly conserved self and non-self Ag, are produced by B-1 cells as well as transitional and MZ B cells, independently of Ag stimulation, and include IgM, IgA, and IgG3. 2) Adaptive Abs targeted against a specific Ag, post-activation by that Ag in either a TI or TD manner, produced by B-2 cells, which include follicular and MZ B cells (Panda & Ding 2014). Various studies in mice, have highlighted a role for iNKT cells in the provision of indirect and cognate help to B cells for Ab production against protein and lipid Ags respectively (see 4.1 introduction). However, there are no publications to confirm the role of iNKT cells in Ab production in humans. To assess whether CD4⁺, CD8⁺, or DN iNKT cells influence the secretion of IgM, IgA, or total IgG by purified B cells *in vitro*, both cell types were co-cultured for 10 days then supernatants were analysed for the levels of each Ab. Although all iNKT cell subsets caused an increase in the three Ab levels when compared to B cells alone, there was a more significant increase in IgM, IgA, and IgG production in the presence of CD4⁺ iNKT cells. Such influences observed by iNKT cells were not only independent of α -GalCer stimulation but were partially abrogated in the presence of the glycolipid. Furthermore, blocking of CD1d, but not IL-4 or IL-13, caused a significant reduction in iNKT cell mediated IgA secretion. Our findings so far suggest that iNKT cells, in particular CD4⁺ iNKT cells, provide help to B cells for Ab production, in a manner that is CD1d dependent. A more recent study confirmed iNKT cell provision for Ab production by B cells, in patients with SLE. Spontaneous IgM, total IgG, and specific auto-reactive IgG production by B cells from SLE patients was significantly reduced upon blocking CD1d or the iNKT cell receptor. The frequencies of plasma cells were also significantly reduced upon iNKT cell depletion or CD1d blocking,

suggesting that iNKT cells have a role in plasma cell differentiation (Shen et al. 2015).

Aside from Ab production, a less understood role for B cells is one of immunoregulation. Primarily through the production of IL-10, B cells have been involved in the control of autoimmunity and inflammation, in both mice and humans. The current technical challenge of isolating IL-10 producing B cells, has given rise to great interest from researchers to identify a specific phenotype of B cells that are specific for at least contain the immunosuppressive B cells. A number of B cell populations have been identified in mice to be enriched for IL-10 producing B cells, including CD5⁺CD1d^{hi} B cells (Yanaba et al. 2008). In humans both CD5 and CD1d^{hi} B cells have been shown to correlate with IL-10 production and to be immunosuppressive in MS, respectively (Gary-Gouy et al. 2002; Correale et al. 2008). CD5⁺CD1d^{hi} B cells have also been shown in humans to be enriched for IL-10 producing B cells, are contained within the transitional CD24^{hi}CD38^{hi} B cells compartment, and possess immunosuppressive function (Blair et al. 2010). iNKT cells are known to play a role in immuno-regulation (Matsuda et al. 2008). However, it is unclear whether iNKT cells can modulate immune function by influencing regulatory B cells differentiation or function. In this study, both autologous and allogeneic iNKT cells lines were co-cultured with B cells. In both cases CD4⁺ and to a lesser extent DN iNKT cells were able to significantly increase the frequencies of both CD5⁺CD1d^{hi} and CD24^{hi}CD38^{hi} B cells. These changes were observed independent of α -GalCer stimulation.

However, when IL-10 productions was analysed, no changes were observed in the presence of iNKT cells. Nor was there a change in IL-4 or IFN- γ production by B cells. These finding suggest that iNKT cells do not promote immuno-suppression through IL-10 production by B cell. However, because human regulatory B cells are thought to carry out their immunosuppression only partly through IL-10 production (Blair et al. 2010), CD4⁺ iNKT cells may induce regulatory B cell functions by increasing the frequencies of both CD5⁺CD1d^{hi} and CD24^{hi}CD38^{hi} B cells. In order to confirm if iNKT cells increase the immunosuppressive capacity of B cells, a T cell suppression assay would need to be carried out on each of the co-cultures.

So far, iNKT cells have been shown to influence B cell differentiation, and Ab production in a CD1d dependent manner. Cognate interactions between both cells involve the interplay of a number of important surface receptors and their ligands, among which are CD1d, CD40, and CD86 on B cells. Upon activation of the B cells, molecules such as CD69 and IL-21R may be upregulated. The expression of such markers were assessed in both allogeneic and autologous iNKT cell and B cell co-cultures. Contrary to a previous study that found CD1d expression to diminish post-activation, in a manner similar to moDCs (Allan et al. 2011), we found that CD1d expression was upregulated on B cells co-cultured with autologous CD4⁺ and DN iNKT cells, while in comparison HLA-DR expression was unchanged. The co-stimulatory CD40 was upregulated on B cells in the presence of CD4⁺ iNKT cells and α -GalCer for both autologous and allogeneic co-cultures, while CD8 and DN iNKT cells induced CD40 expression but to a lesser extent and only on allogeneic B cells. CD40 upregulation was CD1d dependent. However, CD86 did not seem to change on B cells in the presence of iNKT cells especially when compared to B cells co-cultured with conventional T cells. A previous study reported that B cells positive for CD86 expression have lower CD1d expression (Allan et al. 2011). A negative correlation between CD86 and CD1d may explain why CD86 is unchanged but CD1d is upregulated on B cells co-cultured with iNKT cells. Furthermore, B cells appeared to be activated based on a significant upregulation of both CD69 and IL-21R in the presence of CD4⁺ iNKT cells. This CD4⁺ iNKT cell specific upregulation of CD69 is in agreement with previous findings by Lin et al (Lin, Nieda, Rozenkov, et al. 2006). Overall CD4⁺ iNKT cells, although not exclusively, appear to be the most effective iNKT cell for providing cognate help to B cells, bringing about its activation, possible differentiation into MZB cells, CD5⁺CD1d^{hi} and CD24^{hi}CD38^{hi} B cells, and inducing IgA, IgM, and IgG production in a CD1d dependent manner.

When B cells were co-cultured with autologous vs allogeneic iNKT cells, there were no major differences in the findings, suggesting that pure B cell and pure iNKT cell co-cultures can be used as an alternative to autologous co-cultures as preliminary tools in understanding the relationship between both immune cells. This is beneficial from a practical research point of view, as autologous B cell and iNKT cell co-cultures are technically difficult to achieve.

This chapter has focused on investigating and comparing the roles of CD4⁺, CD8⁺, and DN iNKT cells on various B cell functions. This study has provided preliminary confirmation of some the findings in mice studies. iNKT cells are important players in modulating protective Ab secretion by B cells, and hence these innate T cells may be used as vaccine adjuvants or for potential immunotherapy for patients with Ab deficiencies. iNKT cells promote MZB cell differentiation, a B cell population thought to require iNKT cell help, and known in mice to be required for lipid-specific Ab production in an IL-21 dependent manner. Moreover, the receptor for IL-21 on B cells (IL-21R) was upregulated in the presence of iNKT cells.

Alongside the ability of iNKT cells to induce B cell activation, and stimulate the release of Abs by B cells. There is a possibility that iNKT cells promote the differentiation of a population of B cells believed to hold immuno-suppressive properties. Although in this study no functional immuno-suppression was demonstrated, a recent study in mice has demonstrated that iNKT cell induce the differentiation of IL-10 producing Breg cells *in vivo* (Vomhof-DeKrey et al. 2015). The idea that iNKT cells can promote various facets of B cell function is both intriguing and promising for clinical research. However, more research is required for a more tailored and targeted outcome for B cell function.

Chapter 6 iNKT cells in patients with Chronic Lymphocytic Leukaemia (CLL).

6.1 Introduction.

CLL is a B cell neoplastic disease affecting the BM, blood, and LNs. The disease manifests by the infiltration of BM and blood (i.e. leukemia), with small mature monoclonal B lymphocytes, with a characteristic CD5⁺ CD23⁺ phenotype (Jaffe et al. 2001). In healthy individuals CD5⁺ B cells constitute less than 20% of total B cells (Lavabre-Bertrand et al. 1994), but in patients with CLL the predominant B cell phenotype is CD5⁺. Along with immunophenotyping for CD5⁺ CD23⁺ cells in the peripheral blood, a blood cell count of $> 5 \times 10^6$ B cells / ml of blood is diagnostic of CLL (Hallek et al. 2008). 90% of CLLs in Europe and the united states occur in individuals over 50 years old with median age of 65 years, and a male:female ratio of 2:1 and an incidence rate of 0.72 cases /100,000 per year (Jaffe et al. 2001). There are two systems for staging CLL: Rai (0-IV) and Binet (A-C) classifications. Patients with less than 3 organs or lymph nodes involved are considered Binet A, while those with 3 or more involved sites are Binet B, and patients with anaemia and thrombocytopenia irrespective of organ involvement are Binet C. Newly diagnosed patients with asymptomatic stable disease (Binet A) represent around 25 % of all CLL cases and do not require treatment, but are monitored for emerging symptoms and disease progression. Binet stage A patients that have progressive disease, and stage B and C patients are commenced on treatment. Types of treatment available include chemotherapy using purine analogues such as fludarabine and cyclophosphamide, and the more recently introduced alkylating agent, bendamustine. Immunotherapies such as monoclonal Ab therapies using Retuximab (anti-CD20) and Alemtuzumab (anti-CD52) are also available. Lastly, autologous stem cell transplantation may be given to patients with high risk CLL in indicated cases (Hallek 2005). Guidelines for the treatment of CLL in Ireland indicate a combination of fludarabine, cyclophosphamide, and rituximab (FCR), which result in complete remission in 70 – 80 % of patients under 60 years of age (Lymphoma Forum of Ireland 2010). With the standard treatment for CLL including chemotherapeutic agents that target all dividing cells, causing a plethora of side effects in patients, a more targeted treatment regimen is needed.

iNKT cells are multifunctional lymphocytes, as described in previous chapters. They recognize lipid Ags presented in the context of CD1d molecules, enabling

them to activate and regulate immune responses by means of direct and indirect communication with other effector and regulatory cells of the immune system. One of the effector functions that iNKT cells exhibit is direct cytotoxicity, elicited through various mechanisms, including the release of cytolytic granules such as granzyme and perforin, and the expression of surface apoptosis inducing ligands such as FASL resulting in target cell death (Bassiri et al. 2014; Metelitsa et al. 2001). Another hallmark effector activity of iNKT cells is cytokine production. Through the production of cytokines such as: IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IFN- γ , TNF- α , TGF- β , and GM-CSF, iNKT cells can modulate the function of almost any immune cell, hence iNKT cells are considered a major regulator of the immune system (Matsuda et al. 2008). Defects in iNKT cell frequencies and function in many human diseases could be a cause or consequence of that disease. However, despite the lack of direct clinical and scientific evidence, it is speculated that iNKT cell involvement in human disease can be explained by two possible scenarios: 1) iNKT cell numbers are reduced in frequency or function, leading to an overall failure of immune stimulation and regulation resulting in conditions such as cancer, autoimmunity, and infectious diseases. 2) Inappropriate response to self or non-self glycolipid Ags leading to immune activation and conditions such as asthma, allergic reactions, GVHD, and psoriasis (Berzins et al. 2011).

Extensive research in murine models of cancer, have provided strong evidence to support an important role for iNKT cells in tumour immunosurveillance. The first milestone was made in 1995, when the life span of B16 melanoma bearing mice were significantly prolonged, with an IV injection of α -GalCer (Kobayashi et al. 1995). Many studies since have investigated the roles of iNKT cells in murine tumour immunosurveillance by giving tumour-bearing mice α -GalCer and other iNKT cells Ags, inducing tumours in mice deficient in iNKT cells, and then adoptively transferring iNKT cells back into the tumour-bearing mice. These studies have collectively shown that enhancing iNKT cell responses in tumour-bearing mice can protect against tumour challenge and significantly reduce tumour load. The mechanism by which iNKT cells exerted their anti-tumour responses is primarily by indirect means, where tumour recognition by iNKT cells is not required. iNKT cells trans-activate the professional cytotoxic NK cells and CD8⁺ T cells, through cognate DC activation and subsequent release of IL-12, as well as

IFN- γ . Although iNKT cells have not been conclusively demonstrated to directly kill tumour cells *in vivo*, *in vitro* studies have shown that primary murine iNKT cells can directly kill, in a CD1d-ligand dependent manner, a variety of tumour cells (McEwen-Smith et al. 2015; Bassiri et al. 2014; Tarazona & DelaRosa 2003). Important to note, is that anti-tumour effector function by iNKT cells are exerted by CD4⁻ subsets, while CD4⁺ iNKT cells have been shown to dampen anti-tumour responses and cause tumour progression (Crowe et al. 2005). *In vitro* studies investigating the anti-tumour activity of human iNKT cells have shown that iNKT cells can elicit directly cytotoxic killing on a range of human tumour cell lines (Kawano et al. 1999). This process was dependent on both CD1d expression by target cells and CD1d loading with ligand (e.g. α -GalCer). Along with direct iNKT cell killing of tumour cells, upon CD1d- α -GalCer recognition, IL-2 is produced by iNKT cells along with IFN- γ , which activate NK cells and CD8⁺ T cells (Metelitsa et al. 2001). These studies suggest a potential role for human iNKT cells in tumour immunosurveillance (Bassiri et al. 2014; Vivier et al. 2012).

Ex vivo analysis of iNKT cells in cancer patients has revealed a reduction in both frequencies and absolute numbers of iNKT cells in patients with multiple myeloma (Dhodapkar et al. 2003), prostate cancer (M. A. Tahir et al. 2001), and breast cancer (Crough et al. 2004). Despite a lower level of responsiveness to α -GalCer, iNKT cells from various cancer patients were capable of substantial expansion in response to α -GalCer *in vitro* (Crough et al. 2004). When cytokine production was assessed, iNKT cells from patients with advanced solid tumours appeared to have impaired IFN- γ production (Dhodapkar et al. 2003; Tahir et al. 2001) and after *in vivo* mobilisation with α -GalCer (Chang et al. 2005). These studies suggest that various cancer patients have a reduction in PB iNKT cells, and that these iNKT cells may have impairments in cytokine secretion and/or responses to α -GalCer-CD1d stimulation. However, iNKT cells from cancer patients are capable of expanding *in vitro*, hence giving potential for iNKT cell based immunotherapy.

Many clinical trials have attempted to target iNKT cell function *in vivo*, to enhance prognosis of patients with various types of solid tumours. The most basic approach was to administer IV α -GalCer to patients with no alternative therapy. iNKT cells in these patients disappeared from circulation, with no improvement in clinical outcome for the patients (Giaccone et al. 2002). To try and avoid iNKT cell

anergy caused by α -GalCer administration, immature monocyte-derived DCs (mo-DCs) were generated *ex vivo* from patients, pulsed with α -GalCer and IV administered back to patients with metastatic tumours; Mo-DCs migrated to the liver, lungs, and spleen within hours of administration. Such treatment caused the activation of NK, T, and B cells, as well as a sharp increase in serum IFN- γ levels, responses which were heightened on administration of a second dose (Nieda et al. 2004). iNKT cell numbers were also increased from baseline in patients treated with α -GalCer pulsed mo-DCs; a response that was improved when mo-DCs were matured before administration, resulting in sustained CD4⁺, CD8⁺, and DN iNKT cells in PB and tumour bed of patients with various solid tumours (Chang et al. 2005). Improvements in clinical outcomes was observed for some patients (Nieda et al. 2004). As mentioned in chapter 3, clinical trials have also introduced *ex vivo* expanded iNKT cells to patients with solid tumours, with no significant clinical outcome (Motohashi et al. 2006). Following from this, in patients with head and neck squamous cell carcinoma, combination therapy of *in vitro* expanded iNKT cells and α -GalCer loaded APCs was found to significantly improve anti-tumour responses, improve patient outcome, and cause tumour regression associated with tumour-localized increases in iNKT cell numbers (Kunii et al. 2009; Uchida et al. 2008; Yamasaki et al. 2011). These studies suggest that impairments in iNKT cells, as well as the potential for their use in cellular immunotherapy, may be dependent on the type of tumour in question.

Although the majority of studies have focused on solid tumours in humans, a small number looked at iNKT cells in haematological malignancies. However, studies looking at iNKT cells in haematological malignancies, which have thus far focused on multiple myeloma and myelodysplastic syndrome, have yielded conflicting results (Chan et al. 2010; Chan et al. 2014; Fujii et al. 2003). Similarly, our knowledge on iNKT cells in CLL is extremely limited, because previous studies have focused on iNKT-like cells such as CD56⁺ T cells (Güven et al. 2003; Jadidi-Niaragh et al. 2012).

While carrying out investigations for this part of the study, a paper was published reporting on iNKT cells in CLL. The study analysed PB samples from 30 untreated patient and 30 age-matched controls. PBMCs were phenotyped by flow cytometry for V α 24-J α 18⁺ cell frequencies, and CD1d expression on B cells and

DCs was examined. iNKT cell frequencies from CLL patients were reduced, but no changes were found in absolute numbers, phenotype, or cytotoxicity against CD1d-transfected C1R cell lines compared to healthy control. iNKT cells from patients with CLL were reported to expand normally, and patient-derived iNKT cell lines as well as *ex vivo* analysed iNKT cells produced normal levels of various cytokines when compared to healthy controls (Weinkove et al. 2013).

Weinkove et al, also examined the levels of CD1d on APCs in 20 patients with CLL; CD1d expression on CD5⁺ CLL cells was reduced compared to CD5⁻ B cells from patients with CLL, and monocytes also showed reduced CD1d expression compared to healthy controls. Despite the reduction in CD1d expression, α -GalCer pulsed CLL cells were capable of expanding both autologous and allogeneic iNKT cells *in vitro* (Weinkove et al. 2013). Previously, other studies have also found a reduction in CD1d on CLL cells. One study found a significant reduction in CD1d RNA transcripts in CD5⁺ CLL cells compared with healthy controls (Zheng et al. 2002). Another study by Kotsianidis et al, showed that a reduction in CD1d expression on CLL cell could be used as a marker for the differential diagnosis of CLL from a range of B-cell chronic lymphoproliferative disorders (B-CLPDs) (Kotsianidis et al. 2011). However, one study found no reduction in CD1d expression on CD5⁺ CLL cells compared to healthy control B cells and that allogeneic iNKT cells lines were able to efficiently kill α -GalCer pulsed CLL cells (Fais et al. 2004).

All *trans* retinoic acid (ATRA) is an active metabolite of vitamin A and is produced in the body to aid in cellular growth and development. Retinoid acid (RA) acts by promoting the expression of various genes involved in cellular development and function. RA binds its the retinoic acid receptor (RAR), which then dimerises and binds the retinoid acid response element (RARE) in the promoter region of the target gene, promoting gene transcription (Chen & Ross 2007). ATRA is synthetically produced and has been used as a pharmaceutical compound for the treatment of skin conditions (e.g. acne) and as a cancer therapeutic. It has many names including: tretinoin, vitamin A acid, and retinoic acid. ATRA has been researched and trialed for the treatment and prevention of many solid tumours (Siddikuzzaman et al. 2011). ATRA treatment, although considered non-toxic, can result in side effects collectively called the RA syndrome, and ATRA resistance

can occur from long term treatment (Zhang et al. 2000). However, ATRA is currently an established part of the treatment regimen for acute promyelocytic leukaemia (APL). ATRA works by selectively inducing cell cycle arrest and cellular differentiation, causing APL cells to terminally differentiate and naturally undergo apoptosis (Zhang et al. 2000; Degos & Wang 2001). More recently, in CLL patients with fludarabine resistance, the *ex vivo* treatment of CLL cells with ATRA increased their sensitivity to fludarabine-induced apoptosis (Fernández-Calotti et al. 2012).

AM580 is a retinobenzoic acid derivative that was synthesized as a retinoic acid receptor alpha (RAR α) agonist, and has been shown to act in a similar way to ATRA and induces APL cell maturation. ATRA has been shown to up-regulate CD1d expression on rodent and human monocytic cells via binding to RARE found upstream the CD1d gene (Chen & Ross 2007). RAR α agonists such as ATRA and AM580, were also reported to upregulate CD1d on human tonsillar B cells (Allan et al. 2011). No studies to date have reported on CD1d upregulation on normal or CLL B cells, when treated with RAR α agonists. Considering that ATRA is used alongside chemotherapeutic agents as an established treatment for APL, and the suggestion that ATRA may increase the effectiveness of fludarabine for CLL patients, the role of RAR α agonists on CD1d upregulation in CLL warrants further investigation.

6.2 Objectives.

The aim of this part of the study is to investigate the effect of RAR α agonists on CD1d expression by CLL cells, and consequently the potential for improved iNKT cell targeted killing of CLL cells using iNKT cell ligands such as α -GalCer. We hypothesise that up-regulation of CD1d expression by CLL cells via treatment with RA may improve iNKT cell cytotoxic killing of CLL cells.

- To measure the frequencies of iNKT cells and iNKT cell subsets in CLL patients and healthy age matched controls.
- To assess the ability of iNKT cells from CLL patients to expand *in vitro* and to examine CD31 expression as an indicator of their *in vivo* replicative history.
- To generate iNKT cell lines from CLL patients and healthy age matched controls.
- To measure CD1d expression on CD5+ and CD5- B cells, and monocytes from CLL patients and healthy age matched controls.
- To assess the ability of ATRA and AM580 to upregulate cell surface expression of CD1d on CLL B cells.
- To test the ability of α -GalCer and 7DW8-5-pulsed CLL cells to induce cytolytic degranulation by healthy allogeneic iNKT cells.
- To investigate whether CD107a, IFN- γ , or IL-4 responses by healthy allogeneic iNKT cells in response to α -GalCer or 7DW8-5-pulsed CLL cells can be improved by ATRA or AM580 treatment.
- To examine the difference in CD4⁺, CD8⁺, and DN iNKT cell responses to CLL cells pulsed with α -GalCer and 7DW8-5 in the presence and absence of RA.

6.3 Results.

6.3.1 CLL patient demographics.

32 patients diagnosed with CLL, with a median age of 67 [45 - 87], were recruited from St. James's Hospital, Dublin. 23 of the patients had stable disease, were given a clinical score of Binet A, and the remaining were Binet B or C. 23 of the patients had not undergone any previous treatment, while 11 patients were treated and discontinued treatment more than one year prior to sampling. This study was granted ethical approval from St. James's Hospital and Adelaide and Meath incorporating the National Children's Hospital Ethics Committee; all patients and healthy controls provided written informed consent. EDTA-anticoagulated venous blood samples with a median volume of 3.5 ml [0.7 – 9.7 ml] were obtained from 32 patients and 18 healthy controls. Each sample was processed within 24 h of collection; Peripheral blood mononuclear cells (PBMCs) were isolated and stained for immunophenotyping, stimulated in culture for iNKT cell expansion, and frozen in -20°C for further *in vitro* manipulations. For the purpose of age matching, only 14 patients and 12 age matched healthy subjects were included in this study (see Fig. 6.1).

6.3.2 iNKT cells are depleted in CLL.

A previous study found that CLL patients have decreased frequencies but increased absolute numbers of PB iNKT cells, along side increases in total CD3^{+} counts (Weinkove et al. 2013). PBMCs were isolated from 1 – 3 ml of PB taken from 13 CLL patients and 12 age-matched control subjects aged 45 – 65 years. PBMCs were stained with mAb against CD3, CD4, CD8, and $\text{V}\alpha 24\text{-J}\alpha 18$ TCR (6B11) (Fig. 6.2). Flow cytometric analysis showed a decrease both in frequencies ($P = 0.015$) and absolute numbers ($P = 0.118$) of iNKT cells from CLL patients compared to age-matched controls (Fig. 6.2.B), while total CD3^{+} cell numbers were increased (Fig. 6.2.C). Analysis of CD4 and CD8 expression by iNKT cells (Fig. 6.2.D) from 7 CLL patients showed a slight decrease in DN and a slight increase in CD8^{+} and CD4^{+} iNKT cells compared to age-matched controls (Fig. 6.2.E). The results indicate that iNKT cells are selectively depleted in the PB of patients with CLL.

6.3.3 Proliferation of iNKT cells from CLL patients.

iNKT cells are selectively decreased in patients with CLL (see 6.3.1). Impairments in iNKT cell numbers may be resolved by inducing their proliferation *in vivo* using α -GalCer. The ability of iNKT cells to proliferate was assessed from both CLL patients and age matched controls. PBMCs were stimulated *in vitro* with α -GalCer, IL-2, and IL-7 for 30 days; A method that has been optimised for expanding all subsets of iNKT cells from small blood samples (see 2.13.3). iNKT cell frequencies were assessed using flow cytometric analysis of CD3⁺ PBS-57-loaded CD1d tetramer positive cells, before and after stimulation. $(4 - 1400) \times 10^6$ PBMC containing 30 – 700 iNKT cells, from 17 CLL donors, were stimulated in culture. Out of 17 donors only 2 donor PBMC produced iNKT cell lines. As previously published (Weinkove et al. 2013), the two donor samples that expanded iNKT cell lines showed normal population doublings (PDL) when compared to age matched controls (Fig. 6.3.A/B). TRECs or T cell excision circles decrease with each round of post-thymic T cell division. CD31 is a cell surface marker that correlates with TREC levels, hence is an indicator for replication history and residual replicative potential (Junge et al. 2007). Flow cytometric of CD31 levels revealed similar levels of CD31 on CLL iNKT cell compared to age matched controls (Fig. 6.3.C/D). These results suggest that iNKT cells from CLL patients have similar replicative history to iNKT cells from healthy donors and are capable of normal proliferation in response of *in vitro* stimulation.

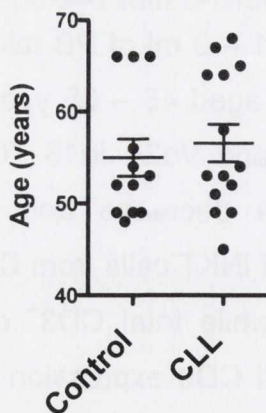


Fig. 6.1. Ages of CLL patients and healthy controls included in this study. Scatter plot of the ages of healthy donor ($n = 12$) and CLL patients ($n = 14$). Data points are plotted as mean $\pm$ SEM.

Table 11. CLL patient demographics.

Patient	Sex	Age	PBMC (cells / ml)	Age matched	Stage	Treatment
2	M	73	6.7	-	A	No
6	F	65	17.1	Yes	A	No
7	M	53	4.7	Yes	A	No
9	F	87	4	-	A	No
10	M	66	33.8	-	A	No
11	F	67	7.7	-	A	No
12	M	68	6.3	-	A	No
13	M	71	6.7	-	A	No
14	M	53	10.3	Yes	A	No
15	M	75	71.8	-	A	No
17	M	58	494.8	-	C	No
20	M	49	51.9	Yes	C	No
21	M	77	6.7	-	A	No
23	F	49	53.7	Yes	A	No
24	F	86	73.8	-	A	No
28	F	69	28.6	-	A	No
29	F	45	3.3	Yes	A	No
30	F	58	12.2	Yes	A	No
31	M	64	21.1	Yes	A	No
32	M	64	5.6	Yes	A	No
1	M	51	/	Yes	/	/
4	F	52	1.5	Yes	A	/
5	M	68	37.4	Yes	C	Yes
8	M	72	32.1	-	B	Yes
26	M	72	2.5	-	A	Bendamustine
18	F	60	56.4	-	C	Campath
19	M	68	/	-	C	Chlorambucil
25	F	70	1.3	-	A	Chlorambucil
27	M	86	11.3	-	A	Chlormabucil
3	M	54	7.9	Yes	A	FCR
16	M	72	75.7	-	B	FCR
22	M	67	8.4	Yes	B	FCR

FCR = fludarabine, cyclophosphamide, and rituximab.

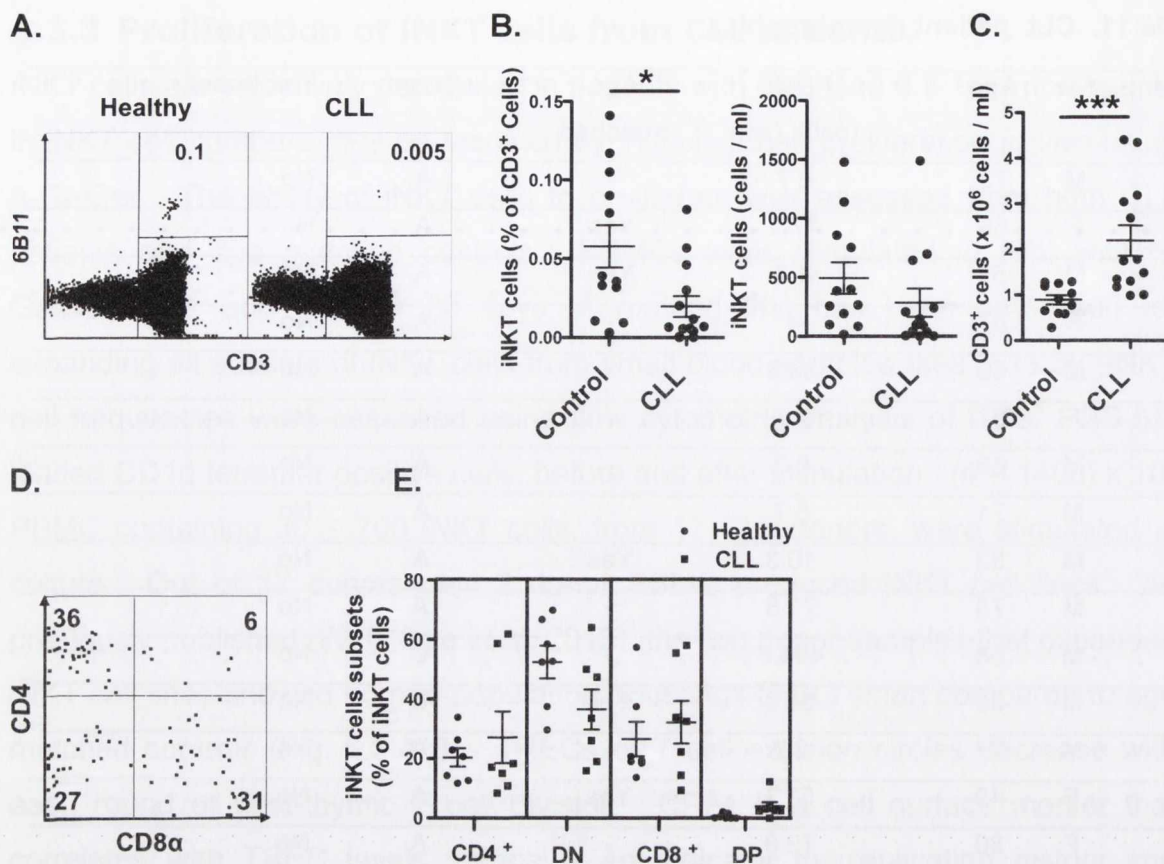


Fig. 6.2. Circulating iNKT cell frequencies in CLL patients and age-matched healthy subjects. 3ml of EDTA treated venous blood were obtained from CLL patients aged 50 – 90 years, and from age-matched healthy control subjects. PBMCs were stained for flow cytometric analysis using mAbs against CD3, CD4, CD8, and Va24-Ja18 TCR. **A**, Representative dot plots showing the detection of iNKT cells (CD3⁺Va24-Ja18⁺) in a healthy donor and CLL patient. **B**, Scatter plots showing the frequencies (left) and absolute numbers (right) of iNKT cells in healthy donors (n = 12) and CLL patients (n = 13). **C**, Scatter plot showing the absolute numbers of CD3⁺ cells in healthy donors and CLL patients. **D**, Representative dot plot of CD4 and CD8 expression by gated iNKT cells. **E**, Scatter plot showing the frequencies of CD4⁺, DN, CD8⁺, and DP iNKT cells from healthy donors and CLL patients (n = 7). Data points are plotted as mean ± SEM. Statistical analysis was carried out using the non-parametric Mann-Whitney test. * P ≤ 0.05, *** P ≤ 0.001 comparing CLL to control.

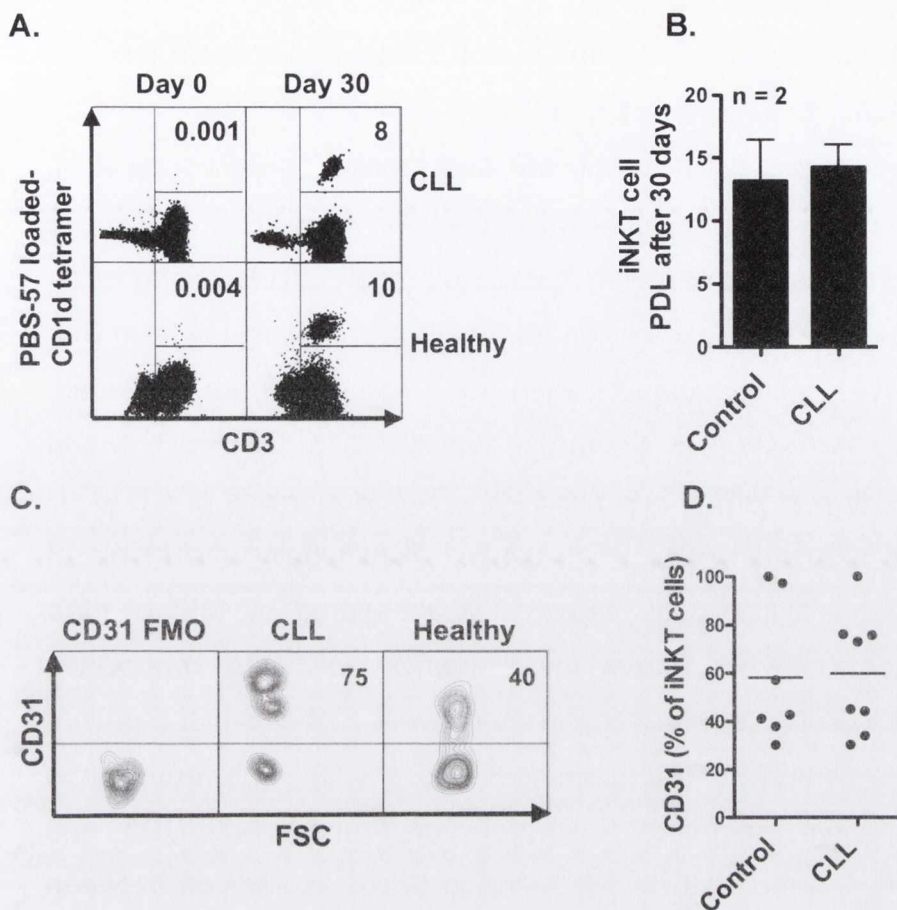


Fig. 6.3. Expansion of iNKT cells from CLL patients and age matched healthy subjects. PBMCs from 2 CLL patients and 2 healthy age matched controls were stimulated *in vitro* with α -GalCer, IL-2, and IL-7 for 30 days. iNKT cell frequencies and CD31 expression were measured by flow cytometry. **A**, Representative dot plots of PBMCs stained with anti-CD3 and PBS-57-loaded CD1d tetramer, before and after 30 days stimulation. **B**, Bar chart showing iNKT cells population doubling (PDL) 30 days post stimulation. **C**, Representative contour plots showing CD31 expression on iNKT cells stained with anti-CD31 and a fluorescence minus one (FMO) control. **D**, Scatter plot showing the frequencies of CD31⁺ iNKT cells. Data points are plotted as mean $\pm$ SEM. Not significant by Mann-Whitney test.

6.3.4 CD1d expression is reduced on B cells and increased on monocytes from CLL patients.

CD1d⁺ B cells are required for iNKT cell growth and proliferation (Bosma et al. 2012). A number of studies looking at CD1d expression on CLL B cells have yielded conflicting results (Fais et al. 2004; Kotsianidis et al. 2011; Zheng et al. 2002). In the present study, CD1d expression on B cells and monocytes from CLL patients and age-matched control subjects aged 45 – 65 years were assessed. Freshly isolated PBMCs were stained with mAbs against CD1d, CD5, CD14, and CD19 and analysed by flow cytometry (Fig. 6.4.A). The frequencies of CD1d⁺ B cells were significantly reduced in CLL (Fig. 6.4.B). Additionally, the levels of CD1d expression by B cells from CLL patients, as measured by MFI of mAb staining, was slightly reduced. In contrast, the frequencies of CD1d⁺ monocytes were significantly increased in CLL, and CD1d levels were higher on CLL patient monocytes compared to healthy control monocytes (Fig. 6.4.C). The reduction in CD1d⁺ B cells in CLL patients compared to controls was found in both CD5⁺ and CD5⁻ B cell populations. There was no difference in CD1d expression between CD5⁺ and CD5⁻ CLL B cells (Fig. 6.4.D). These results suggest a global reduction in CD1d expression on B cells, with a possible compensatory increase in CD1d expression on monocytes from patients with CLL.

6.3.5 iNKT cells show weak cytotoxic activity against CLL cells.

The glycolipid α -GalCer has been used in several clinical studies to modulate iNKT cell activity *in vivo* (see 3.1). The recommended concentration of α -GalCer, which has become standard for *in vitro* stimulation of iNKT cells, is 100 ng/ml. 7DW8-5, an α -GalCer derivative, was shown to be more potent than α -GalCer at inducing cytokine production by iNKT cells *in vitro* (Li et al. 2010). The ability of CLL and healthy B cells to present both α -GalCer and 7DW8-5 to iNKT cells and induce CD107a expression, a hallmark of degranulation / cytotoxic activity, was investigated in this study. Firstly, CD19⁺ B cells were sorted from 3 healthy donor PBMCs using magnetic bead cell sorting, pulsed with various concentrations of α -GalCer and 7DW8-5 O/N, then co-cultured with 3 allogeneic iNKT cell lines in the presence of a fluorescently labeled mAb against CD107a. Flow cytometric analysis of surface CD107a expression on iNKT cells was carried out. 7DW8-5

was more potent than α -GalCer at inducing CD107a expression on iNKT cells; 1000 ng/ml of α -GalCer was needed to give a significant response, but only 10 ng/ml of 7DW8-5 was required (Fig. 6.5.A). Secondly, CD19⁺ CLL cells from 16 patients were pulsed with 1000 ng/ml of α -GalCer, 10 ng/ml of 7DW8-5, or vehicle control, and co-cultured as above with 16 allogeneic healthy donor iNKT cell lines. α -GalCer pulsed CLL cells failed to induce significant CD107a expression on iNKT cells, while 7DW8-5 pulsed CLL cells caused a significant upregulation of CD107a (Fig. 6.5.B). The significant CD107a response by iNKT cells in response to 7DW8-5 was common to the DN, CD8⁺, and DP iNKT cell subsets (Fig. 6.5.C). As a positive control, allogeneic moDCs (n = 2) were compared side by side with CLL cells (n=2) for their ability to present α -GalCer and induce CD107a expression on allogeneic iNKT cells (n = 2). While α -GalCer pulsed moDCs induced CD107a on iNKT cells, CLL cells were incapable of such activity. No synergistic effect was seen from co-culturing CLL cells with moDCs (Fig. 6.5.D). The results obtained suggest that B cells require higher concentrations of glycolipid to induce significant CD107a responses in iNKT cells, and CLL B cells are incapable of inducing a CD107a response even at higher α -GalCer concentrations. Furthermore, the glycolipid 7DW8-5 is a potent antigen capable of inducing CD107a on DN, CD8⁺, and DP iNKT cells in response to pulsed CLL cells.

6.3.6 RA induces CD1d expression on B cells from CLL patients.

Retinoic acid receptor alpha (RAR α) agonists such as all *trans* retinoic acid (ATRA) and AM580, a benzoic derivative of RA, are capable of upregulating CD1d on tonsillar B cells (Allan et al. 2011). The ability of such compounds to upregulate CD1d expression on CLL B cells was investigated in this study. Highly enriched populations of CD19⁺ cells from 4 CLL patients were treated with various concentration of ATRA and AM580 for 2 days. Flow cytometric analysis showed an increase in the frequencies of CD1d⁺ CD19⁺ CLL cells when treated with as little as 1 nM of RA (Fig. 6.6.A/B). To investigate the optimal treatment time using both ATRA and AM580, CD19⁺ cells from 4 CLL patients were treated with one concentration (100 nM) of RA for 1, 2, or 3 days. Flow cytometric analysis revealed that 2-3 days of RA treatment was enough to upregulate CD1d expression relative to untreated CLL cells (Fig. 6.6.C). In addition, the various

concentrations of RA did not affect the viability of CLL cells after 2 days in culture (data not shown). The results suggest that treating B cells from CLL patients with as little as 1 nM of either ATRA or AM580 for 2 days can result in the upregulation of surface CD1d expression (Fig. 6.6).

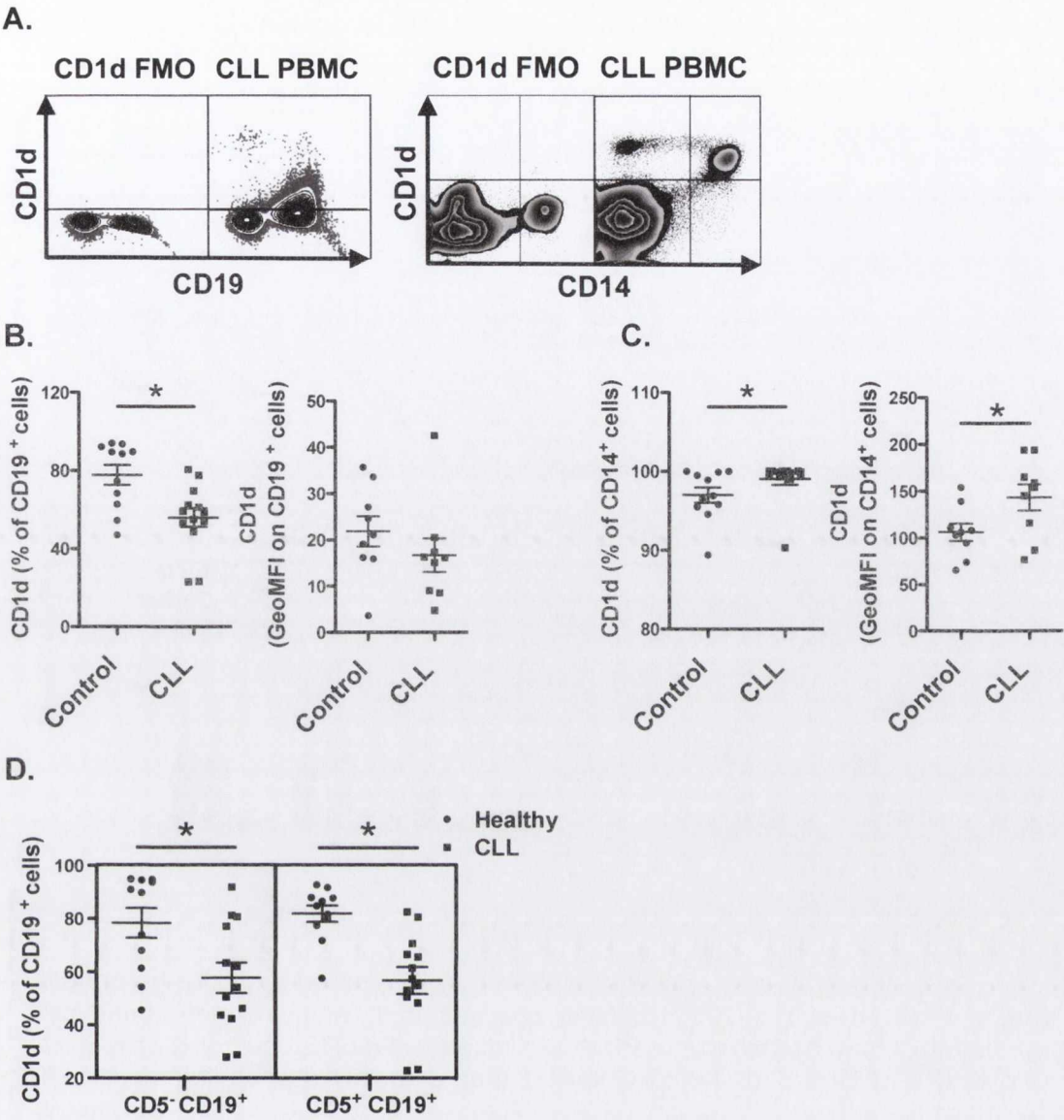


Fig. 6.4. CD1d expression by B cells and monocytes from CLL patients and age matched healthy donors. 3 ml of EDTA treated venous blood was obtained from CLL patients aged 50 – 90 years, and from age matched healthy control subjects. PBMCs were stained for flow cytometric analysis using mAbs against CD1d, CD5, CD14, and CD19. **A**, Representative Zebra plots of CLL PBMCs showing CD1d expression on CD14⁺ and CD19⁺ cells. **B** and **C**, Scatter plots showing the frequencies and geometric mean fluorescence intensity (GeoMFI) (n = 9) of CD1d on B cells (**B**), and monocytes (**C**) from healthy donors (n = 11) and CLL patients (n = 13). **D**, Scatter plot comparing the frequencies of CD1d expression on CD5⁺ and CD5⁻ B cells from healthy donors (n = 11) and CLL patients (n = 13). Data points are plotted as mean ± SEM. Statistical analysis was carried out using the non-parametric Mann-Whitney test (B & C) and Kruskal-Wallis test with post hoc Dunne multiple comparison tests (D). * P ≤ 0.05 comparing CLL to control.

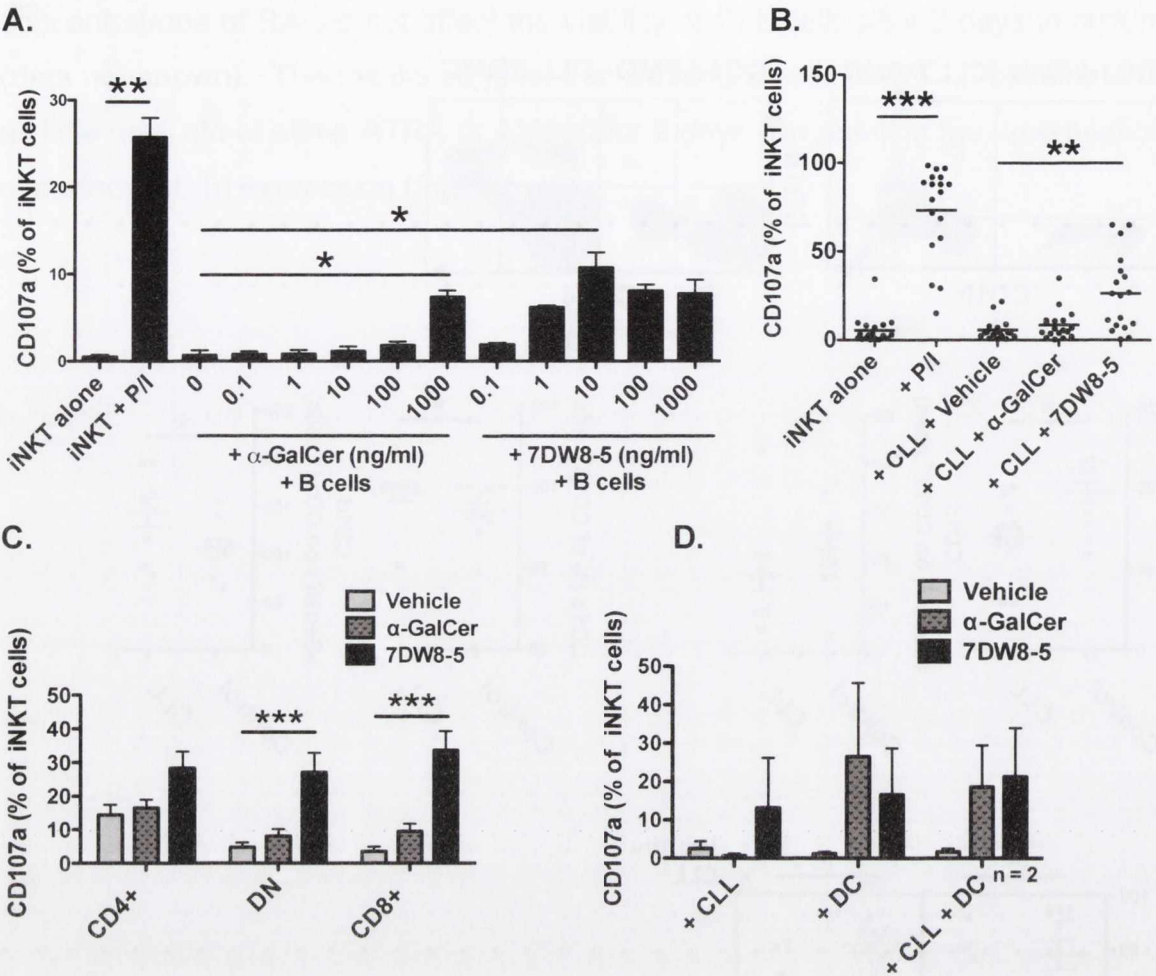


Fig. 6.5. iNKT cells degranulate in response to CLL cells pulsed with the glycolipid 7DW8-5 but not α-GalCer. CD19⁺ B cells were isolated using magnetic bead cell sorting, from both CLL and healthy donor PBMCs. Allogeneic moDCs and/or B cells were pulsed with α-GalCer, 7DW8-5, or vehicle (DMSO), then co-cultured with allogeneic iNKT cell lines for 4h in the presence of anti-CD107a. Cells were stained for flow cytometric analysis using mAbs against CD3, CD4, CD8, and CD19. **A**, Bar chart showing the frequencies of CD107a⁺ iNKT cells in response to α-GalCer or 7DW8-5 pulsed healthy donor B cells (n = 3). **B**, Scatter plot showing the frequencies of iNKT cells that express CD107a in response to 1 μg/ml α-GalCer and 10 ng/ml 7DW8-5 pulsed CLL B cells (n = 16). **C**, Bar chart showing the frequencies of iNKT cells subsets (CD4⁺, DN, CD8⁺, DP) that express CD107a in response to α-GalCer, 7DW8-5, and vehicle pulsed CLL B cells (n = 16). **D**, Bar chart showing the frequencies of iNKT cells that express CD107a in response to α-GalCer, 7DW8-5, and vehicle pulsed CLL B cells and healthy moDCs. Data points are plotted as mean ± SEM. Statistical analysis was carried out using the non-parametric Friedman test with post hoc Dunne multiple comparison tests. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

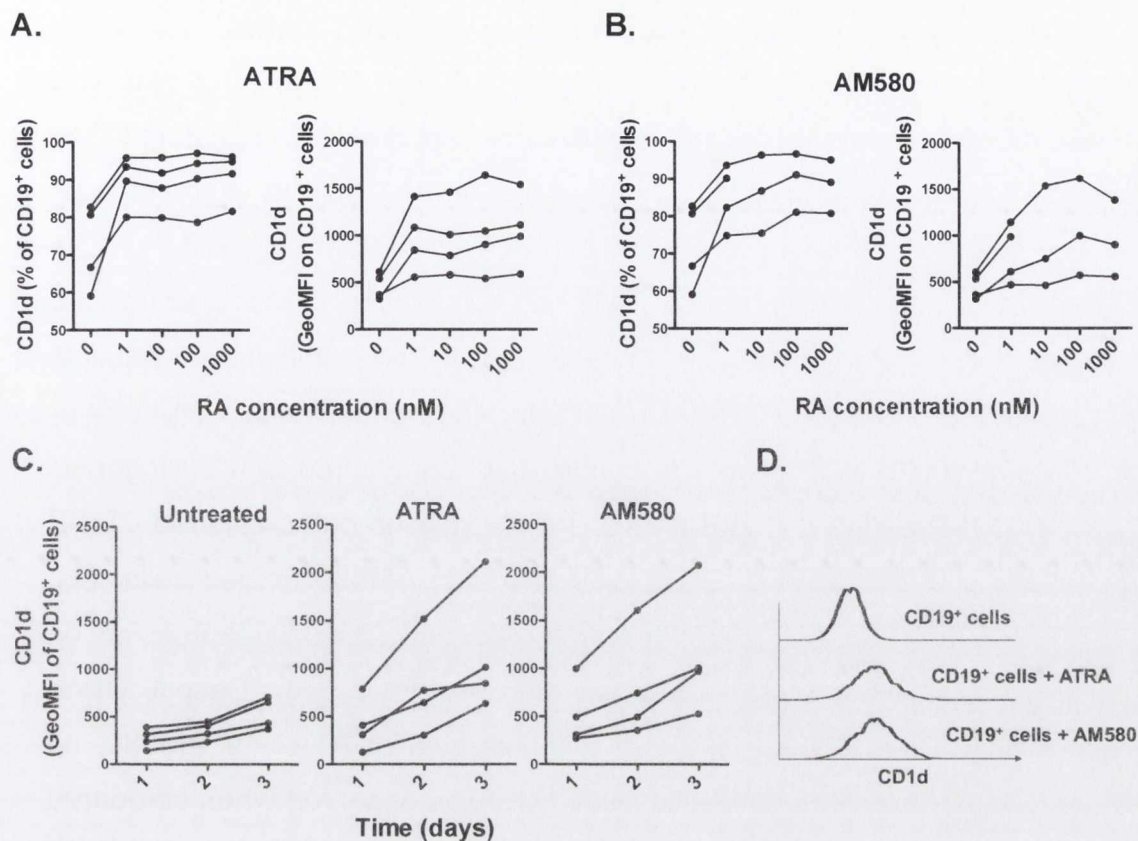


Fig. 6.6. Increased CD1d expression on B cells from CLL patients when treated with retinoic acid (RA). CD19⁺ B cells were isolated using magnetic bead cell sorting from CLL PBMCs, treated for 3 days *in vitro* with 0 – 1000 nM of ATRA or its benzoic derivative AM580. CD19⁺ CLL cells were then stained with anti-CD1d mAb and FVD eFluor® 780, and analysed by flow cytometry. **A** and **B**, Graphs showing the frequencies of CD1d⁺ CLL cells and geometric median fluorescence intensity of CD1d staining on CD19⁺ cells from CLL patients, after 48 h treatment with 1 – 1000 nM of ATRA (**A**) and AM580 (**B**). **C**, CD1d expression on CLL cells after 1, 2, and 3 days of no treatment, 100 nM of ATRA, and 100 nM of AM580. Connected data points represent independent CLL donors (n = 4). **D**, Flow cytometry histogram showing CD1d expression on CLL cells after 48h culture in the absence and presence of 100 nM AM580 or 100 nM ATRA. FVD; fixable viability dye, ATRA; all *trans* retinoic acid.

6.3.7 RA primes CLL cells for killing by CD8⁺ iNKT cells.

Retinoic acid receptor alpha (RAR α) agonists such as all *trans* retinoic acid (ATRA) and AM580, a benzoic derivative of RA, are capable of upregulating CD1d on tonsillar B cells (Allan et al. 2011). In the present study, CD1d expression on CLL cells was enhanced when treated with ATRA or AM580 (see 6.3.6). The ability of ATRA or AM580 to enhance iNKT cell cytotoxicity in response to CLL cells was also investigated in the present study. CD19⁺ CLL cells from 3 patients were pre-treated with 100 nM ATRA or AM580 for 48 h, pulsed with 1 μ g/ml of α -GalCer, 10 ng/ml of 7DW8-5, or vehicle control, then co-cultured with 3 allogeneic healthy donor iNKT cell lines. Measuring CD1d expression on co-cultured CD19⁺ cells revealed an upregulation of CD1d on ATRA and AM580 treated cells (Fig. 6.7.A). 7DW8-5 pulsed CLL cells significantly induced CD107a expression on iNKT cells, while α -GalCer pulsed CLL cells did not (Fig. 6.7.B). Despite CD1d upregulation on CLL cells (Fig. 6.7.A) in the presence of ATRA or AM580, no increase in CD107a expression on total iNKT cells was observed when compared to untreated CLL cells (Fig. 6.7.B). However, when CD4⁺, CD8⁺, and DN iNKT cells were cultured separately, CD8⁺ iNKT cells showed a significant upregulation in CD107a expression in the presence of AM580-treated, as well as a modest upregulation in the presence of ATRA-treated CLL cells. The enhanced CD107a response by CD8⁺ iNKT cells was only observed when CLL cells were pulsed with α -GalCer (Fig. 6.7.C) but not 7DW8-5 (Fig. 6.7.D). These results suggest that AM580 can selectively enhance the cytotoxic activity of CD8⁺ iNKT cells in response to α -GalCer-treated CLL B cells.

6.3.8 IFN- γ and IL-4 production by iNKT cell subsets is not affected by RA treated CLL cells.

iNKT cells are rapid and potent producers of cytokines such as IFN- γ and IL-4. While IL-4 is a Th2 cytokine and is important for Ab mediated responses, IFN- γ is a Th1 cytokine that plays a role in pro-inflammatory and cytotoxic anti-tumour responses (Vivier et al. 2012). The effect of RA on the ability of CLL cells to induce cytokine production by iNKT cells was investigated. CD19⁺ CLL cells were pre-treated with 100 nM ATRA or AM580 for 48 h, pulsed with 1000 ng/ml of α -GalCer, 10 ng/ml of 7DW8-5, or vehicle control, then co-cultured with allogeneic

healthy donor iNKT cell lines. Intracellular IFN- γ and IL-4 were detected using flow cytometry (Fig. 6.8.A). No changes were observed in either IFN- γ or IL-4 production by CD8⁺, CD4⁺, and DN iNKT cells in response to CLL cells in the presence and absence of α -GalCer and 7DW8-5 (Fig. 6.8.B/C). Neither ATRA nor AM580 significantly altered cytokine production by iNKT cells. However, a modest increase in IFN- γ expression was observed in CD4⁺ iNKT cells in response to RA-treated CLL B cells (Fig. 6.8.B). These results suggest that CLL cells are not capable of inducing significant IFN- γ or IL-4 production by iNKT cells.

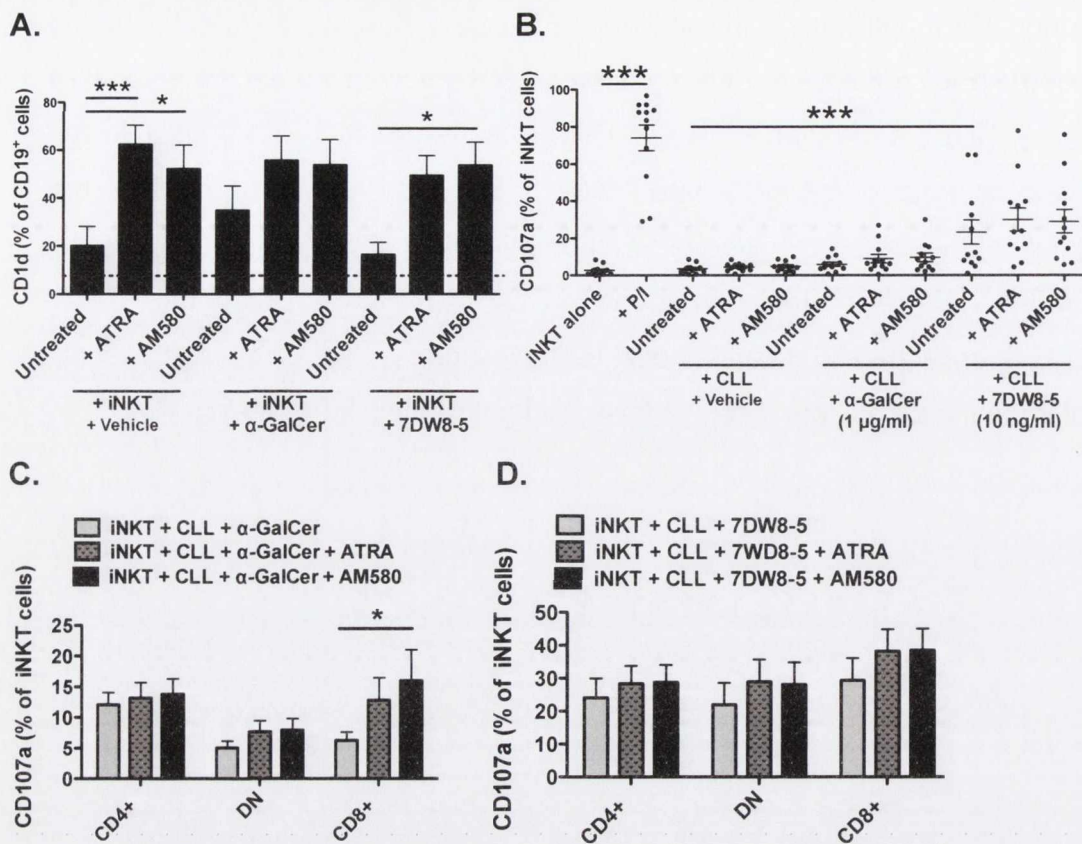


Fig. 6.7. Treatment of α -GalCer pulsed CLL cells with retinoid acid (RA) enhances degranulation of CD8⁺ iNKT cells. CD19⁺ CLL cells were isolated using magnetic bead cell sorting then treated with 100 nM ATRA or AM580 for 48 h before pulsing with α -GalCer, 7DW8-5, or vehicle (DMSO), and then co-cultured with allogeneic healthy donor iNKT cell lines for 4h in the presence of anti-CD107a mAb. Cells were stained for flow cytometric analysis using mAbs against CD1d, CD3, CD4, CD8, and CD19. **A**, Bar chart showing the frequencies of CD1d expression on CLL B cells treated with RA and co-cultured with iNKT cells ($n = 9$). **B**, Scatter plot showing the frequencies of iNKT cells expressing CD107a from co-cultures of different healthy donor lines, in response to RA treated CLL B cells ($n = 12$). **C** and **D**, Bar charts showing CD107a expression on gated iNKT cell subsets co-cultured with α -GalCer pulsed (**C**) and 7DW8-5 pulsed (**D**) RA-treated CLL cells ($n = 12$). Data points are plotted as mean $\pm$ SEM. Statistical analysis was carried out using the non-parametric Friedman test with post hoc Dunne multiple comparison tests. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

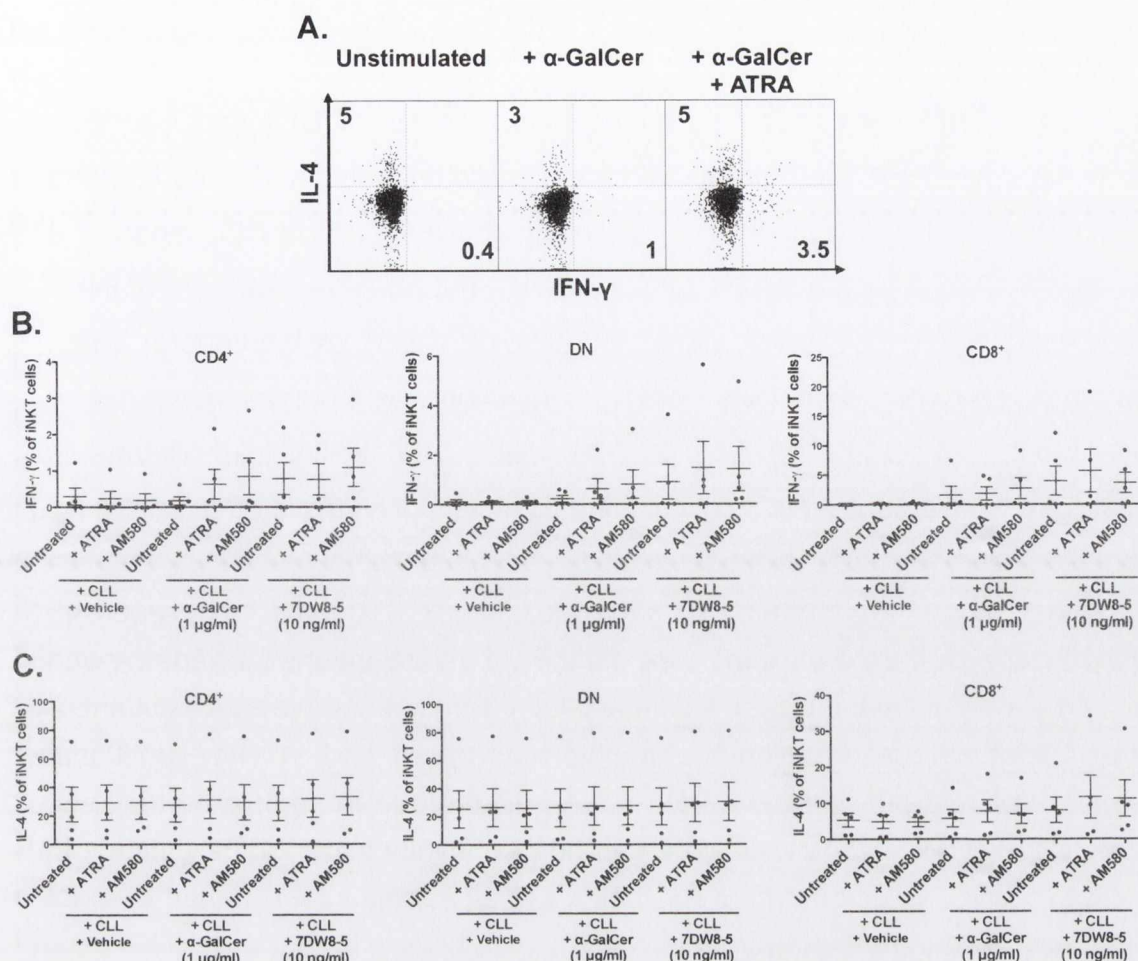


Fig. 6.8. Treatment of CLL cells with RA does not enhance IFN- γ or IL-4 production by CD8⁺ iNKT cells. CD19⁺ CLL cells were isolated using magnetic bead cell sorting then treated with 100 nM ATRA or AM580 for 48 h before pulsing with α -GalCer, 7DW8-5, or vehicle (DMSO), then co-cultured with allogeneic healthy donor iNKT cell lines for 5h in the presence of brefeldin A. Cells were stained for flow cytometric analysis using mAbs against CD3, CD4, CD8, IFN- γ , and IL-4. **A**, Dot plots showing IFN- γ and IL-4 expression by iNKT cells after co-culturing with CLL cells in the presence or absence of α -GalCer, and ATRA. **B** & **C**, Scatter plots showing the frequencies of IFN- γ ⁺ CD4⁺, DN, and CD8⁺ iNKT cells (**B**) and IL-4⁺ CD4⁺, DN, and CD8⁺ iNKT cells (**C**) from healthy donor iNKT cell lines, in response to RA treated CLL B cells. Data points are plotted as mean $\pm$ SEM from 2 independent experiments.

6.4 Discussion.

The roles that iNKT cells may play in the pathogenesis of CLL and / or their potential as targets for the treatment of CLL are yet to be elucidated. The first and only study of its kind by Weinkove et al, looking at iNKT cells in CLL reported a decrease in iNKT cell frequency (% of CD3⁺ cells), but absolute numbers of iNKT cells were increased due to a global increase in CD3⁺ cell numbers, when compared to age-matched controls. iNKT cell function was intact as measured by cytokine production, CD107a responses, as well as the ability to proliferate in response to α -GalCer loaded APCs, including CLL cells (Weinkove et al. 2013).

In the present study, peripheral blood samples from 32 CLL patients between 40 and 90 year of age were analysed. The number of investigations carried on each patient sample were limited due to the small 1.5 - 3 ml PB sample sizes obtained. For the majority of patients, both phenotypic analyses and *in vitro* functional studies were carried out. However, for some patients due to sample restrictions, only one of the two investigations were carried out. While some of the patients in our cohort received prior treatment for their CLL, all samples were taken at least one year after finishing their treatment.

The frequencies of iNKT cells in PB of CLL patients was significantly lower than those of age matched controls. This along with an overall increase in CD3⁺ cell numbers was in agreement with findings by Weinkove et al. However, the cohort tested in this study showed that despite the increased T cell numbers, a reduction in absolute iNKT cell numbers in CLL patients was found compared to controls, contrary to the increase seen by Weinkove et al., despite using the same technique for identifying iNKT cells (i.e. the anti-iNKT cells receptor 6B11 clone). The proportions of CD4⁺, DN, CD8⁺, and DP iNKT cells did not significantly change in patients compared to age matched controls. However, a non-significant decrease in the proportion of DN iNKT cells was observed in CLL patients. PBMCs from 17 patients and 12 controls were stimulated *in vitro* with α -GalCer and cultured for 30 days in the presence of IL-2 and IL-7. After 30 days in culture, expanded populations of iNKT cells were produced from all 12 healthy control samples, but from only 2 CLL patient samples. Enriched PBMCs were then sorted and further expanded into pure total, CD4⁺, DN, and CD8⁺ iNKT cell lines.

Representative CLL samples (n = 7) were measured by flow cytometry for starting iNKT cell frequencies and absolute numbers prior to *in vitro* stimulation with α -GalCer. The starting iNKT cell numbers and frequencies were comparable

between the CLL samples that responded to α -GalCer stimulation ($n = 2$), and those that did not respond ($n = 5$), as well as control samples. Hence, excluding starting iNKT cell numbers as a sole factor affecting their expansion from CLL patients. The ages of patients can be excluded as a factor influencing iNKT cell expansion, because stimulated PBMCs were from CLL patients ranging in age 53 – 87 years. The likelihood of a high CLL burden having a negative effect on iNKT cell proliferation is possibility, however, a previous study has demonstrated that iNKT cells expand in the presence of α -GalCer pulsed irradiated CLL B cells (Weinkove et al. 2013). Furthermore, previous treatment history is not likely to affect iNKT cell expansion, as half of the patient samples unresponsive to α -GalCer stimulation were completely treatment naïve. However, the significant reduction in CD1d on CLL B cells from our patient cohort may have a role to play in the lack of iNKT cell expansion. For this reason, iNKT cell expansion after CD1d upregulation should be considered in future studies. Due to the limitation in patient blood sample size and numbers, the question of whether iNKT cells from CLL patients can expand normally *in vitro* is inconclusive.

As previously described in section 3.4 (page 103 – 104), CD31 is a cell surface marker that correlates with TREC levels, hence is an indicator for replication history and residual replicative potential (Junge et al. 2007). To investigate whether iNKT cells from our patient cohort show any evidence of reduced proliferative potential, CD31 surface expression was measured *ex vivo*. There was no difference found between CD31 expression on iNKT cells from patients and age matched controls, which suggests a similar replicative history *in vivo* for both patient and control iNKT cell populations. For this reason the failure in iNKT cell expansion from some patients cannot be attributed to replicative senescence, and further investigations would need to be carried out using a much larger starting numbers of patient samples. As for the 2 patient samples that did expand iNKT cells, these iNKT cells showed similar population doublings to those from age-matched controls after 30 days in culture. These findings suggest that although iNKT cells are found at a lower frequency and numbers in the PB of CLL patients, they have the potential to expand *in vitro*, based on CD31 expression, and in accordance with Weinkove et al. they can also carry out normal functions such as cytokine production, and cytolytic killing of glycolipid pulsed target cells.

As previously demonstrated by others (Zheng et al. 2002; Kotsianidis et al. 2011), CD1d expression on CD19⁺ CLL cells is significantly reduced in patients compared to age matched controls. However, where others have shown that the reduction in CD1d expression occurs more significantly in CD5⁺ leukaemia cells compared to CD5⁻ B cells, our patient cohort did not show any preferential depletion of CD1d

expression on CD5⁺ B cells. On the other hand, monocytes from CLL patients showed a significant increase in CD1d expression compared to age matched controls, which contradicts the finding by Weinkove et al. that CD1d expression on monocytes is reduced. These findings suggest that CD19⁺ cells in patients with CLL have reduced CD1d expression, which may contribute to a reduction in CD1d dependent iNKT cell responses.

Restoring CD1d expression on CLL B cells to normal levels might serve to improve iNKT cell responses to CLL cells *in vivo* and consequently contribute to iNKT cell mediated cytotoxic killing of CLL cells. ATRA and AM580 have both been reported to increase CD1d expression on tonsillar B cells *in vitro* (Allan et al. 2011). Purified populations of CD19⁺ cells from 4 CLL patients were treated for various time points and concentration of both RA compounds. Treatment with either ATRA or AM580 resulted in CD1d upregulation on CLL cells, and 100 nM of either RA for 48 h was chosen as an optimal treatment. Interestingly, normal plasma concentrations of ATRA is around 5 nM (Napoli et al. 1991). According to our finding, the amount of ATRA required to increase CD1d expression *in vitro* was only marginally higher than that of normal plasma concentrations of ATRA (10 – 100 nM). Low ATRA doses would potentially minimise any RA side effects as well as avoiding resistance to RA.

Before testing the effects of RA treatment on iNKT cell responses to CLL cells, the ability of CLL cells to present glycolipid to healthy allogeneic iNKT cells was investigated. The optimal concentrations of α -GalCer and its derivative 7DW8-5 were determined using healthy B cells in a CD107a assay using allogeneic healthy iNKT cells lines. In order for B cells to induce a significant CD107a response by iNKT cells, 1000 ng/ml of α -GalCer (i.e. 10-100 times the concentration used when DC or CD1d-transfected cells are used as targets) was required, while only 10 ng/ml of 7DW8-5 was sufficient. The ability of CD19⁺ CLL cells to induce a CD107a response by healthy allogeneic iNKT cells was then tested using 1000 ng/ml of α -GalCer and 10 ng/ml of 7DW8-5. α -GalCer failed to induce a CD107a response to CLL cells, while 7DW8-5 significantly increased CD107a expression on iNKT cells, in particular in CD8⁺ and DN iNKT cell subsets. To eliminate other factors that may influence the performance of α -GalCer in a given *in vitro* assay, both CLL cells and healthy moDCs were used as APCs in the same experiment. Despite moDCs inducing CD107a expression in the presence of both glycolipids, CLL cells failed again in the presence of α -GalCer. These findings suggest that healthy B cells require higher concentrations of α -GalCer to induce a CD107a response by iNKT cells, while CLL B cells completely fail to do so. However, the α -GalCer derivative 7DW8-5 significantly increased CD107a response to CLL cells

by iNKT cells, making it superior to α -GalCer as a possible tool for immunotherapy in CLL. In addition, when CLL cells were pulsed with the glycolipid OCH, they were able to upregulate CD107a expression on CD8⁺ iNKT cells similar to OCH-pulsed healthy donor B cells (Appendix Fig. 8.1). This observation was made using 5 μ g/ml of OCH, which is half the optimal concentration of 10 μ g/ml as later determined using healthy donor B cells (Fig. 4.7). Investigations into the potential of the glycolipid OCH in enhancing iNKT cell cytotoxic killing of CLL cells was limited in this study. More work is required to confirm these provisional findings.

The ability of ATRA or AM580 to enhance CD107a responses by iNKT cells to either α -GalCer or 7DW8-5 pulsed CLL cells was investigated. Overall iNKT cell response was not affected by RA treatment of CLL cells. However, CD8⁺ iNKT cells gave significant CD107a responses in the presence of AM580 treated CLL cells pulsed with α -GalCer. Furthermore, α -GalCer pulsed CLL cells pre-treated with RA showed an upregulation in CD1d, which presumably contributed to enhancing CD107a responses by CD8⁺ iNKT cells. Intracellular cytokine staining for IFN- γ and IL-4 was carried out to assess any changes in cytokine production by CD8⁺ iNKT cells in response to RA treated CLL cells. CD8⁺ iNKT cells as well as other subsets showed minimal IFN- γ production in response to both glycolipids, which was not enhanced by RA. Similar to IFN- γ , IL-4 showed little change in response to RA treated CLL cells. These findings suggest that CLL B cells treated with RA have increased CD1d expression, and are better capable of selectively inducing cytotoxic responses by CD8⁺ iNKT cells.

This study has highlighted the potential for RA as an immunotherapeutic agent for patients with CLL, especially if combined with α -GalCer to selectively induce CD8⁺ iNKT cell cytotoxicity. Another interesting finding was the ability of the iNKT cell ligand 7DW8-5 to induce cytotoxicity without the need for RA treatment; the potential use of 7DW8-5 in targeting iNKT cells for cancer immunotherapy requires consideration.

The use of iNKT cell based immunotherapy has shown promise for patients with solid tumours, and have been shown to be safe therapies (Kunii et al. 2009; Uchida et al. 2008; Yamasaki et al. 2011). Patients diagnosed with CLL and requiring treatment, undergo regimens of chemotherapy and biologic therapies that are not specific and result in a multitude of side effects. Harnessing and boosting the patient's anti-tumour defenses may prove more promising. Boosting CD1d-iNKT cell responses, which are lacking in these patients may contribute to reducing CLL burden. More work is required to investigate the potential for *ex vivo* expansion of iNKT cells with the aim of transferring larger numbers back into the

patients. α -GalCer pulsed APCs may be combined with expanded iNKT cells, as well as administration of RA to improved iNKT cell responses to CLL cells.

Chapter 7 General discussion.

iNKT cells play central roles in immunity against microorganisms and tumours. They recognise self and microbial glycolipids, by which they can detect pathogens and pathogenic changes to host tissues. Their ability to rapidly kill target cells, to release cytokines that stimulate and regulate adaptive immune responses, and to promote Ag presentation by providing adjuvant signals to DC, place iNKT cells as central players in innate and adaptive immune responses. iNKT cells mediate antitumour immunity in murine models and appear to be deficient or impaired in human cancers. These findings have led to multiple clinical trials involving the adoptive transfer of *ex vivo*-expanded autologous iNKT cells and DC from patients with various cancers (Berzins et al. 2011).

iNKT cells also provide help for lipid-reactive and protein-reactive B cells. They are required for the generation of protective Ab responses against some murine pathogens and they dramatically augment Ab responses to co-administered Ags *in vivo* (Dellabona et al. 2014). However, despite their name, iNKT cells are multifunctional in nature. Human CD4⁺, CD8⁺, and DN iNKT cells display different cytolytic activities and cytokine secretion profiles, and differentially activate DC. Furthermore, CD8⁺ iNKT cells appear to exhibit the most potent antitumour activity, while CD4⁺ iNKT cells may suppress antitumour immunity (O'Reilly et al. 2011). These variable effector activities prompted us to compare the relative abilities of CD4⁺, CD8⁺, and DN iNKT cell lines to provide help for B cell differentiation, Ab production, and cytokine production *in vitro*. We also looked at the reciprocal ability of B cells to induce iNKT cells degranulation and cytokine production through the presentation of various glycolipid Ags. In view of the well-documented roles that iNKT cells play in antitumour immunity and in B cell differentiation, we also investigated their role and treatment potential in patients with CLL.

iNKT cells are present in various human tissues and vary in frequencies depending on the tissue. The lymphocytes in human liver are 1% iNKT cells, while omentum contains 10% iNKT cells. Removal of solid tissues such as liver or omentum is not warranted when in comparison, obtaining peripheral blood holds less risk for the donor and is extremely easy from a technical perspective. Since iNKT cells account for less than 1% of peripheral blood T cells, the first objective of this study was to optimize protocols for generating pure lines and their individual

CD4⁺, CD8⁺, and DN subsets, for use in functional studies and ultimately for use in iNKT cell-based cancer immunotherapies. iNKT cells are typically expanded by activation with mitogen or glycolipid Ag presented by CD1d⁺ cells in the presence of IL-2. This expands all subsets of iNKT cells, but CD4⁺ iNKT cells preferentially expanded (O'Reilly et al. 2011). Several groups have examined the ability of various cytokine cocktails to expand total iNKT cells (Nishi et al. 2000; van der Vliet et al. 2001). A combination of IL-2, IL-7 and IL-15 was found to work in synergy to improve total iNKT cell yields from PBMCs cultured for 7 days in the presence of α -GalCer, giving an average 40-fold increase in iNKT cell numbers (Nishi et al. 2000). An improvement on this method was reported when iNKT cells were co-cultured with α -GalCer-pulsed moDCs in the presence of both IL-7 and IL-15 for 7 days, yielding a 76-fold increase in total iNKT cell numbers (van der Vliet et al. 2001). However, when we cultured α -GalCer-pulsed PBMCs in the presence of IL-2 and IL-7, we observed an average 270-fold increase in iNKT cell yields after 7 days in culture, which is equivalent to 1 PDL per day, considerably higher than those reported in previous studies. In addition, the method described selectively improved yields of CD8⁺ iNKT cells, the most difficult subset to expand using previously described methods (O'Reilly et al. 2011). Moreover, using the method described in the present study, we were able to expand significant numbers of iNKT cells from as little as 1.5 ml of PB, as well as producing pure primary cell-lines of CD8⁺, CD4⁺, and DN iNKT cells. The expanded cells were fully functional as measured by their ability to produce and secrete IFN- γ and IL-4 upon activation with α -GalCer presented by CD1d-HeLa cells, as well as their ability to upregulate cell surface expression of CD107 as a measure of cytotoxic degranulation.

Taking into consideration the superior anti-tumour effector activities of human CD8⁺ iNKT cells, compared to CD4⁺ and DN iNKT cells, demonstrated *in vitro* (O'Reilly et al. 2011), and the safety and efficacy of *ex vivo* expanded iNKT cell-based immunotherapies for solid tumours (Kunii et al. 2009; Uchida et al. 2008; Yamasaki et al. 2011), we believe that our method for expanding pure CD8⁺ iNKT cells may provide the basis for preparing autologous cells for a more anti-tumour targeted iNKT cell-based immunotherapy.

Activation of iNKT cells requires the recognition of lipid Ag presented by APC in the context of CD1d. Understanding the stimulatory capacity of different glycolipid Ags as well as of the different CD1d⁺ APC for modulating iNKT cell functions is pivotal to our ability to manipulate iNKT cell functions for the propose of immunotherapies. moDCs are professional APCs, which are well known for their abilities to potently activate iNKT cells (O'Reilly et al. 2011). iNKT cells are capable of fully activating moDCs *in vitro* resulting in their maturation into APCs capable of presenting and cross-presenting Ag to T cells (Kitamura et al. 1999).

B cells are also known for their ability to act as APC to conventional T cells (Lund & Randall 2010). Recent findings suggest that human B cells can activate iNKT cells through the presentation of α -GalCer in a CD1d dependent manner (Allan et al. 2011), and are potentially required for normal iNKT cell proliferation (Bosma et al. 2012). Furthermore, iNKT cell frequencies are reduced in a number of B cell related disorders, including systemic lupus erythematosus (SLE), MM, and CLL, where therapies restoring B cell function in some conditions resulted in restoration of iNKT cell frequencies (Dhodapkar et al. 2003; Bosma et al. 2012; Weinkove et al. 2013). However, there have been no reports on the ability of human B cells to present different glycolipid Ags to iNKT cells, or on the differential responses of CD4⁺, CD8⁺, and DN iNKT cells to antigen presentation by B cells. The results from the current study highlight differences in iNKT cell responses to various glycolipids when presented by B cells compared to those presented by other APC such as CD1d-transfected HeLa cells. B cells induced less iNKT cell degranulation than CD1d-HeLa cells. However, the glycolipid OCH induced more degranulation by iNKT cells when presented by B cells compared to α -GalCer, while this did not hold true when each glycolipid was presented by HeLa cells. However, a much more significant response by iNKT cells was obtained when α -GalCer concentrations were increased 10 fold. These results suggest that the same glycolipid Ags can be presented to iNKT cells by different APCs and may result in different outcomes. In addition B cells required high concentrations of α -GalCer in order to elicit an equivalent response by iNKT cells as those observed using other APC. Furthermore, we can speculate that B cells may be better able to present OCH compared to α -GalCer, as at low concentrations of each glycolipid, iNKT cell responses to B cells presenting OCH was superior to those presenting α -GalCer. These results suggest the need to consider increasing the

dose of α -GalCer or to consider the use of other therapeutic glycolipids when used for patient treatment, especially when the desired outcome relies on cognate interaction of iNKT cells with B cells.

We investigated the novel synthetic α -GalCer analogue 7DW8-5 for its ability to activate iNKT cell subsets when presented by B cells. We found 7DW8-5 to be at least 100 times more potent than α -GalCer for the induction of iNKT cell degranulation as well as IFN- γ and IL-4 secretion. While 7DW8-5 is undergoing development for future clinical trials in humans, the most recent study tested the effect of 7DW8-5 on rhesus macaques as a malaria vaccine adjuvant. As well as reporting on the safety and tolerance of 7DW8-5, this glycolipid was found to induce a 9-fold increase in Ag-specific CD8⁺ T cells when compared to α -GalCer (Padte et al. 2013). Based on our findings, 7DW8-5 is a considerably more potent Ag than α -GalCer for inducing cytotoxicity and cytokine production by iNKT cells, and in particular when modulating iNKT cell activity using B cells as APC. This provides further evidence in humans on the potency of 7DW8-5, as well as being the first study to report on the effects of 7DW8-5 on each of the separate iNKT cell subsets. Further investigations are required to compare 7DW8-5 to the well established α -GalCer with regards to its effects on human iNKT cell function and in particular when using different APCs. Ultimately, 7DW8-5 may prove to be one of greatest novel contributions to human cancer immunotherapies targeting iNKT cell function.

The reciprocal side of the cognate interaction of iNKT cells and B cells is the influence that activated iNKT cells have on B cell activation, differentiation, and effector functions. Studies in both mice (Lee et al. 2005; Lisbonne et al. 2003; Belperron et al. 2005) and humans (Galli et al. 2003) have highlighted the importance of iNKT cells in the induction of humoral responses, and iNKT cells have accordingly been targeted as adjuvants for vaccinations in mice (Ko, Ko, & Chang, 2005; Galli et al., 2007; Lang et al., 2006). Furthermore, a recent study has confirmed that in patients with SLE, iNKT cells provide help for auto-Ab production by B cells (Shen et al. 2015), further emphasising the importance of iNKT cells in humoral immunity. The present study also confirmed that iNKT cells provide help for Ab production by human B cells. We also looked at the differential effect of CD4⁺, CD8⁺, and DN iNKT cells on B cell activation, differentiation, as

well as Ab production. CD4⁺, CD8⁺, and DN iNKT cells were compared for their ability to enhance total IgG, IgA, and IgM secretion by B cells *in vitro*. iNKT cells induced significant increases in Ab production by B cells in a CD1d-dependent manner. While this effect was not subset-specific, CD4⁺ iNKT cells were the most effective at enhancing Ab production by co-cultured B cells. Interestingly, unlike conventional T cells, CD8⁺ iNKT cells were capable of providing help to B cells for the production of IgG, IgA, and IgM. These findings provide further support for the use of iNKT cells as vaccine adjuvants. However, our results suggests that α -GalCer is not required, and to an extent was inhibitory, for the provision of B cell help by iNKT cells, which is in agreement with previous findings (Galli et al. 2003). However, we also found that B cells require higher concentration of α -GalCer than other APC to bring about significant iNKT cell degranulation and cytokine production (Fig 4.7 & 4.8), making it possible that higher concentrations of glycolipid may augment Ab production by B cells. For this reason, these experiments should be repeated using higher concentrations of α -GalCer before ruling out its adjuvant effects on Ab production by human iNKT cells. Furthermore, 7DW8-5 should be investigated as possible adjuvant for enhancing iNKT cell help to B cells.

CD4⁺ iNKT were not only the most effective at inducing Ab secretion by B cells, but they also induced significant upregulation of the activation markers CD69 and IL-21R, as well molecules necessary for glycolipid Ag presentation and co-stimulation, CD1d and CD40. However, such phenotypic changes to B cells were not exclusively induced by CD4⁺ iNKT cells, but were induced to a lesser extent by CD8⁺ and DN iNKT cells. These result suggest that iNKT cells, and in particular CD4⁺ iNKT cells are capable of activating B cells, inducing the upregulation of molecules required by B cells for the presentation of lipid Ags to iNKT cells, ultimately leading to polyclonal secretion IgG, IgA, and IgM. We hypothesised that iNKT cell help may also induce cytokine production by B cells, which may contribute to iNKT cell activation by B cells. However, when CD4⁺, CD8⁺, or DN iNKT cells were co-cultured with autologous B cells, there was no induction of IFN- γ , IL-4, or IL-10, both in the absence or presence of the exogenous glycolipid Ag α -GalCer. These results suggest that B cells do not produce these cytokines in response to iNKT cell stimulation. However, we cannot rule out any positive

effects of higher concentrations of α -GalCer, and indeed 7DW8-5, on cytokine production by B cells. Hence, these experiments should be repeated accordingly.

Having investigated the differential effects of CD4⁺, CD8⁺, and DN iNKT cells on various B cell functions, we then investigated if different iNKT cell subsets can influence the differentiation of various B cell subsets. We found that CD4⁺, CD8⁺, and DN iNKT cells were all capable of increasing the proportion of unswitched memory B cells as well as MZB cells, independent of α -GalCer, when co-cultured with total human B cells *in vitro*. However, although it is unclear if the proportional changes are due to B cell proliferation or differentiation, we found that in the presence of iNKT cells, MZB cell numbers are significantly increased. A recent study in mice has also shown that cognate help provided by iNKT cell induces the expansion of MZB cell population *in vivo* (Vomhof-DeKrey et al. 2015). MZB cells have constitutively higher levels of CD1d expression, making them better targets for iNKT cell stimulation.

In recent years, a particular functional B cell subset has emerged with immunosuppressive properties that are dependent on IL-10 production in mice, but do not require IL-10 production in humans. A number of B cell phenotypes enriched for Breg cells include CD1d^{hi}CD5⁺ B cells and CD24^{hi}CD38^{hi} (Rosser & Mauri 2015; Mauri & Menon 2015). We investigated the ability of CD4⁺, CD8⁺, and DN iNKT cells to induce these B cell phenotypes *in vitro*. iNKT cells, and in particular CD4⁺ iNKT cells, were capable of inducing a Breg phenotypes in co-cultured B cells. It is unclear if Breg cells proliferate in response to the iNKT cells or if these phenotypes are induced as a result of interactions with iNKT cells. However, a recent study in mice reported an expansion of IL-10 producing CD1d^{hi}CD5⁺ B cells upon the provision of cognate iNKT cell help to B cells (Vomhof-DeKrey et al. 2015), which further points to a possible role for iNKT cells in regulating immune responses through the induction of Breg cells. Future work is required to confirm an immunosuppressive function in the Breg cell induced by iNKT cells in our co-cultures.

It is possible that iNKT cells induce an effector function or regulatory function in different B cell subsets, rather than multiple effector functions on the same B cell subsets. Further work involving the sorting of various phenotypic B cell subsets

and studying the effects of iNKT cells on multiple B cell functions is required to resolve this issue. Furthermore, understanding the effects that CD4⁺, CD8⁺, and DN iNKT cells have on B cell functions is vital to determining the possible consequences of targeting iNKT cell function for the purpose of immunotherapy.

Cognate B cell help is provided by T_{FH} cells, classically CD4⁺ conventional T cells, and more recently iNKT cells (NKT_{FH} cells) were shown to provide help for Ab production in mice (King et al. 2012). We investigated whether B cells can induce a NKT_{FH} phenotype on human CD4⁺, CD8⁺, or DN iNKT cells *in vitro*. Because the experimental donor number was low (n = 2), no conclusive findings regarding NKT_{FH} cells was made, but speculations can be made based on general trends observed when iNKT cells from 2 donors were cultured with autologous B cells. Our preliminary findings suggest a preferential induction of NKT_{FH} phenotype on a small proportion of CD8⁺ NKT cells when co-cultured with B cells, when compared to CD4⁺ and DN iNKT cells. Interestingly, this induction was independent of α -GalCer stimulation, which could be due to the requirement of higher α -GalCer concentrations when presented by B cells.

A recent study looking at patients with Gaucher disease, a disorder of lipid metabolism, found that a population of type 2 NKT cells that recognise 2 major sphingolipids, accumulate in these patients. These type 2 NKT cells were found in both humans and mice, they constitutively expressed CXCR5 and PD-1, and contained a significantly higher population of CD8⁺ cells than their α -GalCer reactive type 1 counterparts. They were able to provide help for lipid-specific Ab production *in vivo* in mice, and *in vitro* in humans. These novel type 2 NKT cells were better able to provide help for Ab production by human B cells *in vitro* compared to α -GalCer-reactive type 1 NKT cells (Nair et al. 2015). While our results are preliminary, the possibility of CD8⁺ iNKT cells holding a preferential NKT_{FH} function cannot be ruled out. However, because of the low frequencies of NKT_{FH} cells within the total iNKT cell population, it is likely that iNKT cell provision of help for B cells is independent of this phenotype. To confirm this, further work is required to determine if a prototypic T_{FH} phenotype is necessary for the provision of B cells help by iNKT cells, where different clones of iNKT cells with and without a T_{FH} phenotype are co-cultured with autologous B cells and compared in their ability to provide help for Ab production *in vitro*. Specifically identifying iNKT cells

with distinctive phenotypes that hold particular effector functions, can allow us to further tailor autologous iNKT cell immunotherapies to obtain a disease-specific desirable therapeutic outcome.

The results of this investigation suggest that B cells can present glycolipids to iNKT cells resulting in their activation. Reciprocally, iNKT cells induce polyclonal Ab production, activation, as well as up-regulation of CD40 and CD1d on B cells, the most likely target of which are MZB cells. Furthermore, un-switched memory B cells, MZB cells, as well as CD24^{hi}CD38^{hi} B cells, are B cell phenotypes that predominate in the presence of iNKT cells. While iNKT cells provide help to B cells, the proportion of iNKT cells expressing a T_{FH} phenotype are minor, indicating iNKT cell help to B cells may be independent of this phenotype.

The final part of this study focused on investigating the roles of iNKT cells and their possible clinical applications as targets for immunotherapy in patients with the B cell malignancy CLL. Knowing that iNKT cells play an important role in modulating B cells functions, we investigated the phenotype and function of iNKT cells and B cells in patients with CLL. Our findings highlighted that patients with CLL have a selective depletion of iNKT cells when compared to age matched controls. No significant alterations to the distribution of iNKT cell subsets based on CD4 and CD8 expression was observed. However, the latter finding is inconclusive due to the limited number of patients and controls that were available for analysis and age matched. We also demonstrated that B cells from CLL patients but not monocytes had a selective and significant reduction in surface CD1d expression. We speculate that this reduction in iNKT cells may be a result of CD1d down regulation on CLL B cells, and that modulation of CD1d expression on B cells may lead to restoration of iNKT cell frequencies in the PB of patients with CLL, ultimately leading to increased antitumour activities by iNKT cells, and finally improved prognosis or disease resolution. The RAR α agonists ATRA or AM580 act on the RAR α receptor on B cells, causing the upregulation of CD1d (Allan et al. 2011). We investigated the ability of such compounds to influence CD1d expression on CLL cells. We found that CD1d expression was significantly enhanced on CLL cells following treatment with RAR α agonists ATRA or AM580, which in turn enhanced the cytotoxic degranulation response of CD8⁺ iNKT cells, potentially leading to enhanced CLL killing. Furthermore, the glycolipid 7DW8-5

was superior at inducing iNKT cell degranulation in response to pulsed CLL cells, which make it a prime candidate for use for CLL immunotherapies. From our findings, CLL patients are prime candidates for iNKT cell targeted immunotherapy trials, where patients could be given ATRA, an already established treatment for PML (Degos & Wang 2001), to enhance CD1d expression by CLL cells. Coupling the adoptive transfer of α -GalCer-pulsed moDCs with *ex vivo* expanded iNKT cells has improved upon iNKT cell-based cancer immunotherapy (Chang et al. 2005). We suggest coupling ATRA with α -GalCer-pulsed moDC, as well as using autologous expanded CD8⁺ iNKT cells, as a potentially more effective treatment for cancer patients. The use of 7DW8-5 would need to be clinically tested on patients before consideration for use. So far iNKT cell targeted immunotherapy trials have focused on end-stage cancer patients with solid tumours, where both immunosuppression and end-stage illness may be playing a major role in dulling the potential outcome of iNKT cell activity in such patients. Nonetheless, some patients have benefited from these trials, and further tweaking the regimens to better harness iNKT cell function may drastically improve patient prognosis.

Overall the present study has highlighted that iNKT cells are capable of influencing B cell phenotype and function. We also confirmed that B cells can present glycolipid Ags to iNKT cells, but are less potent and require higher concentrations of Ag than other APC to elicit a significant iNKT cell response. We suggest 7DW8-5 as a potent iNKT cell Ag that should be considered when trying to modulate iNKT cell responses, in particular when using B cells as APC. We demonstrated an overlap in the effector functions of CD4⁺, CD8⁺, and DN iNKT cells with regard to their response and effects on B cell functions. Furthermore, we suggest that iNKT cells may provide a primary target for the treatment of CLL patients, either directly via cellular infusions of CD8⁺ iNKT cells, indirectly through the modulation of CD1d expression on CLL cells, or both. Finally, we found that each iNKT cell subset has a distinct role to play, with CD4⁺ iNKT cells providing superior B cell helper activity or Ab production, while CD8⁺ and DN iNKT cells displaying the greatest anti-tumour cytotoxic activity. These distinct roles of iNKT cell subsets need to be considered when optimizing protocols for cancer immunotherapies.

iNKT cells, while they represent a relatively minor population of innate T cells in humans, they are equipped with an array of effector and regulatory activities that

influence a multitude of other immune cells. Highlighted by the present study, iNKT cells influence a number of B cell functions, placing them at the heart of adaptive immune responses.

Chapter 8 Appendix

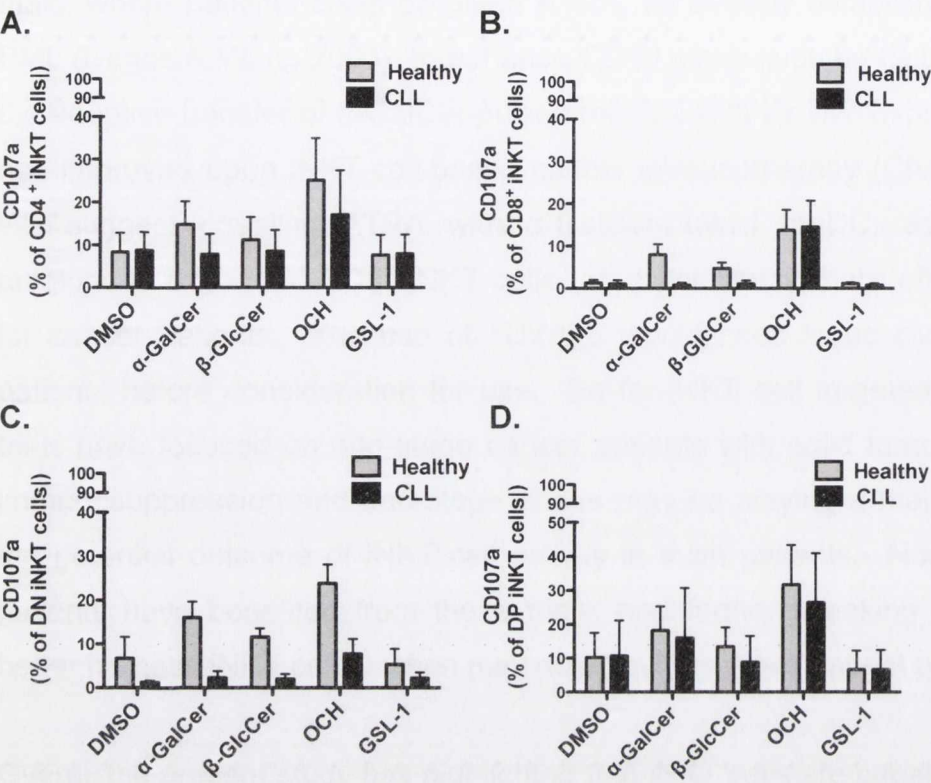


Fig. 8.1. The glycolipid OCH enhances the susceptibility of CLL cells to cytotoxicity by CD8⁺ iNKT cells. CD19⁺ cells were isolated from CLL patient and healthy donor PBMCs using magnetic bead cell sorting. B cells were cultured with DMSO (vehicle) or pulsed with 50 ng/ml of α -GalCer, 5 μ g/ml of β -GlcCer, 5 μ g/ml of OCH, or 5 μ g/ml of GSL-1 over night at 37°C. B cells were then co-cultured with 3 different allogeneic iNKT cell lines for 4 h in the presence of anti-CD107a mAb. Cells were stained with mAbs against CD4 and CD8 then analysed by flow cytometry. **A, B, C, and D,** Bar charts showing the frequencies of CD4⁺ (A), CD8⁺ (B), DN (C), and DP (D) iNKT cells expressing CD107a after co-culturing with CD19⁺ cells pulsed with various glycolipids, from healthy donors and patients with CLL. Data points are plotted as mean $\pm$ SEM.

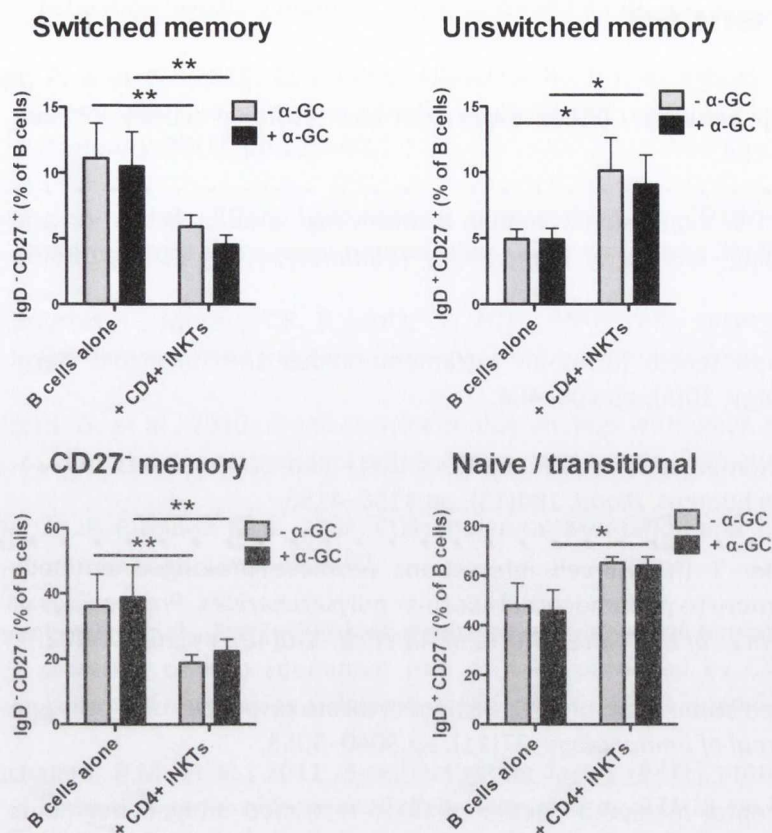


Fig. 8.2. iNKT cell influence the differentiation of allogeneic B cells. iNKT cell lines were prepared from healthy donor PBMC. CD19⁺ cells were isolated from fresh PBMC from allogeneic donors, using magnetic bead cell sorting. B cells were cultured alone (-α-GC) or with 100 ng/ml of α-GalCer (+α-GC) over night at 37°C. B cells were then cultured alone or co-cultured with iNKT cells for 7 days. B cells were then stained using mAb against IgD and CD27, and analysed by flow cytometry. Displayed are frequencies of IgD⁺CD27⁻ naïve/transitional B cells, IgD⁺CD27⁺ unswitched memory B cells, IgD⁻CD27⁺ switched memory B cells, and IgD⁻CD27⁻ CD27⁻ memory B cells, after co-culture with allogeneic iNKT cells in the absence and presence of α-GalCer (n = 4-8). Data points are plotted as mean ± SEM. Statistical analysis was carried out using Friedman test with post hoc Dunne multiple comparison tests. * P ≤ 0.05, ** P ≤ 0.01.

Chapter 9 References

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