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A study of invariant NKT cell biology in health and disease

by

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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Doctor of Philosophy

A STUDY OF INVARIANT NKT CELL BIOLOGY IN HEALTH AND DISEASE

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Invariant NKT cells (iNKT cells) have been increasingly recognised as an important cell type in regulating certain immune responses. Advances in the understanding of iNKT biology have provided opportunities for manipulating their functions for combating malignant, infective and autoimmune diseases. During the tenure of my thesis I have explored aspects of iNKT function in healthy individuals, assessed the activity of novel iNKT compounds in an ex vivo human system and evaluated this important conserved T cell subset in a human disease setting.

iNKT cells were initially evaluated in healthy individuals to explore whether there were differences in their function and development that co-associated with an enumeration that extended over a three log range. We performed phenotypic and functional assessments of ex vivo, polyclonal iNKT cells in 47 healthy donors. We established that those individuals with low numbers of iNKT cells ($<200/10^5$ T cells) displayed a different iNKT phenotype and functional profile to those with a high numbers of iNKT cells ($>500/10^5$ T cells).

Following the establishment of a robust screening platform for human iNKT responses a series of 14 novel iNKT cell ligands were designed and analysed for functional distinctiveness from the prototypic α-GC ligand. We identified ligands that had been modified in the acyl and sphingosine chains, which both dissociated iNKT proliferation from cytokine production and skewed effector responses in a Th1, Th2 or Th17 direction. We similarly identified novel compounds that were able to reduce iNKT anergy after initial activation. Finally we showed that these functional differences were modified by CD1d affinity and the nature of the antigen presenting cell, co existent cytokines and donor phenotype.

Lastly, we applied our iNKT investigative tools to a clinical disease where the link between iNKT cells and B cell help for antibody production was a possibility. We showed that in the commonest antibody deficiency syndrome, common variable immunodeficiency, iNKT were absent or functionally impaired in the majority of cases and that their number was correlated with presence of memory B cells.

Table of Contents

List of Figures and Tables

DECLARATION OF AUTHORSHIP

I, Yifang Gao

declare that the thesis entitled

A study of invariant NKT cell biology in health and disease

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
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- none of this work has been published before submission, **or** [delete as appropriate] parts of this work have been published as: [please list references]

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Abbreviations

α-GalCer - α-galactosylceramide

AP – Adaptor Protein

APC – antigen presenting cells

BAFFR – B cell activating factor of the tumour necrosis Factor Family Receptor

BCR – B Cell Receptor

BTK – Brutons Tyrosine Kinase

Con A – Concanavalin A

CTL – Cytotoxic T Lymphocytes

CVID – common variable immunodeficiency

DC –Dendritic Cells

EAE – Experimental Autoimmune Encephalomyelitis

Grb2 – Growth Factor Receptor-Bound protein 2

HD – high donor

HSV – Herpes Simplex Virus

ICOS – Inducible T cell costimulator

Ig – Immunoglobulin

IL – interleukin

iNKT cells – invariant Natural Killer T cells

IP3 - Inositol trisphosphate

ITAMs – Immunoreceptor Tyrosine-based Activation Motifs

ITIMs – Immunoreceptor Tyrosine-based Activation Motifs

KIR – Killer Immunoglobulin-like Receptors

KRN7000 – α-galactosylceramide

LD – low donor

LPS – Lipopolysaccharide

MAIT – Mucosal-associated invariant T cells

mDC – Myeloid Dendritic Cells

MHC – Major Histocompatibility Complex

MS – Multiple Sclerosis

MTP – Microsomal Triglyceride Transfer Protein

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NK cells – Natural Killer cells

NKT cells – Natural Killer T cells

PAMPs – Pathogen Associated Molecular Patterns

PBMC – Peripheral Blood Mononuclear Cell

PD – programme death

PID – Primary Immunodeficiency

PIP2 - Phosphatidylinositol 4, 5-bisphosphate

PKC - Protein kinase C

PLCγ1 - Phospholipase C γ 1

PLZF – Promeylocytic Leukemia Zinc Finger

PRR – Pattern Recognition Receptor

RAG – Recombinase-Activating genes

SAP – SLAM Associated Protein

SCID – Severe Combined Immuno Deficiency

SLAM – Signalling Lymphocytic Activation Molecules

SLE – Systemic Lupus Erythematosus

TACI – Transmembrane Activator and calcium modulating Cyclophylin ligand Interactor

TCR – T Cell Receptor

Thelp – Helper T cells

TLR – Toll Like Receptor

Tmem – Memory T cells

TNF – Tumour Necrosis Factor

Tregs – Regulatory T cells

WAS – Wiskott-Aldrich syndrome

XIAP – X-linked inhibitor of apoptosisprotein

XLA – X linked agammaglobulinaemia

XLP – X Linked Proliferative Disease

Chapter 1 Introduction

The immune system is designed to defend the host against a variety of pathogenic microbes, including bacteria, fungi and viruses. An effective immune system can protect the host against disease by identifying, targeting and eliminating such pathogens.

The immune system has developed a wide variety of immune responses from soluble factors to cellular activities. The different immune responses can be broadly defined into two types, namely innate and adaptive immune responses. The innate immune responses have a broad range of recognition, and can normally give rise to immediate responses; whereas the adaptive immune components are highly specific, usually with a delay between the exposure to antigen and the maximal response. The adaptive immune response leads to a persistent immune memory for that particular antigen. In contrast, the innate immune responses do not develop any immunological memory after repeated exposure to the foreign antigens.

The bridging of innate and adaptive immune responses is very important for establishing an effective immune response (1).

1.1 The innate immune system

Pathogenic microbes can enter the body by various ways, through many different sites. Various barriers are present that initially protects against infection. There are three main types of surface barrier including mechanical, chemical and biological (1).

Microorganisms may first encounter the epithelial barrier of the skin or those of the lungs, intestines and the genitourinary tract. In the lungs, pathogens may be mechanically removed from the respiratory tract by means of coughing and sneezing. Likewise, the respiratory and gastrointestinal tracts can secrete mucosal substances that serve as traps for invading microorganisms (2).

Biological barriers protect the host from the pathogens in the form of antimicrobial peptides. An example of such peptides include the β-defensin proteins (3). The majority of human β defensins are produced by mucosal epithelial cells, which are active against a wide range of pathogens. They have direct anti-microorganism effects and may aid the recruitment of additional innate, cellular and humoral immune responses (4). Some inflammatory mucocutaneous diseases such as Crohn's disease and atopic dermatitis may be related to abnormalities in this area of immunity and be targets for the therapeutic development of these agents (4).

Besides biological and mechanical barriers, chemical barrier may eliminate microorganisms by altering the pH of the invasion site of bacteria or limiting available food resources i.e. the availability of iron (1). Once microorganisms breach this initial line of barrier defence, invasive infectious can occur, leading to the development of disease.

The key feature of the innate immune system is its rapid reaction to pathogens once they enter the body. Failure to develop an efficient innate immune response

may lead to lethal infections, as has been demonstrated in murine and human single gene defects (5, 6). The immediate innate response is important in establishing an inflammatory environment that can direct the appropriate form of an adaptive immune response (7). For example:

1) Mycobacterial pathogens, TH1 responses and IFN-γ

The effective control of mycobacterial pathogens is mainly reliant upon adaptive Th1 responses. The initial priming of naïve T cells is particularly dependent on the innate immune response. Successful priming responses require the engulfing of mycobacterial pathogen derived antigens and their presentation by dendritic cells. This process additionally requires the recognition of mycobacterial signatures by specific toll like receptors as well as a competent IFN-γ response from the host (8).

2) Fungal pathogens, TH17 responses and IL-17

Recent studies have suggested that a specific CD4 T cell subset is important for protecting against candidiasis. These Th17 cells are notable for their production of IL-17A, which can support neutrophil recruitment. This subset of cells can be activated by APCs following contact with a number of fungal components that set up a distinctive cytokine milieu that includes IL-6, TGF-β and IL-23 (9).

3) Parasitic pathogens, TH2 responses and IgE

It has been recognized for many years that parasite infections, such as helminths, can prime naïve T cells for a Th2 response and IgE production from antigen specific B cells. It has been suggested that the priming to a Th2 response is highly dependent on the initial signal received from the parasite to the innate immune response. In contrast to the activation conditions that are typical for priming Th1 responses, optimal Dendritic Cell (DC) conditions for Th2 differentiation are correlated with down regulation of costimulatory molecules. The process of limited DC activation also leads to a reduction in Th1 type T cells in such infections.

1.1.1 Cells comprising the innate immune system

The range of cells involved in the innate immune response is broad and includes monocytes, macrophages, neutrophils, basophils, mast cells, eosinophils, dendritic cells (DC), natural killer cells (NK cells), and natural killer T cells (NKT cells). Amongst these, NK and NKT cells arise from lymphoid progenitor cells; whereas monocytes, macrophages, neutrophils, basophils, mast cells, eosinophils and dendritic cells arise from myeloid progenitor cells (7).

1.1.1.1 The professional phagocytes

The common origin of monocytes, macrophages and neutrophils suggest there might be a functional similarity between these cell types. Indeed, these cells share many characteristics including their functional categorization as professional phagocytes (10). One common feature is that all utilize defense mechanisms that involve the production of cytotoxic oxidants (11). The nicotinamide adenine dinucleotide phosphate (NADPH) complex produces reactive oxygen species following phagocytosis of pathogens in these cells. In addition, nitric oxide can combine with reactive nitrogen species produced by the phagocytes, to become highly toxic to pathogens (10). Secondly, these cell types have very similar chemokine and cytokine profiles. Neutrophils and monocytes are both recruited to the sites of infection by chemotactic agents such as interleukin-8 (IL-8). Upon engulfing the foreign pathogen, they produce similar chemokines such as CXCL1/2/3, CXCL8, CCL2, and CCL3/4 (10, 12, 13). These responses are then further amplified by the release of inflammatory cytokines such as IL-1, IL-6 and TNF-α. Thirdly, these cells all express pattern recognition receptors (PRRs); a portfolio of receptors that can identify foreign components that are distinctive to invading pathogens. PRRs include the toll like receptors (TLRs 1-9), the mannose receptor and the scavenger receptors. The PRRs recognize a limited set of patterns that are present in foreign pathogens, namely pathogen associated molecular patterns (PAMPs). These PAMPs may be on bacterial cell walls i.e. lipopolysaccharide (LPS). The TLRs may also

recognize certain intracellular features, such as motifs on DNA (i.e. TLR9 and CpG) and single stranded RNA (i.e. TLR 7,8). The recognition of PAMPs by TLRs triggers an inflammatory response that induces expression of chemokine receptors and intergrins, facilitating the recruitment of additional phagocytic cells to the site of inflammation (10).

1.1.1.2 "Killers" in the innate immune system

NK cells are another early defender, first characterized more than 20 years ago (14). They are important in viral infections, responding quickly after the initial exposure through expansion and immediate effector functions. Cytotoxic killing is undertaken through the release of perforin and production of cytokines such as IFN-γ and TNF-α (15). NK cells are also becoming recognised as pivotal in shaping the sequential adaptive response (15). Rather than undergoing somatic rearrangement of their receptors, NK cells have a limited number of activation and inhibition receptors. These recognize classical MHC class I complexes as well as non classical MHC class I molecules such as HLA-E and G (16). The killer immunoglobulin-like receptors (KIRs) recognise the classical MHC class I complexes; leading to either activation or inhibitory signals depending on the intracellular domain. The intracellular domains with a short tail contain the activation motifs (ITAMs) and those with long intracellular tails contain inhibitory motifs (ITIMs). The activation of NK cells is dependent upon the combinatorial signal received by their surface receptors, when there is an imbalance between the activation and inhibitory signals, these cells may become activated (17). NK cells can also respond to MHC class I independent signals provided by the environment, for example cytokines such as IL-7 and IL-15, mediating NK responses (18).

In humans, there are two major NK cells subsets; defined with the phenotypic markers CD56 and CD16. These different NK cell subsets have different functions aligned to their $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ status (19). The majority of the NK cells present in the peripheral blood and spleen are

$CD56^{dim}CD16^{+}$. This population of NK cells is known to express perforin and exhibit potent cytotoxicity. The $CD56^{bright}CD16^{-}$ population is known to lack perforin but possess marked cytokine production (20).

More recently, NK cells have been purported to acquire a 'memory' function as an innate effector cell. A recent study showed that adaptive transfer of phenotypically similar pre-activated or control NK cells (including CD69, CD11b and B220 expression) into RAG knockout mice led to significant differences in IFN-γ production. It was noted that upon re-stimulation, pre-activated NK cells showed a two times higher IFN-γ production compared to their counterpart control. The study demonstrated that the NK "memory" lasted a minimum of three weeks (21, 22). This "memory" was considered to be long standing since the half-life of the NK cells is less than three weeks (23).

The cytokine portfolio of NK cells has also been expanded recently with the identification that they are also programmed to produce Th17 type cytokines, i.e. IL-22. The IL-22 cytokine production in human NK cells is activated by IL-23. This type of NK cell has now been named NK-22, mainly $CD3^{-}CD56^{bright}$ and residing around mucosal sites (24). This subset expresses several Th17 expression factors including RORγ and RORα (25, 26) and is derived from cells expressing CCR6 (26).

1.2 Adaptive Immune system

Unlike innate immune responses, the adaptive immune system is more specific and finely-tuned. B cells and T cells are the key players in the adaptive immune system and memory cells will typically develop after the first exposure to the foreign antigen (1).

The adaptive immune system is thought to have first arisen in jawed vertebrates. Compared to its evolutionary older innate counterpart it is more specialized in function and responsiveness. It generally builds upon the conserved innate immune system of vertebrates and non-vertebrates to provide a more robust, finely tuned immune response that possesses transferable memory for offspring protection and neutralising herd immunity (1). The distinguishing features of the adaptive immune system are:

1) Cooperation with the innate system, to generate functionally specialised immune responses that are best suited to nature of the invading pathogen, often leading to sterilising immunity (1).

2) Development of immunological memory that provides a quicker and enhanced response to future infections as well as offering protection to offspring (1).

1.2.1 T cell development and activation

T cells originate from bone marrow and move to thymus for development and maturation (27). IL-7 is the primary cytokine that commits T cells precursors to further differentiation, facilitating the early expansion of thymocytes by signalling through the common γ chain (27). In humans, mutations in this common γ chain lead to X-linked severe combined immunodeficiency with an absence of T and NK cells (28). The expression of Notch-1 is an additional key step in committing lymphoid precursors to T cell lineage development. It has

been proposed that Notch is essential for the early development of T cells, whilst in the later stages it plays a role in CD4/CD8 lineage choice. The current model of early T cell development, suggests that the Notch complex binds to a transcriptional regulator CSL. Upon binding, Notch may then bind to Deltex and the IKK complex (29, 30). Following the expression of these early signalling molecules, the pre-T cells subsequently undergo TCR gene rearrangement (31).

The TCR locus contains variable region (V), diversity region (D) and joining region (J) genes. All the TCR loci contain the V and J region, but the D region is only present in the β and δ TCR loci. The V, D and J regions are randomly arranged together by the recombines-activating genes 1 and 2 (RAG1/RAG2), which initiate the rearrangement process. The VDJ random rearrangement allows various combinations to occur contributing to the great variability of TCR antigen specificity. Successful rearrangement of two TCR genes with either an αβ or γδ TCR configuration marks the end of the pre-T cell stage and the beginning of double positive T cell stage. These T cells now express both CD4 and CD8 co-receptors together with a fully functional CD3 receptor with γ, δ, ε, and ζ chain components (32, 33).

The double positive T cells then further differentiate to single positive T cells through positive and negative selection, guided by MHC I and II affinities. The T cells are positively selected when the TCR binds to the MHC complex with low avidity. A high avidity of TCR and MHC binding, indicating potential autoreactivity, will lead to negative selection i.e. deletion of the T cell clone. If the double positive T cells have successfully passed through both positive selection and negative selection, those restricted to MHC class I will become CD8 T cells. Those restricted to MHC class II will become CD4 positive T cells (33). Such mature T cells then leave the thymus to circulate as naïve T cells.

T cells become fully activated upon interaction of their TCR with the MHC/antigenic peptide complex in association with CD4/CD8 co-receptor

ligation. CD8 positive T cells will engage with MHC class I complexes (HLA-A, B and C) bearing short peptides (9-11 amino acids), whereas CD4 positive T cells will recognize MHC class II (HLA-DR, DQ and DP) complexes with longer peptides. The aggregation of the TCR and co-receptor complex at the immunological synapse leads to the recruitment of early signalling molecules for T cell activation. Lck is recruited early by the co-receptor complex and subsequently activates a further tyrosine kinase, ZAP-70, which binds to the tyrosine-based activation motifs of the CD3 chains. This leads to the downstream phosphorylation of several other signalling molecules including Lymphocyte cytosolic protein 2 (SLP-76), Phospholipase C γ 1 (PLCγ1), Phosphatidylinositol 4, 5-bisphosphate (PIP2) and Inositol trisphosphate (IP3). These ultimately lead to Protein kinase C (PKC) activation (34, 35). The PKC further activates transcription factors AP2 and Nfka, leading to the production of cytokines (35).

It has been long recognized that calcium signalling is important for T cell maturation. Studies have shown that failure to convert from double positive T cells to single positive T cells was observed in the presence of calcineurin inhibitors. Studies have also found that defects in the catalytic subunit, regulatory subunit as well as early signalling molecules such as NFATc3 and Bcl-2 may restrict double positive T cell development (36-39). The calcium signalling is orchestrated by the binding of PI3 to the calcium store membrane, and this allows the release of calcium from endoplasmic reticulum to cytoplasm by a process called store depletion. This allows the opening of the cell membrane calcium channel. The activated store operated calcium channel is the main source of calcium influx in lymphocyte development. The opening of the calcium channel allows further signalling transduction pathways to occur (40).

The production of cytokines and calcium dependent processes allows naïve cells to initially proliferate and then differentiate to activate phenotypes. These phenotypes are shaped by the innate system to allow a T cell response to be appropriate for the activating pathogen.

1.2.2 Population of T cells

The classical description of T cells in humans comprises $CD4^+$, $CD8^+$ and double-negative cells within both TCR αβ and γδ compartments.

$CD4^+$ T cells are the most abundant T cells present in human, performing many helper functions to both $CD8^+$ T cells and B cells, hence the name Thelper (Th). Depending on the activating conditions and environment, Th cells can produce a wide range of cytokines. Initially, two different types of helper cells were identified 20 years ago, namely Th1 and Th2 (41). These were characterized by their mutually exclusive cytokine production with IFN-γ in Th1 and IL-4 in Th2 cells. We now recognize at least five different types of Th cells broadly characterized by their predominant cytokine profiles and functional associations (42-45). The different subsets of Th cells are summarized in Fig 1.1.

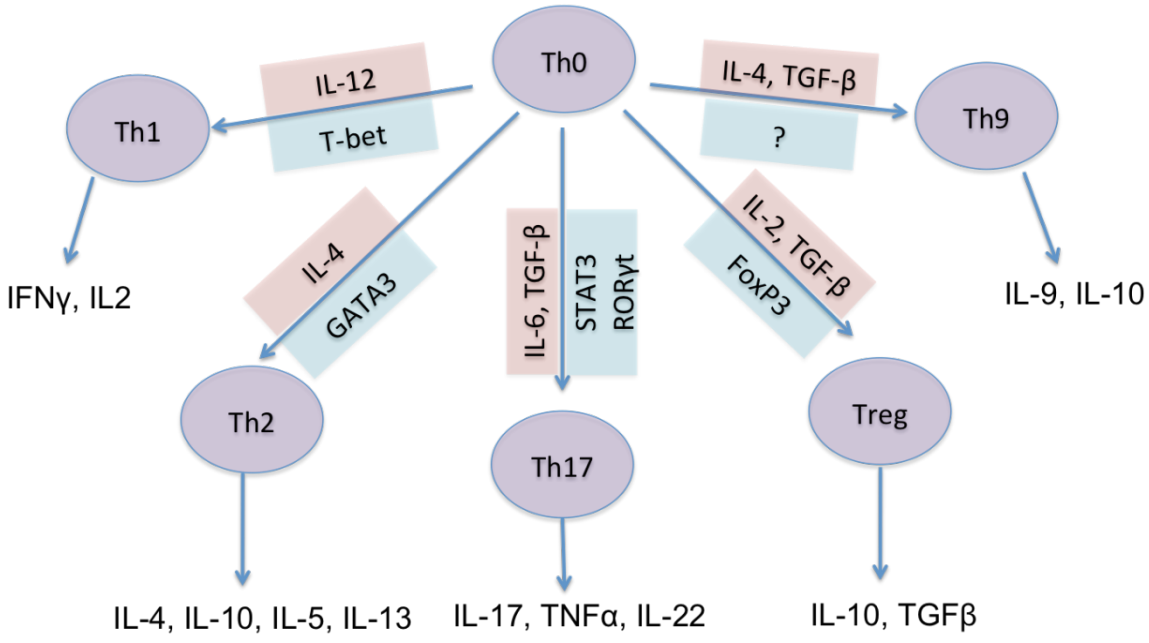


Figure 1.1 Major CD4 T cell subsets. T cell subsets are indicated as purple, the key transcription factors for driving the T cell differentiation are indicated as blue, the differentiating cytokines are indicated as pink and the effector cytokines produced by the T cell subset are indicated as black.

Th1, Th2 and Th17 cells secrete different cytokines and mediate different types of responses. Th1 type cells mainly produce pro-inflammatory cytokines such as IFN-γ, TNF-α and lymphotoxin. These cytokines are important for mediating inflammatory responses and promoting the activation of CD8 cytotoxic T cells. In contrast, Th2 type cells produce IL-4, IL-5, IL-10 and IL-13. These cytokines are regulatory cytokines and contribute significantly to development of the humoral immune response. Th17 can secrete IL-17, IL-21, IL-22 and IFN-γ. These cytokines are important inflammatory cytokines, and appear to have a major role in many autoimmune diseases (46).

Another subset of $CD4^+$ T cells is the $CD4^+CD25^+$ regulatory T cell (Treg). Treg cells are an auto-reactive T cell subset that can regulate and suppress host immune responses. This subset of $CD4^+$ T cells expresses the critical transcription factor Foxp3. Treg cells regulate the immune responses by a variety of mechanisms including the release of suppressive cytokines such as IL-10 and TGF-β (47). These cytokines are additional to other mechanisms of suppression including cell contact mediated inhibition (i.e. CTLA-4) that can suppress effector CD4 and CD8 populations (48).

IL-9 producing Th9 cells are a newly discovered cell type (44), described two years ago by two independent groups (45, 49). The production of IL-9 by CD4+ T cells was described more than 10 years ago (50). Until recently, the IL-9 producing cells have not been recognized as an unique subset due to the fact that they do not share a unique cytokine signature compared to other T cell counterparts, as summarized in Figure 1.1. The exact function of Th9 T cells is currently unknown. Early studies have suggested they might be involved in the defence against helminth infection (51). Recent studies have shown that Th1 type cytokines are inhibitory towards the development of Th9 cells; whilst the presence of IL-21 promotes the differentiation of this Th subtype (52).

$CD8^+$ T cells are the 2nd major subset of T cells, often termed cytotoxic T cells (CTL) secondary to their primary role as an effector cytotoxic T cell to MHC class I restricted targets. The CTL kills target cells through cell-cell contact following the recognition of foreign peptides presented by MHC class I molecules, and the release of perforin and granzymes that activate an apoptosis programme in the target cell. CTL may also release cytokines such as IFN-γ, and this can lead to inhibition of viral production and increase the activation of other cell types involved in host defence (33).

Double negative T cells are not as abundant as $CD4^+$ and $CD8^+$ T cells, however, they too posses very important functions. The major cell types include γδ T cells as well as certain subtypes of αβ T cells, including iNKT cells and mucosal-associated invariant T (MAIT) cells. γδ T cells are represented at approximately 1/10th the number of αβ T cells. These cells may be selected upon peptide loaded MHC class I or II molecules or upon Group 1 CD1 molecules (53). MAIT cells are distinguished by having an invariant Vα chain and a restricted Vβ chain. These T cells are selected upon a distinctive MHC class I like molecule termed MR1 and require the presence of peripheral B cells for full maturation/survival. Invariant NKT cells are discussed in detail in a following section.

1.2.3 B cell development and activation

Unlike T cells, which originate in the bone marrow and develop in the thymus, B cells develop in the same place as they originate i.e. bone marrow. Several non-redundant transcription factors are necessary to ensure that the development of B cells is under tight control. These include zinc finger family molecules IKAROS, early B cell factor 1 and interferon regulatory factor 8 (54, 55). The gene rearrangement of B cells is very similar to the TCR rearrangement. The heavy chain consists of four segments including VH, D, JH and CH; the light chain consists of variable, joining and constant domains of either kappa or lambda loci. Successful rearrangement of heavy and light variable gene segments leads to the

formation of a surface immunoglobulin. Immature B cells that reside in the BM and can be further divided into Pro, Pre and Transitional subsets. IgM is the first antigen detectable in the early B cell subset. It can be detected in the cytoplasm as early as the pre-B cell stage. Surface expression of such subtype can be identified in the late immature B cell stage. IgD is expressed at the mature B cell stage marking the mature, naïve step in development (56). B cells move out the bone marrow in their immature stage, which can be identified as $IgM^{+}IgD^{-}CD27^{-}$. The development of immature B cells to mature B cells is a antigen-independent process (57).

1.2.4 Populations of B cells

In mice, CD5 has been a very useful tool to identify sub populations of mature B cells. It was found that the population of $CD5^{+}$ B cells, namely B1 B cells; tend not to undergo somatic hypermutation and to largely respond to T cell independent antigens (58). The $CD5^{-}$ B cell subset is considered to represent conventional B cells (B2). Although CD5 is expressed on some B cells in man, this approach has not been found to be as useful as in mice (58).

In humans, there are several subsets of B cells that have been identified based on the cell surface expression of certain phenotypic markers. The subsets of B cells broadly include, $IgM^{+}IgD^{-}CD27^{-}$ - immature, $IgM^{+}IgD^{+}CD27^{-}$ naïve, $IgM^{+}IgD^{+}CD27^{+}$ marginal zone memory, $IgM^{-}IgD^{-}CD27^{+}$ switched memory (germinal center), $CD38^{high}IgM^{high}$ transitional/activated and $CD27^{low}$ $CD38^{high}IgM^{-}$plasmablasts (33).

Naïve B cells are present in the secondary lymphoid organs and circulate in peripheral blood. Marginal zone memory B cells are thought to be important in responding to polysaccharide antigens and may form short-lived plasmablasts that produce IgM. B cells that have been activated within the germinal centre undergo somatic hypermutation and class switching to become memory cells, plasmablasts and eventually plasma cells. Plasma cells lose expression of surface

immunoglobulin and home to the bone marrow where they remain as long-lived memory B cells producing high affinity antibody (33). There are nine different types of immunoglobulins based on the isotypes of heavy chain; they are IgM, IgD, IgG1-4, IgA1-2 and IgE) (33).

1.3 Bridging innate and adaptive immunity

For effective immune function, 'cross talk' between innate and adaptive immunity is extremely important. It is essential that the adaptive immune system is suitably informed by the innate immune system regarding the most appropriate effector response (i.e. Th1>Th1 or IgA>IgE). Whilst many innate cells may interact with pathogens, dendritic cells are uniquely placed in being able to capture antigens, contribute to the inflammatory milieu and present the foreign material to the naïve T cells within secondary lymphoid organs. Furthermore, they can provide the requisite co-stimulatory signals to naïve T cells to instruct them for further division and differentiation. For these reasons they have been considered as the most important professional antigen presenting cell linking innate and adaptive immunity.

1.3.1 The master of directing – dendritic cells

The term "Dendritic Cell" was first introduced in 1973 by Ralph M. Steinman and Z.A. Cohn (59, 60). Soon after the initial description of dendritic cells, they were then identified in lymphoid and some non-lymphoid tissues i.e. skin (61). Dendritic cells (DCs) have since become further subtyped either according maturational status i.e. immature or mature or origin i.e. myeloid or plasmacytoid (62, 63). Dendritic cells are also distinguished from other cells by their high expression of adhesion molecules, which include CD11c, ICAM-1, 2 and 3 and CD11a, and co-stimulatory molecules i.e. CD80/CD86 (64, 65). In mice, myeloid DCs cells can be further classified to several other 'subclasses' i.e. $CD8^{+}CD4^{-}$, $CD8^{-}CD4^{-}$ and $CD8^{-}CD4^{+}$ DCs. These phenotypic divisions align with functional differences such that $CD8^{+}$ dendritic cells are thought to be responsible for cross presentation of cellular antigens, for cross-priming and cross-tolerance. In contrast, $CD4^{+}$ dendritic cells do not cross present as well as $CD8^{+}$ dendritic cells, but they are the most abundant cell type in the spleen (66).

Dendritic cells may act as an initial bridge between innate and adaptive immunity due to their residence in most organs and their pathogen recognition receptor portfolio allowing them to respond to most "danger signals" (67). Dendritic cells are able to secrete a variety of cytokines that influence naïve T cell development including IL-2, IFN-γ, IL-12 and IL-4 (68). The different types of dendritic cells have also been found to have different functions in Th1/Th2/Th17 priming. $CD8^{+}CD4$ DC have been noted to promote Th1 responses whilst $CD8^{-}CD4^{-}$ DC promote Th2 responses (Figure 1.2).

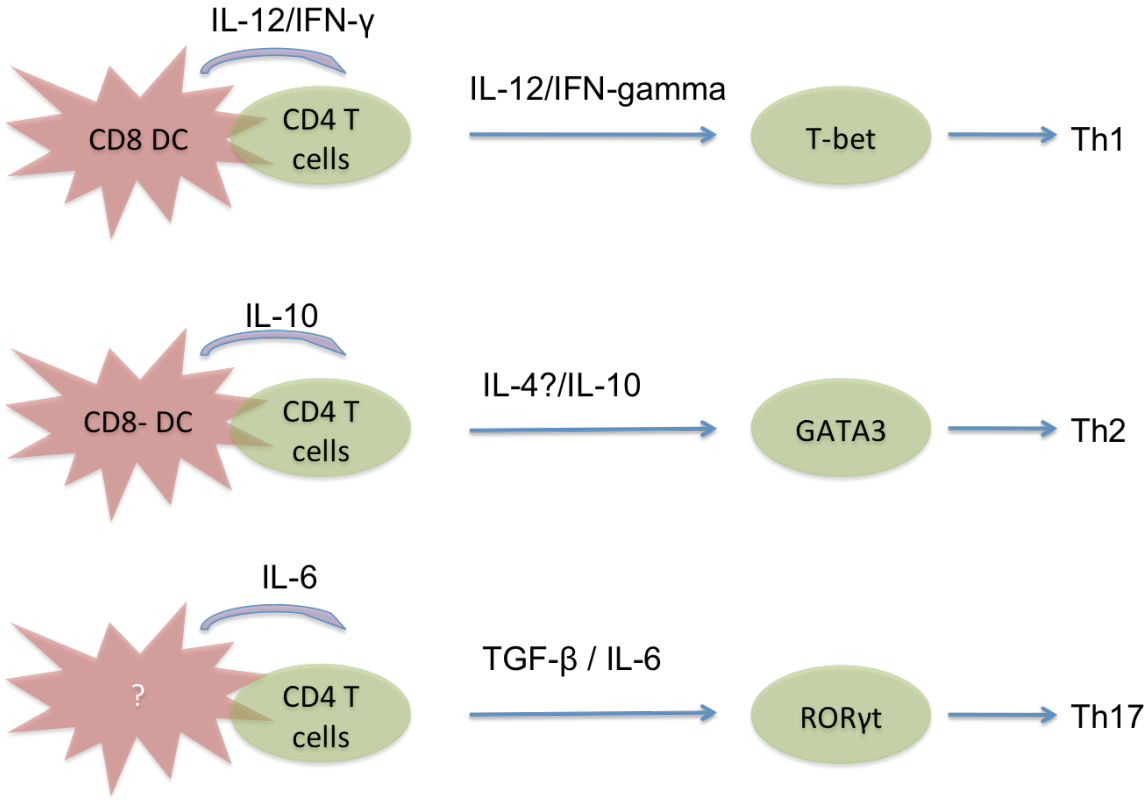


Figure 1.2. Subsets of dendritic cells and cytokine production. Adapted from (68).

In addition to MHC class I and class II presentation, DC's may present antigens upon CD1d. The glycolipid antigen cargo of CD1d is presented to a subtype of T cells termed NKT cells. Recent studies have suggested that cooperative activity between NKT cells and dendritic cells is central to an effective immune responses (69).

1.3.2 Natural Killer T cells

Natural Killer T cells (NKT) are a subpopulation of T cells. The name "Natural Killer T cells" was first used in 1995 (70) and was initially derived from murine studies through the analysis of T cells bearing the NK cell marker NK 1.1. The terminology has been further complicated by the description of other T cells subsets as NKT cells when cell surface antigens such as CD56 and CD57 are seen on such cells. These T cells with NK markers ('TNK') are fundamentally different as they are restricted by conventional MHC class I and II molecules and have a variable TCR αβ usage (71, 72). NKT cells recognize glycolipids presented by a MHC class I like molecule, CD1d. Recently a new classification has been proposed and this considers at least three different subsets of NKT cells. These three subsets of cells are Type I classical NKT cells (also sometimes referred to as invariant NKT cells), Type II Non-Classical NKT cells and NKT like CD1d-independent T cells (72) (Table 1.1).

	Type I NKT cells	Type II NKT cells	NKT like cells
Restriction	CD1d	CD1d	MHC / others
T cell receptor expression	Vα14-Jα18 (mice) Vα24-Jα18(human)	Diverse	Diverse
CD161 expression	Yes (resting mature) No (immature or post-activation)	Yes/no	Yes
α–GalCer reactivity	Yes	No	No
Subsets	$CD4^+$, DN and $CD8^+$	$CD4^+$, DN	$CD4^+$, DN and $CD8^+$
Major Cytokine produced	IL-4, IFN-γ	IL-4, IFN-γ	IFN-γ

Table 1.1 NKT subset classfication

1.4 The glycolipid responsive immune system.

1.4.1 The extended CD1 family

The CD1 family represent a group of antigen presentation molecules that unlike the peptide accommodating MHC class I and II complexes, accept lipid antigens. This family of molecules were first detected by monoclonal antibodies that recognized these structures on human thymocytes (73). Historically these were the first immune molecules to be named within the cluster differentiation system (74). Two groups of CD1 molecules are identified in humans. Group 1 CD1 molecules include CD1a, CD1b, CD1c and CD1e; the only group 2 CD1 molecule is CD1d. In contrast to the human system, the genes encoded for group 1 CD1 molecules are deleted in the mouse, hence, only CD1d is present in the mouse CD1 family (74). CD1 family members are type I integral membrane proteins with α1, α2, and α3 domains (75, 76). In contrast to MHC complexes the CD1 family members are non polymorphic (77). More than 200 donors were sequenced from a wide range of ethnic groups showing that CD1b and CD1c possessed silent mutations across all the individuals, and only two CD1a, CD1c and CD1d alleles were seen secondary to amino acid changes (78, 79). Further studies have suggested that these few allelic forms do not contribute to a greater susceptibility to infection (80).

1.4.2 Group 1 CD1

The group 1 CD1 molecules are found on professional antigen presenting cells including dendritic cells and B cells. In contrast to MHC class I and II complexes they are not expressed on circulating monocytes, unless activated to become a DC. This makes them an excellent marker for the differentiation of DC (74, 81). CD1a and CD1b have been used widely to identify monocyte derived dendritic cells (82, 83), $CD34^{+}$ precursor dendritic cells (84, 85), and also Langerhans cells (86). The expression of group 1 CD1 molecules on dendritic cells, further supports the key role of DCs as the professional antigen presenting cell bridging

innate and adaptive immunity. Interestingly, besides the phenotypic differences, CD1a has been found to be an important functional marker. Several studies have identified that in DC populations, when CD1a is deficient, there will be a reduction in IL-12 production, and an upregulation of IL-10 production compared to their $CD1a^{+}$ counterpart (87-89). Studies have also found that in plasmacytoid DC, unlike myeloid DC and monocyte derived DC described above, there is no expression of CD1a, CD1b or CD1c (90). CD1c has also been found to be expressed on certain subsets of B cells, both circulating (91) and in the marginal zones of the spleen (92).

αβ T cells represent the majority of T cells that recognize the group 1 family (83, 93), with some CD1 reactive γδ T cells also recognized (94-96). Sequence analysis from these αβ CD1 reactive T cells has identified a high level of diversity in the V-J junction and template independent N nucleotides. Currently, no crystal structure of a group 1 CD1 antigen complex has been resolved, thus the interaction between CD1 and the TCR are currently predicted through mutagenesis studies on the TCRβ CDR3 and CDR1 loops (97, 98). These studies suggest that the CD1 reactive T cell receptors dock diagonally, similar to that for MHC class I and Class II complexes. For the subset of γδ CD1 reactive T cells, most were found to be CD1c reactive, with a Vδ1 predominant usage (99).

1.4.2.1 Lipid recognition by group 1 CD1 molecules

The first member of the group to be shown to recognize lipid antigen was CD1b, presenting a lipid antigen, myolic acid, from the cell wall of *M.tuberculosis* (100). A selection of subsequently described group 1 CD1 antigens is shown below.

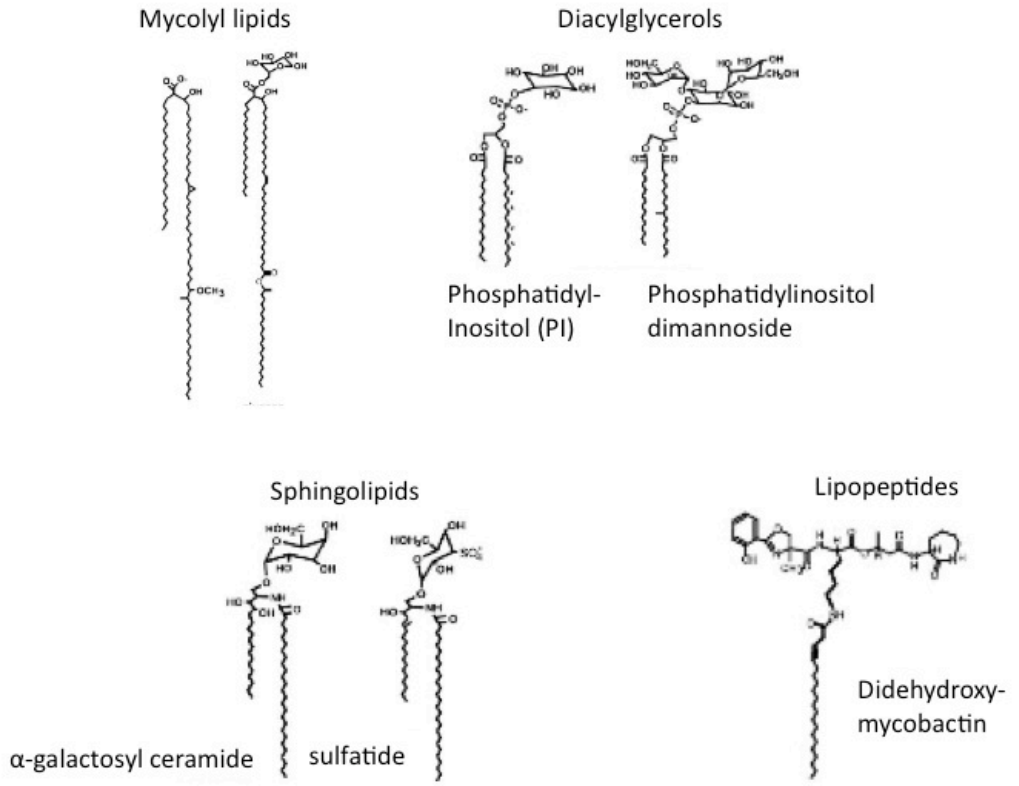


Figure 1.3. Lipids recognized by CD1 molecules. Adapted from (81). Lipids recognize by CD1a, CD1b, CD1c and CD1d.

As shown above, the main group of lipids recognized by group 1 CD1 molecules include mycolic acids, mycobactin, and acylated sulfoglycolipids (81). Many of these lipids are only present at low levels in humans but are critical in pathogens. For example, mycobactin is essential for bacterial survival due to its iron-scavenging properties (81) with its precursor (DDM) being presented and recognized by CD1a (101). This promotes CD1a restricted cytotoxic responses resulting in the killing of the infected cells (102, 103). Lipoarabinomannan (LAM) is another lipid, which plays a role in microbial pathogenesis; maintaining the structure of the microbial membrane and promoting cell binding to enable cell infection. This lipid is presented by CD1a and leads to antigen specific cytotoxicity.

Additional components of the mycobacterial wall can also be recognized by the other CD1 members, such as CD1b restricted Ac_2SGL (104) and CD1c restricted Hexoysl-1-phosphoisoprenoid lipids (105).

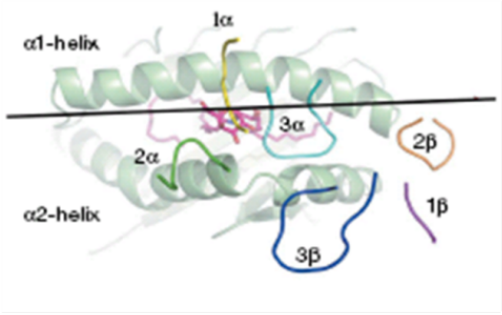
1.4.3 Group 2 CD1 family

CD1d is the only member of the group 2 CD1 family and it is the only CD1 family member present in mice. Compared to CD1a, b and c, the expression of CD1d is considerably boarder. It is expressed on most professional antigen presenting cells including monocytes, macrophages, dendritic cells and B cells; in addition, it is also present on certain non-lymphoid cells such as epithelial cells, parenchymal cells as well as smooth muscle cells in the gut and liver (106, 107). It was also found to be highly expressed on double positive thymocytes in the thymus, playing a crucial role in CD1d restricted T cell selection.

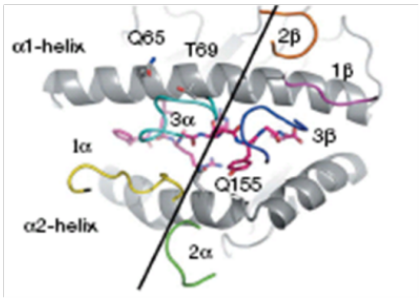
Unlike MHC restricted T cells, invariant NKT cells do not engage with conventional MHC antigen presenting pathways, but recognise the 'MHC class I like molecule' CD1d. As a 'class I like molecule', CD1d has a similarity to MHC class I molecules in terms of protein sequence homology, domain organisation and association with β_2m (108). However, distinctive differences are also observed which makes CD1d only distantly related to MHC Class I molecules. Based on the crystal structure of CD1d, several differences to MHC class I molecules were found. Firstly, the binding groove of CD1d is significantly narrower compared to MHC Class I molecules. Secondly, the docking mode of the TCR to CD1d-glycolipid complex is parallel to CD1d; whilst the TCR is diagonally docked to MHC-Class I molecules (Figure 1.4) (109, 110).

(a)

CD1



MHC class I molecule



b

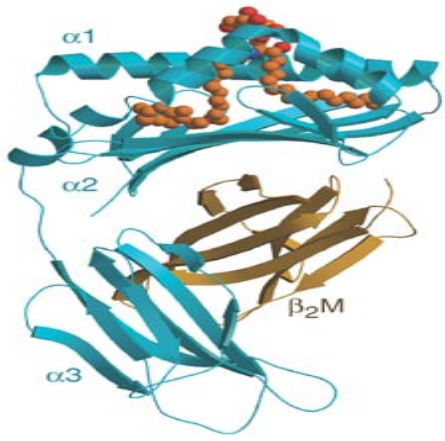


Figure 1.4. Crystal structures of CD1d and MHC class I molecules with TCR docking alignment. Adapted from (109, 110)

(a) TCR docking of CD1d and MHC class I (b) Ribbon diagram of CD1d

CD1d complexes present glycolipid antigens such as the marine sponge derivative α-galactosylceramide (α-GalCer), which has become the archetypal

CD1d ligand for iNKT activation. This was initially identified through an anti-tumour compound screening programme. This revealed the potential of CD1d in anti-tumour responses (111). The glycolipid contains a ceramide with an 18 carbon phytosphingosine chain and a 26 carbon acyl chain. An α-linkage links the two chains to the galactose head. The glycosidic bond found in the marine sponge α-galactosylceramide is not naturally present in mammalian sphingolipids (112). Other than α-galactosylceramide, bacterial glycosphingolipids can also be presented by CD1d including α-glucuronosyl-ceramide and α-galacturonosyl-ceramide (113-115). Most of these glycosphingolipids specifically stimulate NKT cells. Two other glycosphingolipids, namely ganglioside (GD3) and sulphatide, can also be recognized by CD1d and stimulate type II CD1d restricted T cells (116).

CD1d may also present immunogenic lipids of plant origin. Examples of these lipids including phosphatidylcholine and phosphatidylethanolamine, which are also commonly recognized by the group1 CD1 family (112). Many attempts have been undertaken to find the self lipid for CD1d. Several lipids were claimed to be the self lipids for CD1d, including lysophospholipids and sphingomyelin (117). A study by Bendelac's group identified a self lipid that was reactive to iNKT cells in mice, namely iGb3 (118). Interestingly, subsequent studies have failed to identify this ligand, even by mass spectrometry in lymphoid tissue (119).Of additional note was the recent observation of normal iNKT cells in iGb3 synthase deficient mice and the finding that the gene encoding for iGb3 synthase in humans, was not functional (120). Currently, there is no definitive evidence for the identity of the endogenous self lipid proven to be involved in the thymic selection of CD1d restricted T cells.

1.4.4 Processing the fat

Lipids are highly insoluble in water, necessitating the use of carrier proteins that allow them to traffic in the extracellular and intracellular compartments. Lipoproteins are the main elements involved in the presentation of the lipid antigens, with ApoE being identified as important for facilitating the uptake and presentation of lipid antigens for antigen presenting cells (121).

CD1d molecules emerge from the endoplasmic reticulum as a heterodimer destined for the cell surface, where they will be internalized through the adaptor proteins AP-2/AP-3 in the clathrin-dependent pathway. The newly synthesised CD1d molecule is likely associated with a self-lipid during this early trafficking, permitting access to the late lysosome/endosomal compartments. In this compartment, a dedicated chaperone protein, saposin is present which can facilitate lipid exchange. Repeated travel between the plasma membrane and lysosomal/endosomal compartments allows continuous lipid exchange (122). Additional lipid exchange proteins have been identified more recently, including the microsomal triglyceride transfer protein (MTP). Deletion of MTP has led to a down-regulation of CD1d on the cell surface, secondary to its important role in loading lipids onto CD1d in the ER (123).

In the case of exogenous lipids, it was suggested that they enter cells together with the very low density lipid when bound to lipoproteins (122). Foreign microbial lipids may also fuse with the membrane of APC, prior to transportation to other compartments. The trafficking ability is highly dependent upon the polarity, size and physical properties of the lipids (112). Studies have also shown that bacterial lipid antigens can form antigenic complexes with CD1d following traffic through the lysosomes (124) (112).

1.4.5 Factors influencing lipid antigen presentation

Infection is one factor that can influence lipid antigen presentation. One example is infection by Herpes simplex virus-1 (HSV-1), which actively subverts CD1d presentation through an interference with the recycling of CD1d through the endosome (125). In contrast some infections i.e. *salmonella typhimurium* (112) can upregulate CD1d expression on myeloid dendritic cells through the activation of TLR or production of cytokines i.e. interferon-γ. A similar upregulation is also seen when myeloid dendritic cells are recruited to the site of inflammation (126). Alternatively bacterial infections may cause changes in self-lipid metabolism by means of increasing the synthesis of self-glycosphingolipids. This allows CD1 restricted T cells to recognize the infected APC and generate an effective immune response against the intracellular pathogen (127).

One of the major differences in terms of the classical presentation pathway of MHC class II molecules compared to CD1 molecules is the timing of presentation. MHC class II molecules will rapidly traffic to the plasma membrane from the phagolysosomes for loading only upon infection by foreign pathogens. On the contrary, CD1 molecules are continually recycling between the different compartments, which allows for a foolproof mechanism to be immediately available after the infection of a foreign pathogen (112).

1.5 Invariant NKT cells

Type 1 invariant NKT cells are the most well characterised CD1d restricted T cell. They develop in the thymus, and are present at a very low number in most tissues. In human peripheral blood, approximately 0.01-1% of the T lymphocytes are iNKT, characterised by their hallmark TCR- invariant chain Vα24 – Jα18 and variant Vβ11 (128). Besides the expression of the TCR invariant chains, expression of CD161 is also found on most of the resting and mature invariant NKT cells, but it is not seen on immature or post-activated cells (72).

A few different approaches were initially used to identify invariant NKT cells following their original description. The first approach was to sequence a set of mouse hybridomas, which were suggested to be suppressor T cells (129-131). The sequencing results identified a conserved Vα14-Jα18 TCR (129-131). Subsequent studies identified a subset of double negative Vβ8 T cells with the same Vα14-Jα18 sequence (132, 133). Lastly, the conserved Vα24-Jα18 double negative T cells were identified in human in 1994 (134). Following the identification of the CD1d-iNKT ligand α-GC, these cells have attracted increased attention.

iNKT cells were subsequently found to be highly conserved in terms of TCR rearrangement in both human and mice. In mice, the majority of such cells are Vα14-Jα18 paired with Vβ8, 7 or 2. A more conserved rearrangement was found in humans, which were Vα24-Jα18 with Vβ11. Both human and mouse iNKT cells react with α-GC (135).

Unlike other T cells, iNKT cells were considered to be “memory/activated/non naive” when they emerge from the thymus. In mice, these reside in different

lymphoid tissues including the spleen, lymph nodes and liver. The distribution of iNKT cells in human is unclear, but they are found to be 10 times less common compared to murine studies (122).

1.5.1 Invariant NKT cell development

1.5.1.1 Positive selection of invariant NKT cells

The generation of the correct CD1d restricted, semi-invariant TCR (Vα24Vβ11 in human; Jα18 in mouse) is the crucial factor for the development of iNKT cells. It was shown in early studies that complete absence of iNKT cells was found in mice deficient in either RAG or the Jα18 gene segment (136). A similar finding was also shown in patients with Omenn's syndrome, which is caused by hypomorphic mutations of the RAG2 gene (137, 138). Similar to other TCRs, the semi-invariant TCR is generated by the random recombination of the VDJ region (135). Even though the process of VDJ rearrangement is a random selection process, the likelihood of selection of individual TCR genes is highly dependent on the prolonged lifespan of double positive thymocytes (139). Due to this, genes responsible for double positive thymocyte survival (i.e. RoRγt and Bcl-xL) are crucial for the development of iNKT cells. Targeted deletion of these genes again results in diminished iNKT cell numbers (140), whilst overexpression of these genes significantly increases the number of iNKT cells (141).

Following the assembly of the invariant TCR within a double positive thymocyte population, the pre iNKT cell requires a subsequent interaction with CD1d for full development (135). A complete absence of invariant NKT cells is observed in CD1d knockout mice (72). Unlike MHC restricted T cells where thymic epithelial cells are important for selection, iNKT are selected upon CD1d expressed by other thymocytes (142). Self-antigen is also required for their development; and early studies have identified the glycosphingolipid iGb3 as

important in mice (118). However, the equivalent of iGb3 in humans is yet to be identified.

1.5.1.2 Negative selection of invariant NKT cells

Negative selection is a physiological process in the development of T cells that eliminates self-reactive lymphocytes with excessive self ligand reactivity. From the viewpoint of invariant NKT cell development, they emerge from the thymus as post naïve cells (memory or activated), with self ligand reactivity. However, these self-reactive cells are found to be in the resting state in vivo (72, 122). This may suggest that due to the random TCR rearrangement and the diversity of their TCR in the β chain, these cells have undergone a negative selection process sufficient enough for a partial activation phenotype. Several studies have shown that invariant NKT cells do undergo such a negative selection process (135, 143, 144) and that in the presence of high affinity lipid, such as α-GC (144) or over-expression of CD1d in dendritic cells (143), a reduction in the number of iNKT cells is found. Interestingly, when α-GC in used in the above context it was found that the emergent iNKT cells had a bias to Vβ2 usage, which has the lowest affinity for CD1d-α-GC (144). Other studies have shown that mice engineered to only express CD1d on thymocytes appear to bypass negative selection, but are hyper-responsive to α-GC in the periphery (142). This suggests that negative selection of iNKT cells is not principally undertaken on thymocytes, but most occur on other CD1d expressing thymic cells.

1.5.1.3The regulators

SLAM and SAP

In conventional T cells, thymic epithelial cells are responsible for providing the signals for their early development. In contrast to this, thymocytes are believed to be the important cells in the selection and development of iNKT cells, with signalling via lymphocytic activation molecule (SLAM) family member proteins being crucial. The SLAM family member proteins have also shown importance

in Th2 cell priming, the generation of memory B cells, as well as NK activation, MHC-independent NK cell inhibition and iNKT cell development (145).

The SLAM family members include SLAM1, 2B4, CD84, NTBA, Ly9, CD319, SLAM8 and SLAM9 (135, 145). Most of these receptors have two immunoglobulin-like extracellular domains; with the exception of Ly9 which has four such domains (145). Within the family, 2B4 is the only member that does not undergo self-association through its extracellular domain but recognizes a glycosyl phophatidyl inositol anchored cell surface protein CD48, which is widely expressed on immune cells (146). SLAM1 and NTBA are expressed by thymocytes suggesting a role in regulating iNKT cell development (147).

The crucial step played by SLAM proteins in T cell development is their interaction with the SLAM associated proteins (SAP) family, through tyrosine-based motifs present in both the cytoplasmic region of SLAM receptor and the SH2 domain of SAP receptor (148). iNKT cells are uniquely dependent on this compared to other T cell subsets (135). Following a SAP:SLAM engagement phosphorylation events allow the recruitment of FYN and Lck to the TCR complex (149). Both murine and human studies have proven that iNKT cells are highly dependent on signals provided by the SLAM proteins. Mice that lack SAP have shown an absence of iNKT cells, whereas no effect was found in conventional T cells. In agreement with this, a similar absence of iNKT cells has been identified in patients with X linked proliferative disease (XLP) secondary to an absence of the SAP gene (150-152).

A summary of our current understanding of early iNKT signalling events is shown in Figure1.5. Following a productive, CD1d/TCR and SLAM/SLAM interaction a series of key transcription factors are triggered. The early transcription factors initiate three independent but related pathways. The

recruitment of PKC initiates the NF-κB pathway, further ligation of RasGAP leads mitogen-activated protein kinase pathway signalling whilst PLCγ supports the calcium-flux pathway (135).

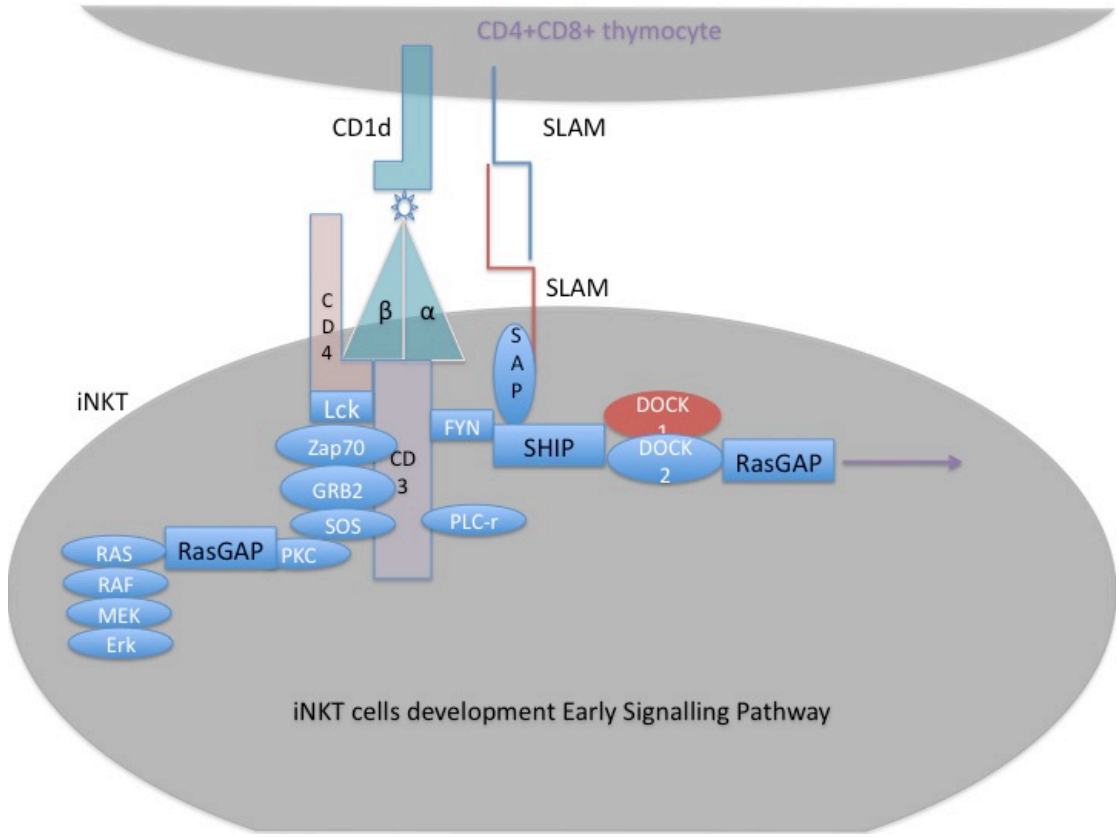


Figure 1.5. Early signalling pathway for iNKT cell development, modified from (135)

c-Myc

Two independent studies have recently shown that c-Myc is another early signalling regulator (153-155). In the study by Gounari et al., they showed that c-Myc is not essential for the selection of the thymic iNKT cells but is key for promoting their proliferation, as well as long-term survival (153). The importance of c-Myc is also apparent for conventional T and B cells, supporting

proliferation and maintaining their homeostasis. It is also responsible for continuously renewing hematopoietic stem cells (156). Selectivity for iNKT cells in c-Myc knockouts could be achieved when a targeted deletion of c-Myc in double positive thymocytes was engineered (153-155).

Promeylocytic Leukemia Zinc Finger (PLZF)

Recent studies have shown that the transcription factor PLZF might be the master control of iNKT cell development (157, 158). PLZF is not present in conventional T cells, but is present in all iNKT cells, regardless of subset. However, the PLZF expression was identified in other unconventional T cells such as MAIT cells and γδ T cells (157, 159). Interestingly, the induction of PLZF in iNKT cells is independent of a SLAM-SAP interaction (157, 158). In PLZF deficient mice a significant reduction of iNKT was observed, with most of the remaining iNKT cells failing to complete maturation, displaying a naïve phenotype, with the expression of CD62L (25, 26). These residual iNKT cells also failed to display the normal activated and memory phenotype upon stimulation (135).

1.5.1.4 Current models for invariant NKT cell development

The prevailing theory in invariant NKT cell development is that they emerge from double positive thymocytes. Two models have been proposed for the development of iNKT cells - the committed precursor model and the TCR instructive model.

The committed precursor model is based on a single study done by Makino et al (70). In this study, they identified that the development of NKT cells occurred much earlier than the development of conventional T cells. NKT cells started to express the Vα chain at day 9.5 of gestation and this committed such cells to NKT development.

The TCR instructive model is more widely accepted compared to the committed precursor model. In mice following Vα TCR rearrangement the Vα14- Jα18 chain will pair with Vβ8.2, Vβ7 or Vβ2. In human, only when both the Vα24 and Vβ11 are expressed can invariant NKT cells undergo 'instructive' differentiation. They then undergo positive selection by the CD1d expressed on DP thymocytes rather than conventional MHC selection upon cortical and medullary thymocytes. This leads to a branch of NKT lineage cell development that may be further attenuated through negative selection. NK1.1 expression can either appear in the thymus or in periphery (160).

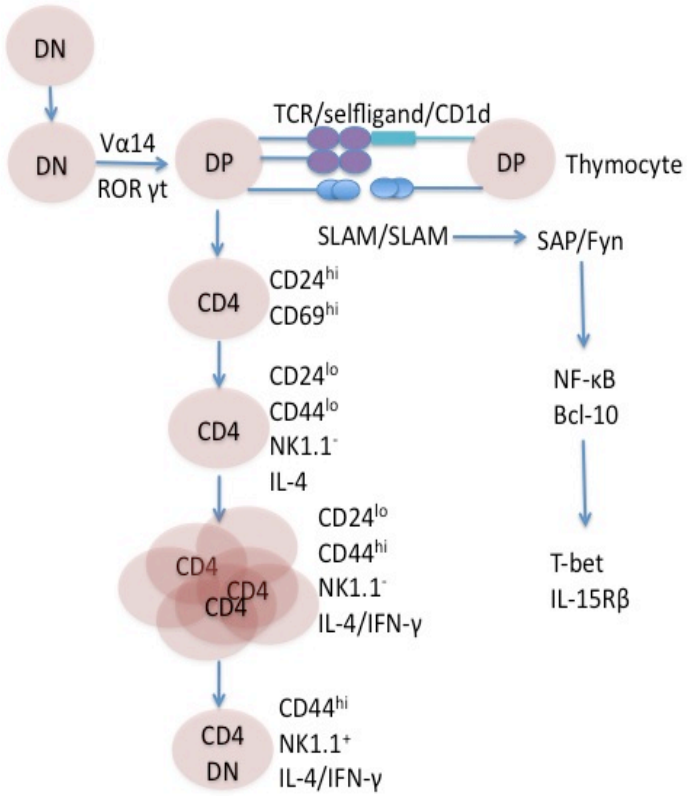


Figure 1.6. The development program of iNKT cells. Modified from (160).

There are three groups of invariant NKT cells in humans, which are characterized as $CD4^+$, $CD8^+$ and double negative (161). The different subsets give rise to the variety of cytokines that NKT cells have been shown to produce (162). One important parameter that segregates with the cytokine production

pattern is the expression of CD4 in invariant NKT cells. Many studies have shown that $CD4^+$ invariant NKT cells favour the secretion of Th2 type cytokines; whereas $CD4^-$ invariant NKT cells mainly secrete Th1 type cytokines (163, 164). This dichotomy influences the role played by invariant NKT cells in their environment.

1.5.2 Function of invariant NKT cells

The ability to restrict and counter pathogens or tumours is one of the main features of the immune system. At the same time, efficient immune regulation is required to prevent excessive self-reactivity through the secretion of cytokines and unrestricted activation of the immune system. Thus, the ability of immunoregulatory cells to appropriately promote activation or suppression is a key feature of the integrated immune response.

Invariant NKT cells are able to produce both Th1 (proinflammatory) and Th2 (regulatory) type cytokines. This multi-cytokine secretion property has made iNKT cells one of the most important cells in bridging innate and adaptive immunity. The diverse cytokines produced by different subsets of invariant NKT cells make them prototypic immunoregulatory cells. Due to this ability, invariant NKT cells have been shown to play an important role in various diseases (Figure 1.7). Clinical trials are ongoing with α-GC pulsed dendritic cells and adaptively transferred invariant NKT cells in patients with cancer (165-167). Studies have also shown that invariant NKT cells are important in certain autoimmune disorders, especially Multiple Sclerosis (MS), Systemic Lupus Erythematosus (SLE), Systemic Sclerosis and Rheumatoid Arthritis (162, 168-170). Other than cancer and autoimmunity, NKT cells have also been demonstrated as playing an important role in anti-bacterial, viral and parasitic immune responses (114, 171-174).

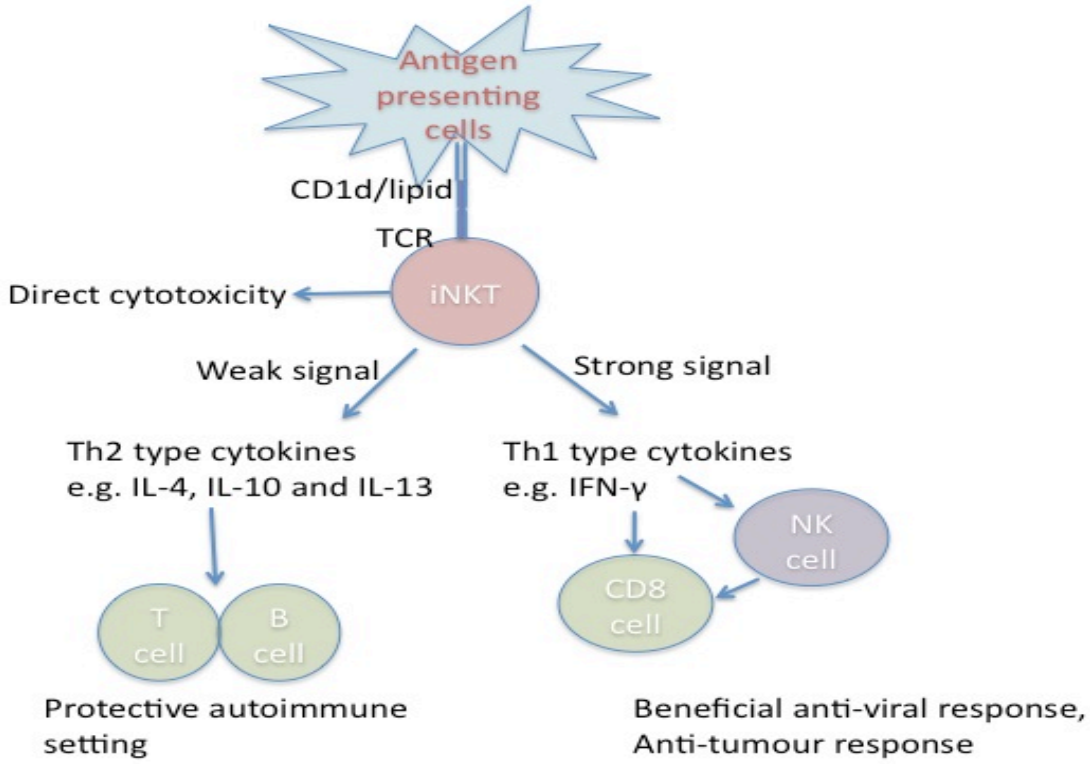


Figure 1.7. Summary of iNKT cell functions. iNKT cells can either promote Th1 or Th2 responses depend on the signal strength receive from CD1d/lipid and TCR interaction

More recently studies have found that not only the types of iNKT cells but also the binding affinity of CD1d and the glycolipids, contributes significantly to the types of cytokine released by iNKT cells (175-178). α-GC has been recognized as the "prototypic" ligand for CD1d binding, possibly mimicking the undefined natural ligand (109). Crystal structures have shown that the lipid component of α-GC fits into the CD1d binding groove by forming α helices (179). The two lipid chains of α-GalCer, a 26 carbon acyl chain and an 18 carbon sphingosine chain (Fig 3) fit tightly into the respective A' and C' pocket of CD1d. Extrapolating from the conformation and stability of α-GC and CD1d, it was predicted that the maximum carbon size that can be accommodated in the A'

pocket is 26 carbon atoms with 18 carbon atoms in the C' pocket. This allows α-GC to become one of the affinity lipids described (110).

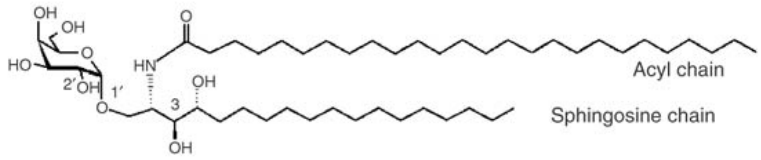


Figure 1.8. Structure of α-GalCer

CD1d interacts with α-GC at the intersection of the polar head and the two alkyl chains by forming three hydrogen bonds (110). The 2'-OH of the galactose ring forms a hydrogen bond with Asp151 in CD1d. The second hydrogen bond is formed between the 3-OH on the sphingosine chain with Asp 80 in CD1d. Several studies have shown that this hydrogen bond is crucial for mouse iNKT recognition of the complex (180, 181). The last hydrogen bond is formed between 1'-O of the α-GC and Thr154 (110). The head group of α-GalCer is then recognized by the invariant TCR by interacting with the CDR1α and CDR3α loops (109).

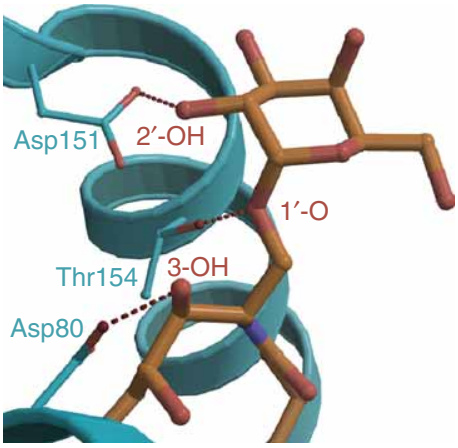


Figure 1.9. Interaction of α-GC with CD1d. Adapted from (110)

1.5.3 invariant NKT cells in health and disease

1.5.3.1 Infectious disease

So far, iNKT cells have been demonstrated to play a role in bacterial, parasitic, fungal and viral infections (182). During the anti-microbial response, iNKT cells can be directly involved in microbial recognition, direct cytotoxicity, and cross talk with the adaptive immune response; or they can modulate the innate response such as influencing NK cells (183).

Glycosphingolipds are commonly found in *Sphingomonas spp.* and they can be efficiently recognized by iNKT cells. In contrast, extracellular bacteria such as *Salmonella spp.* and *E.coli* do not have glycolipids for iNKT cell recognition (184).

Many studies have focused on the relationship of iNKT cells and viral infections. Such studies have found that in human subjects with viral infection, the number of iNKT cells is significantly decreased (185). In EBV associated X-linked lymphoproliferative disease in patients without SAP mutation a second gene defect has been identified in the X-linked inhibitor of apoptosis protein (XIAP), that significantly reduces iNKT cell numbers with no change in T, B and NK numbers (186). Very recently, iNKT-cell deficiency was also demonstrated in another primary immunodeficiency, Wiskott-Aldrich syndrome (WAS). Patients with mutation in the WAS protein also share an increased susceptibility to viral infection (187). In HIV-1 infected individuals, the number of circulating iNKT cells decreases and this may relate to the expression of the CCR5 co-receptor on iNKT cells making the CD4 iNKT cells are more susceptible to the virus than conventional CD4 T cells (188).

1.5.3.2 Cancer

The first glycolipids recognised (α-GC) by iNKT cells were identified through an anti-tumour screening programme (111, 189, 190). iNKT cells can achieve the primary protective role in tumour responses through the release of Th1 type cytokines i.e. IFN-γ. When α-GC is added to certain tumour lines including thymoma, melanoma, sarcoma and carcinomas, iNKT cells can mediate tumour rejection through CD1d recognition (191, 192). In vivo studies have found that adoptive therapy of NKT cells to NKT deficient mice can protect the mice from induced sarcomas (193). Such adoptive therapy may facilitate cooperative responses with dendritic cells for additional conventional T cell responses. In a B16 melanoma mouse model where mice were treated with syngeneic dendritic cells pulsed with α-GC, tumour regression was effectively demonstrated (194). Other studies have also revealed that iNKT cells are necessary for tumour rejection in IL-12 therapeutic settings (136).

Despite numerous studies in mice identifying a role for iNKT cells in cancer, what is the evidence in humans? Several in vitro studies have revealed that human iNKT cells can expand and differentiate to a Th1 phenotype when dendritic cells are pulsed with α-GC, subsequently inducing tumour lysis (195, 196). In vivo studies have also found a link between iNKT cells and tumour status. A defect of functional Th1 NKT cells was found in progressive multiple myeloma patients in both peripheral blood and tumour tissue. Interestingly, the defect was not found in non-progressive myeloma or premalignant monoclonal gammopathy patients (197). Such studies have identified a loss of CD1d on myeloma cells as the malignancy progresses (198), with resistance to iNKT cytotoxicity. Target cell killing could be reinstated after CD1d was restored on the cell surface of myeloma cell lines (198). Other than multiple myeloma patients, prostate cancer patients were also found to be defective in Th1 NKT cells (199). In a cohort of 109 patients with solid tumours, a decrease in the number of iNKT cells compared to the healthy controls was identified (200). Many additional studies have revealed a coexistent defect in IFN-γ production

from the reduce number of iNKT cells in cancer patients (201, 202). More recently, the Van der Vliet group suggested that the lower IFN-γ production in Th1 iNKT cells might be due to their interaction with myeloid dendritic cells. They suggest it this based on their study showing that dendritic cells from cancer patients can suppress the iNKT cytokine production from healthy individuals (203).

In contrast to these studies with deficiencies of iNKT cells, certain tumours have been associated with robust iNKT responses. Isolated iNKT cells from patients with glioma can kill glioma target cells following expansion with α-GC and mediate direct cytotoxicity (204). Interestingly, in colorectal cancer patients, a high number of iNKT cells were found infiltrating the tumour tissue (205), with those patients having a higher NKT infiltration demonstrating a higher overall disease free survival rate (205).

1.5.3.3 Autoimmune disease

The role of iNKT cells in autoimmune disease is controversial; with studies showing that iNKT cells can either have a protective role or potentiate the autoimmune disorder. Studies have been undertaken in several autoimmune disease models. A milder course of Con-A induced hepatitis was found in the absence of iNKT cells (206, 207). Interestingly, Th1 skewed iNKT cells were found in human type I diabetes (208, 209).

The impact of exogenous α-GC in these disease models has similarly not been conclusive. In some disease models i.e. autoimmune cholangitis, the iNKT activation makes the disease worse whilst others show remission i.e. EAE (210). Experimental autoimmune encephalomyelitis (EAE), a murine model of human multiple sclerosis, has undergone extensive research with iNKT manipulation. Immunization with α-GC and myelin antigen enhances the disease in B10 mice; whereas it prevents EAE development in the C57BL/6 mice (170). It has been suggested that the functional consequence of iNKT cells in autoimmune EAE

model is highly dependent on the MHC haplotype and the conventional T cell effector response that is shaped by iNKT cells (170).

1.5.4 Mechanism of invariant NKT cells in cancer

1.5.4.1 Action of iNKT cells in tumour immunity

As suggested above, iNKT cells play an important role in the anti-tumour response, but how might they achieve this?

Even though iNKT cells can release Th1 cytokines and promote tumour cell lysis, they may also orchestrate other cells in the anti-tumour response. The other effector cells such as NK, $CD8^+$ T and dendritic cells may be influenced by iNKT cells. The study of methylcholanthrene – induced tumour suppression by transferred iNKT cells in mice was found to be $CD8^+$ and NK cell dependent. The transferred iNKT cells are seen to induce perforin production in $CD8^+$ and NK cells (193). Several studies have shown that perforin and interferon-γ are important for this tumour rejection, and this is initiated by iNKT cells in an IL-2 and IFN-γ dependent manner (211).

The ability of iNKT cells to mature and induce the release of IL-12 is also important in the anti-tumour responses (Figure 1.10). The production of IL-12 is a mechanism by which iNKT cells can activate and interact with DCs (212). The prototypic ligand α-GC induces the expression of the IL-12 receptor on activated iNKT cells. When DCs pulsed with α-GC are co-administered a robust IL-12 dependent Th1 cytokine production is seen with enhanced activation of conventional $CD4^+$ and $CD8^+$ T cells (213).

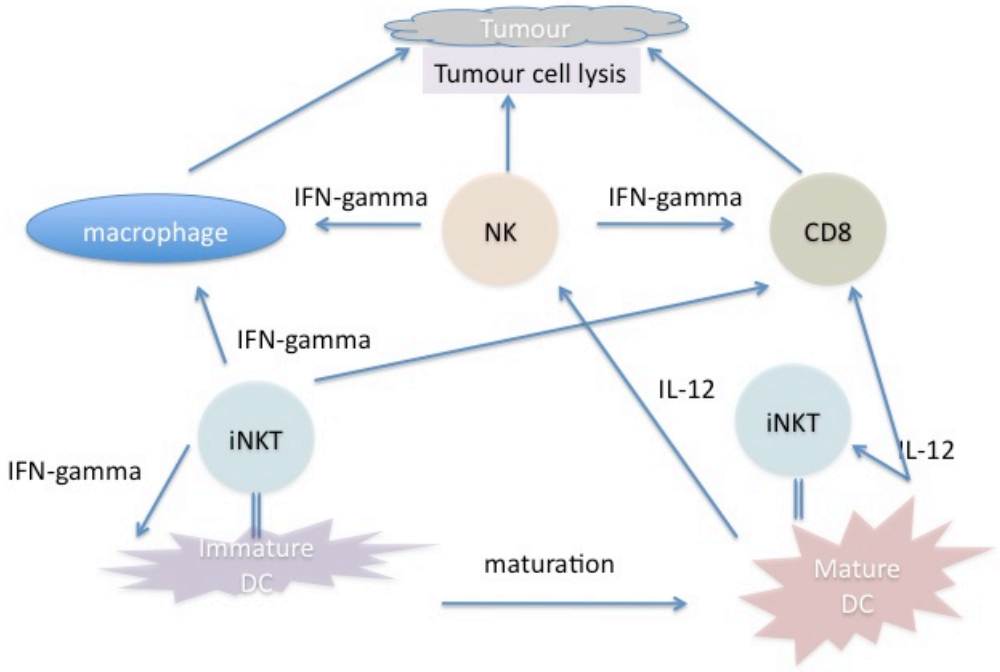


Figure 1.10. iNKT cells in anti-tumour responses

1.5.4.2 Clinical application of iNKT cells in anti-tumour response

The development of iNKT cells as a therapeutic strategy has evolved alongside the basic scientific discoveries and animal models. In one of the earliest murine models of B cell malignancy, co-delivery of α-GC and irradiated tumour pulsed dendritic cells led to improved activation of conventional $CD4^+$ and $CD8^+$ T cells with successful rejection (214). Other studies using pulsed α-GC in B16 melanoma, J558 plasmacytoma and EL4 thymoma models were all shown to provide the anti-tumour responses to the host by cross presentation of tumour antigens through DC to conventional T cells (215, 216). This protection was tumour specific with no cross protection, consistent with a antigen restricted conventional T cell effector response (195). The adoptive transfer of iNKT cells was also shown to protect against melanoma lung metastases in a murine model (217).

In human subjects, studies have also been undertaken to treat cancer patients with either α-GC alone or in combination of autologous dendritic cells. Studies

on solid tumour patients showed that no toxicity was found when they were given α-GC in a 4 weekly cycle. Increasing Th1 cytokine levels were identified in those patients with higher circulating iNKT cells pre-treatment (218). In 2002, the first α-GC pulsed dendritic cell clinical trial was carried out in 4 metastatic cancer patients, with two infusions. The serum level of IFN-γ was found to be significantly increased in the follow up study, but there was no improved outcome (219, 220). A later study with α-GC pulsed mature DC was undertaken to treat five patients with advanced metastatic non-small cell lung cancer. An increase in the iNKT cell number was found even after 6 months of the treatment; together with elevated levels of IL-12 and CMV specific CD8 T cell immunity were also found significantly increased (197). This suggested that adoptive therapy was safe and could mediate bystander effects on existing T cell populations.

However, despite the mouse models and clinical trials showing an increase in iNKT cell number and production of Th1 type cytokines, no human study has demonstrated a complete or partial remission of the tumour (195).

Chapter 2 Methods and Materials

2.1 Methods and Materials for Chapter 3

2.1.1 Antibodies and Reagents

The antibodies for identifying the iNKT cells used in this project were as follows: anti-human Vα24 (clone C15) FITC and PE (Immunotech, Marseille, France), anti-human Vβ11 (clone C21) PE and APC (Immunotech, Marseille, France), anti-human CD161 APC (Becton-Dickinson Pharmingen, California), anti-human CD3 PerCP (Becton-Dickinson Biosciences, San Jose, CA), anti-human CD4 PE-Cy7 (Becton-Dickinson, Pharmingin, California), anti-human CD8 APC-Cy7 (Becton-Dickinson Biosciences, Pharmingin, California) and anti-human 6B11 (BD Pharmingen, Pharmingin, California).

The FACS lysing solution was obtained from Becton-Dickinson Biosciences. The α-galactosylceramide was made by School of Chemistry. The α-galactosylceramide analogues were made by the School of Chemistry, University of Southampton. Ficoll-Plaque Plus was purchased from GE Healthcare. RPMI, Pen-Strep, NEAA and L-glutamine were from Cambrex, UK. IFN-γ and IL-4 ELISPOT sets were purchased from eBioscience. The cytokines IL-2, IL-15, GM-CSF and IL4 were from purchased from Peprotech. PHA was obtained from Sigma-Aldrich, US. Other reagents for specific experiments are mentioned alone with the text.

2.1.2 Study population

Buffy coats from healthy donors containing around 8 – 9 x 10^8 PBMCs were purchased from National Blood Service, UK. Recruitment of healthy volunteers was also carried out at the local laboratory.

2.1.3 Developing a FACS panel for enumerating invariant NKT cells

2.1.3.1 Sample labeling

47 samples from healthy individuals were used for developing and validating the panel for iNKT cells. The volume of antibodies used was determined using a flow cytometry titration assay. The volumes of antibody used in the control were as follows:

	Antibody volume
Vα24 FIT-C	15µl
Vβ11 PE	15µl
CD161 APC	20µl
CD3 PerCP	10µl
CD4 PE-Cy7	5µl
CD8 APC-Cy7	5µl

Table 2.1. Volume of antibody used in experimental samples

For the iNKT TCR binding affinity, the following antibody combination was used.

	Antibody volume
6B11 FITC	20µl
Vα24 PE	15µl
Vβ11 APC	5µl
CD3 PerCP	10µl
CD4 PE-Cy7	5µl
CD8 APC-Cy7	5µl

Table 2.2 Antibody concentrations for clonaltypic panel

500µl of the whole blood sample or diluted buffy coat from each individual was used to identify the iNKT cells. All the antibodies were added to the 500µl whole blood sample. The samples were incubated in the dark for 15 minutes at room temperature, after adding all the antibodies. Two ml of the BD FACS lysing solution was then added to the sample and briefly vortexed. Samples were then incubated for a further 15 minutes to lyse the red cells at 4°C. After this incubation, they were spun down at 1500rpm for five minutes and the supernatants were discarded. Two ml of the FACS washing buffer was added to each tube to remove the excess antibody, and the samples were again centrifuged at 1500rpm for five minutes. A further two ml of FACS washing buffer was added to each sample and vortexed well. They were then left at 4°C in the dark until prior to analysis.

2.1.3.2 FACS panel for identifying iNKT cells

Due to the low number of iNKT cells present in the peripheral blood there is a greater likelihood of non-specific events/debris interfering with accurate enumeration and therefore an appropriate approach was required to analyse the cell population in the peripheral blood. The approach shown in Figure 1 was used to identify the iNKT cells through a process of sequential gating in this project.

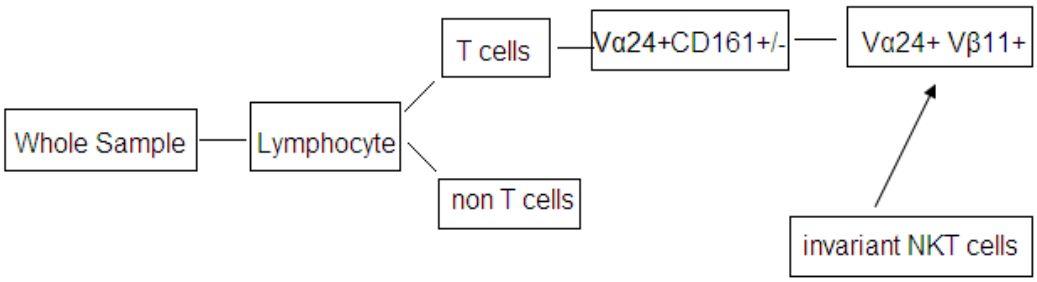


Figure 11. Approach for identifying invariant NKT cells

The antibody stained sample was run on the flow cytometer with a minimum of 100,000 T cells collected for further analysis. The T cells were further positively selected upon the markers CD161 and Vα24. The $V\alpha24^{+}CD161^{+}$ cells were then analysed for expression of Vβ11 expression. Only those cells with both Vα24Vβ11 expression through this strategy were true iNKT cells. This analysis was validated in 18 samples and further applied to 29 samples.

2.1.4 Proliferation assay of invariant NKT cells

2.1.4.1 Lymphocyte isolation

Lymphocytes were isolated from 40ml of whole blood (or alternatively from 250ml 80%PBS+ buffy coat) by Ficoll gradient. 15ml of the Ficoll solution was transferred by pipette into 50ml tubes. The tubes were tilted to a 45^{o}C angle, and the blood was carefully layered onto the top of the Ficoll solution. The samples were centrifuged at 1600rpm for 25 minutes at room temperature, with the brake off. The interphase was carefully removed using pasteur pipettes after centrifugation. The interphase was then washed three times with PBS. The lymphocytes were then suspended in one ml of RPMI. 2 x 10^5 PBMCs were used in every well for IFN-γ and 5 x 10^5 PBMCs were used for the IL-13 and IL-17 ELISPOT.

The proliferation assay of iNKT cells was performed with α-GC for 7 days (221). 5 x 10^6 PBMCs were resuspended in 5ml of complete RPMI1640 culture medium with 100IU/ml of recombinant human IL-2 and 100ng/ml of the α-GC. Proliferation of NKT cells was assessed at day 7 using the six-colour FACS panel. Proliferation index was calculated by comparing the total number of iNKT cells pre-proliferation to the total number of iNKT cells post proliferation.

2.1.5 Cytokine profile of the analogues

The cytokine profile of the PBMCs stimulated with different analogues was measured using IFN-γ, IL-13 and IL-17 ELISPOTs according to manufactures instructions.

2.1.5.1 IFN-γ, IL-13 and IL-17 ELISPOT assay

Cultured ELISPOTs were done with cells cultured for 7 days with the appropriate analogue and pulsed for final 18 hours with the CD1d ligand.

ELISPOTs were performed using a human IFN-γ ELISPOT, IL-13 ELISPOT and IL-17 ELISPOT set according to the manufacturer's protocol. ELISPOT plates were coated with PBS diluted anti-IFN-γ (or IL-13, IL-17) antibody overnight. The plates were then blocked with complete RPMI for 2 hours. The lymphocytes were then counted and incubated overnight at 2 x 10^5/well for IFN-γ or 5 x 10^5/well for IL-13/ IL-17 at 37°C with a series concentrations of the appropriate ligand (either control or experimental) in triplicate. Wells were then washed with deionised water and PBS-Tween. Plates were then incubated with biotinylated anti-human IFN-γ antibody or anti-human IL-13 or anti-human IL-17 for 2 hours. Plates were washed four times with PBS-Tween, and then incubated with streptavidin for 1 hour at room temperature. The plates were then washed again with PBS-Tween and PBS. The plates were incubated with the substrate solution and the spot development was monitored. The reaction was stopped by washing with deionised water. The plates were dried for 2 hours before analysis by ELISPOT analyser.

2.1.6 FACS sorting of invariant NKT cells

PBMC was isolated with Ficoll, followed by labeling antibodies Vα24 FITC, Vβ11PE, CD161APC, CD3 PerCP, CD4 PE-Cy7 and CD8 APC-Cy7 for half an

hour at 4°C. The labeled PBMCs were then washed with FACS wash twice and resuspended in PBMC, and run on the FACSAria. The invariant iNKT cells that were identified with the approach from figure 1 were further divided into CD4 and nonCD4 subsets. The CD4 and non-CD4 components were separately sorted into a different collection tubes with complete medium. In the experiments performed in Chapter3, a set number of 1000 cells were collected in each fraction.

2.1.7 Statistical Analysis

The statistical analysis in the thesis was performed using Graphpad Prism 4 software. Statistical Analysis for pair comparison in Chapter 3 and 7 was performed using Paired t test, as data analysis by prism suggest data show Gaussian distribution. Correlation studies in Chapter 3 was analysed with linear regression to obtain R^2. Chapter 4, 5 where statistics are required for comparing cytokine production, non parametric test (Wilcoxon test) was performed.

2.2 Methods and Materials for Chapter 4

2.2.1 Isolation of antigen presenting cells

Various antigen presenting cells were positively selected using magnetic separation with MACS beads (Miltenyi Biotec, Germany). Cells were incubated at room temperature for ten minutes with anti – CD19 microbeads for B cell depletion, anti-CD14 microbeads for monocyte depletion and anti-BDCA-1 microbeads for myeloid dendritic cells depletion (20µl per 10^7 cells). The cells were then washed with RPMI and resuspended in 100µl MACS buffer (phosphate buffered, 5mM EDTA and 0.5% BSA). At the same time, an LS separation column (Miltenyi Biotec, Germany) was set up by flow through with 500ul of the MACS buffer. The cells were then washed and resuspended in 500µl of buffer. The cells were then applied to the LS separation column and the flow through collected according to manufacturer instructions. The column was washed three times with 500µl MACS buffer and the flow through collected. The purity of the flow through was then checked by cytometry (above 93% purity were obtained with the depletions). The remaining monocyte/B cells in PBMC were also assessed by cytometry (10.4% in total PBMC pre-depletion compared to below 0.5% post-depletion).

2.2.2 iNKT proliferation with separate APC subsets

PBMC or partially depleted PBMC were cultured with 100ng/ml α-GC and 100IU of IL-2. In experiments when IL-15 was added, 20ng/ml of IL-15 was used. The PBMC was cultured for seven days before the proliferation index was determined by FACS. Proliferation index was calculated by comparing the total number of iNKT cells pre-proliferation to the total number of iNKT cells post proliferation.

2.2.3 Titration of IL-15

Cultured ELISPOTs were performed with a series of IL-15 concentrations for seven days. The concentration of IL-15 used for these experiments was 25ng/ml, 20ng/ml, 15ng/ml, 10ng/ml, and 5ng/ml. IFN-γ, IL-13 and IL-17 were performed as described in Chapter 3.

2.2.4 Study of effect of IL-15

2.2.4.1 IL-15Rα western blot

2.2.4.1.1 Cell preparation

For the IL-15 induced IL-15Rα experiments, 20ng/ml of IL-15 was added to the PBMC, and then the B cells and monocytes were then sorted. For intrinsic expression of IL-15, the B cells and monocytes were directly sorted from the PBMC without pre-culturing. The cells were pelleted and washed once with PBS, prior to lysis for 30 minutes (150mM, 5mM EDTA (Sigma, UK), 20mM TriHCL (Sigma, UK), 2mM Phenylmethylsulphonylfluoride (Sigma, UK), 2mM Iodoacetamide (sigma, UK) and 0.5% NP-40 (Sigma, UK)). The lysates were then centrifuged at maximum speed (13,000 rpm) for 5 minutes. The lysates were then mixed in 2x SDS-loading buffer (4% SDS (Sigma, UK), 0.125M Tris (Sigma, UK), 20% glycerol (Sigma, UK), 0.05% Bromophenol blue (Sigma, UK), 5% β-merceaptoethanol (Sigma, UK)). Heated to 95°C for 5 minutes and spun down.

2.2.4.1.2 Western blot

The cell lysates with the SDS-loading buffer were run on a 10% polyacrylamide gels (Invitrogen, UK). The gel was cast in 1mm gel cassettes and run in a X-Cell SureLock Mini Cell System (Invitrogen, UK). The gel was then transferred to a nitrocellulose membrane overnight at 12 Volts.

Once the transfer was complete, the membrane was blocked with 5% milk in PBS at room temperature for an hour or 4°C overnight. The membrane was then added to PBS with a 1:2000 dilution of the IL-15Rα detection antibody and incubated overnight at 4°C. The membrane was then washed 3 times in PBS-0.1% Tween. The membrane was then further incubated in the 1:10,000 anti-goat HRP in PBS for an hour at room temperature. The membrane was then washed 3 times in PBS and detected in ECL solution (Pierce).

2.2.4.2 FACS analysis of IL-15Rβ (CD122) and commonγ (CD132) chain

To look at the expression of IL-15Rβ and commonγ chain in iNKT cells, PBMC, after a seven day culture were labeled with Vα24 FITC, CD122/CD132 PE, CD3 PerCP, CD4 PE-Cy7 and CD8 APC-Cy7 for 15 minutes at room temperature. The cells were then washed twice in FACS buffer and run on the FACSCanto.

2.3 Methods and Materials for Chapter 5

2.3.1 Preparation of Analogues

The analogues were received from collaborators (Dr Bruno Linclau, University of Southampton and Dr. Serge Van Calenbergh, University of Ghent) and all dissolved in PBS with 10% DMSO. The analogues were then aliquoted and stored at -20°C.

2.3.2 Cytokine study of the Analogues

2.3.2.1 Intracellular cytokine study

Cells from seven day cultures with various analogues were collected and washed. The cells were plated in a 96 U bottomed plate. The cells were then incubated with 100ng/ml of the appropriate analogues and 5 x 10^3 irradiated autologous feeder cells and 1uM/well Golgistop were added for 6 hours.

Cells were then labeled with 15ul of 6B11 and 10ul of CD3 for 15 minutes at 4^oC. Cells were then washed twice, fixed and permeabilised with the BD permeabilisation and fixation kit before intracellular labeling with 5ul PE-Cy7 mouse anti-human IFN-γ, 20ul FITC mouse anti-human IL-17 and 5ul APC rat anti-human IL-4 for 20 minutes at 4^oC. After a final wash the cells were resuspended in FACS buffer and run on the FACScanto.

2.3.2.2 Multicytokine analysis

Supernatants from PBMC cultured with α-GC and analogues were sampled at 2-16 hours at every two hours interval to identify the optimal time for cytokine sampling. The samples were then stored at -20°C for use in the multiplex assay.

To perform the cytokine analysis, a human Th1/Th2 multiplex kit was used. 50µl of 1x Assay Buffer was added to each well of the filter plate to pre-wet the plate. 25µl of Standard Mixture were added to the first two rows of the plate to generate a standard curve. The samples were added to appropriate wells. 25µl of the Bead Mixtures were then added to each well, and 50µl of Biotin-Conjugate Mixture was added to the wells. After adding the mix to the plate, it was covered by adhesive film and incubated at room temperature for 2 hours on a 500rpm shaker with protection from the light. The film was removed after 2 hours and the plate was washed three times with 100µl of 1xAssay Buffer. 100µl of 1xAssay Buffer was added to each well after the washes with 50µl of the Streptavidin-PE solution. The plate was again covered and incubated at room temperature for 1 hour on the shaker. The plate finally washed three times with assay buffer and 200µl of the assay buffer was added to each well for mixing the samples and transfer to FACS tube.

The samples were then run on the flow cytometer and each detected cytokine was identified by the size of its conjugation beads. The level of PE intensity indicated the level of the cytokine and the results were analysed on the FlowCytomix Pro2.3 Software.

2.3.4 Anergy study of iNKT cells

To investigate the level of anergy in iNKT cells PBMCs were labeled with PD1 FITC (20µl), 6B11 PE, CD3 PerCP, CD4 PE-Cy7 and CD8 APC-Cy7 for 15 minutes at room temperature pre and post a 7 day culture. The cells were then washed twice in FACS buffer and run on the FACSCanto.

2.4 Methods and Materials for Chapter 6

2.4.1 Specificity killing of iNKT cells

2.4.1.1 Generation of NKT cells line /clones

The NKT cells lines were generated from 1 x 10^7 PBMCs, initially incubated in a T 25 flask with 100ng/ml α-GalCer and 100IU/ml IL2. α-GC pulsed autologous APCs were restimulated every 7 days. The NKT cells were sorted with MACS isolation after 2-3 rounds of restimulation and the lines maintained in IL-2.

2.4.1.2 CD1d expression on Target cells

CD1d expression on the target tumour cells was analysed before each killing assay. The CD1d expression was analysed on Jurkat, K562 and U 937 cell lines with a CD1d PE antibody.

The tumour cells were incubated with the CD1d PE antibody for 15 minutes at room temperature. The cells were then washed twice with FACS wash and analysed on the FACSCanto.

2.4.1.3 Direct killing measurement

The killing assay in this project was established by measuring direct cell death using the DNA binding dye To-Pro-3 (Invitrogen, UK). Each assay counted 5000 target cells that had been pulsed with 100ng/ml α-GC or analogues for 2 hours. The cells were then washed twice with complete RPMI1640. The NKT cells were added to the wells in appropriate effector: target ratios in a 96 well plate. The plate was then incubated at 37˚C for 4 hours. 2ul (1:100) of To-Pro-3 was added into each well and incubated on ice for 30 minutes. After the incubation, the plates were washed twice with FACS wash and analysed on the FACSCanto.

2.4.2 Generation of a CD1d dimer

2.4.2.1 Cloning of signaling peptide-β2m-CD1d-mHeavy chain construct

2.4.2.1.1 Primers for generating CD1d dimer

The primers (Sigma, UK) used for generating the CD1d dimer were as follows:

Signaling peptide – β2m:

- Forward: GGT GCT AGC ATG GAA TGG AGT TGG ATA TTT CTC TTT CTC CTG TCA GGA ACT GCA GGT ATG ATC CAG CGT ACT CCA AAG
- Reverse: ACG AAG CTT AGC ACC TCC TCC AGA ACC TG

CD1d:

- Forward: 5'- CGA AAG CTT GTC CCG CAA AGG CTT TTC C – 3'
- Reverse: 5' – ACG GGT ACC CAG GAC GCC CTG ATA GGA AG – 3'

Heavy Chain.

- Forward: CGT GGT ACC GAG GTC CAG CTG CAG CAG TCT GG
- Reverse: 5' – AGC CTC GAG TCA TTT ACC AGG AGA GTG GG – 3'

Those underlined sequences represent the restriction sites:: GCT AGC – NheI, AAG CTT – HindIII, GGT ACC – KpnI, CTC GAG - XhoI

2.4.2.1.2 PCR reaction for the construct

Individual PCR reactions were carried out to generate different fragments for subsequent ligation into the cloning vector. Human CD1d and signaling peptide were generated from from an image clone (*Clone* ID 39685). The mouse Heavy Chain was generated from a pEE6.1 vector containing mouse variable and constant regions (a gift from Claude Chan, Tenovus, Southampton). The PCR approach utilised was as follows ; 5 minutes at 94°C,33 cycles of 30 seconds @94°C:45 seconds 60°C and 2 minutes @72°C. A 7 minute 72°C final extension was then performed.

PCR products were purified using a QIAquick PCR purification kit (QIAGEN), and performed according to the manufacturer's protocol. For DNA binding, the

mixture was then transferred to a QIAquick spin column, centrifuged for 30-60s and the flow through was discarded. 0.75ml of PE buffer was added to wash the column, prior to a 30-60s centrifugation step. 30µl of elution buffer was added to the centre of the column membrane. The flow through was collected and retained.

2.4.2.1.3 Clone PCR product into pGMT vector

All PCR products were cloned individually into pGMT vector. 5µl of the PCR product were mixed with 2µl of the pGMT vector, 1µl DNA ligase and 2µl of T4 ligase buffer. The reactions were ligated at 16°C overnight.

2 µl of each ligation preparation were transferred to a tube containing competent *E.coli***.** The tubes were left on ice for 30 minutes. The tubes were then heat shocked at 42^{o}C for 30 seconds. They were left on ice for a further 2 minutes after heat shocking. Then, 250µl of SOC medium was added to each tube. The tubes were left at 37^{o}C in a shaking incubator for one hour. After incubation, the tubes were centrifuged for 20 seconds to spin down the bacteria. The liquid was tipped out of each tube and the bacteria were resuspended in the remaining medium

A blue/white screening was performed for pGMT cloning with 50µl of X-gal and 50µl β-galactosidase spread on ampicillin-LB agar plates. 200µl of the transformed culture was plated and incubated at 37°C overnight. White colonies were picked and grown on ampicillin LB medium at 37°C overnight.

After overnight incubation, the bacterial culture was tipped into a 1.5ml eppendorf tube and the remaining cultures kept for future use. The eppendorf tubes were spun for 1 minute at 13000rpm and the medium was removed. The bacterial cells were resuspended in 250µl of buffer P1 (resuspension buffer) and 250µl of buffer P2 (lysis buffer). 350µl of P3 was then added and mixed by tube inversion. The culture tubes were then centrifuged for 15 minutes at 13000rpm.The supernatant was added to the QIAspin column, centrifuged for 1 minute. And 0.5ml of the binding buffer (PE) added for 1 minute. The column

was washed using 0.75ml PE and centrifuged for 1 minute. 50µl of elution buffer was added to elute the DNA; the DNA was collected by centrifugation for 1 minute.

To screen the colonies, 5µl of the purified DNA was digested with the appropriate enzymes in a total volume of 20µl. The digested plasmids were then run on a 1% agarose gel to check for the presence of an insert.

The DNAs with the correct insert were sent for sequencing by GeneService (Oxford, UK).

2.4.2.1.4 Clone into pcDNA3.1

The DNA containing the correct sequence was cut with the appropriate enzyme to release signaling peptide-β2m, CD1d and heavy chain. The three fragments were cloned into the pcDNA3.1 by multiple-restriction enzyme ligation. The cloning was performed by three step ligation. First, the signaling peptide-β2m sequence was inserted into the plasmid, followed by CD1d and heavy chain. Double digestion was performed after every new insertion to check the correct insert has been placed.

The final product was sent for confirmatory sequencing. A Maxiprep was then performed following the sequencing confirmation of our correct vector. The overnight culture in 250ml LB-amp was centrifuged and the bacterial pellet was resuspended in 10ml of P1 buffer. 10ml of buffer P2 was added into the mixture and left at room temperature for 5 minutes. 10ml of P3 were added to the culture and this was inverted 4-6 times. The lysate was then poured into a QIAfilter Cartridge and left for 10 minutes. The QIAGEN – tip 500 was equilibrated by adding 10ml QBT buffer and left to flow through. After 10 minutes, the cap of the QIAfilter Cartridge was removed, and the plunger was inserted into the QIAfilter Maxi Cartridge. The flow through from Maxi Cartridge was collected into the equilibrated QIAGEN tip. The QIAGEN – tip was then washed with 2 x 30ml QC. 15ml of QF were used for eluting DNA and the DNA was collected in a Beckman tube. 10.5ml isopropanol was added to the eluted DNA, in order to

precipitate it, mixed and centrifuged at 15000g for 30 minutes. The supernatant was decanted and the pellet washed in 70% ethanol and dried at room temperature for 30 minutes, prior to redissolving in 200µl of elution buffer.

2.4.2.2 Generation of CD1d dimer

2.4.2.2.1 Transfection of CD1d dimer to J558L cells

The cells line for expressing the CD1d dimer construct is a murine myeloma cell line J 558L which is heavy chain deficient but capable of producing Igλ chain (a gift from Professor Aymen Al-Shamkhani). The cells were grown on serum free medium and 2µl of pcDNA3.1 containing construct was transfected to 1 x 10^7 cells by nucleofector (Lonza Cologne AG, Cologne, Germany). Cells with the transfected DNA were grown in the presences of 0.5mg/ml of G418.

The supernatant produced by transfected J558L cells was collected after 2 weeks incubation. The culture supernatant was then applied to protein G sepharose column and washed with 0.1M glycine buffer until there was no detection of protein on the flow through, by nanodrop. The CD1d dimer was then eluted by adding 0.1M glycine buffer and a series of 500µl elutions collected.

2.4.2.2.2 Validate CD1d dimer

The purified protein was then used as the protein target of a western blot with a primary rabbit anti-human β2m antibody and secondary anti-rabbit HRP antibody.

The CD1d dimer construct was also validated by FACS analysis by intracellular staining of non-transfected and transfected J558L cells with an anti CD1d-PE labeled antibody.

2.4.2.3 Competitive ELISA assay with CD1d dimer

The competitive ELISA assay followed the protocol developed by Shiratsuchi et al as discussed in Chapter 6. To calculate the Kd of the conditional ligand a 96 well Nunv MaxiSorp plate was coated overnight with 100µl of 1:500 mouse monoclonal antibody (a gift from Dr. Natalia Savelyeva). The plates were then washed three times with PBS-Tween and a titration series of the conditional lipid (Biotinyl PE) was added to 8µl/ml of CD1d dimer. The half maximal concentration was calculated to give the Kd. For competitive ELISAs a similar approach was taken with a titration series of analogues added to a CD1d dimer conditional ligand mix of 2µl/ml CD1d: 0.8µl/ml of Biotinyl PE. This was incubated overnight at 37°C to facilitate the competition between the reporter Biotinyl PE and index analogues. The plates were washed three times with PBS-Tween and the HRP-labeled avidin was added to detect the residual binding of the reporter. The Ki of each analogue was then calculated using the formula Ki = IC50 / (1 + [B-PE] / Kd), where IC50 is the concentration of index analogue that reduces maximal signal by 50%, [B-PE] the concentration of reporter ligand and Kd reflecting that previously calculated for B-PE.

2.5 Methods and Materials for Chapter 7

2.5.1 Details of patient samples

37 CVID patient samples and 4 X linked agammaglobulinaemia samples, 20 ml of lithium heparin blood, were sent from our clinical collaborator (Professor Bodo Grimbacher) at the Royal Free Hospital, London. The methods used for the enumeration and functional studies of these patients were identical to those used Chapter 3. Ethics Approval was obtained in Royal Free Hospital , London.

Chapter 3 Invariant NKT cells in healthy individuals

3.1 Introduction

Studies in recent years have shown that the number of iNKT cells can vary enormously in healthy individuals (163, 168, 222, 223). Most studies report that the number of iNKT cells in the peripheral blood among healthy individuals, ranges from 0.01 -1% of total T cells, with a mean of around 0.1% (224). This three-log range for a conserved T cell subset is unique to the iNKT lineage and poorly understood.

3.1.1 Approaches for identifying iNKT cells

Traditionally, this cell type in humans was identified using two-color flow cytometry with antibodies directed against the conserved T cell receptor Vα24 and Vβ11 (222). The generation of CD1d/α-GC tetramers have provided a more specific way to identify the iNKT cells in both humans and mice (225). However, in comparison to monoclonal antibodies, the tetramer staining can lead to a higher background staining, especially in the presence of monocytes (222, 226). Recently a clonotypic monoclonal (6B11) antibody has been established as a useful tool for identifying iNKT cells. This antibody was generated against the conserved CDR3 Vα24 chain of iNKT cells (227). In comparison to the previous technique, the advantage of using the 6B11 is that it can identify iNKT cells with a single antibody rather than a combination of two and that it is specific for a TCR sequence signature. However it again shows a higher background staining as compared to the dual antibody approach.

3.1.2 Factors influencing the number of iNKT cells

Currently, little is known as to the factors that contribute to the differences in iNKT cell numbers amongst the heterogeneous human population. Several candidate genes have been identified that are influential in their development. SH2D1A is one of the genes that is thought to have an impact in iNKT development. SH2D1A encodes for the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), whose absence is responsible for X-linked lymphoproliferative disease (XLP). In SAP deficient patients, their B, T and NK cell numbers are within normal range, whilst their iNKT cells are deficient (151, 228). Reciprocal experiments in mice with engineered knockouts of SAP confirm this selective requirement for iNKT cell development. Studies have also shown that the activation of PKCθ by Fyn is essential for the generation of NKT cells (229, 230). It is now known that the SAP-Fyn- PKCθ pathway is essential for the NKT cells development.

Peripheral factors may also shape the iNKT repertoire as it is known that low levels of iNKT cells are present in cancer patients, as well as individuals with viral infections (231, 232).

3.1.3 iNKT cells – a number's game?

Invariant NKT cells are also believed to influence certain autoimmune diseases. Some studies have shown that the number of iNKT cells is significantly reduced in patients with Multiple Sclerosis (MS), Systemic Lupus Erythematosus (SLE), Systemic Sclerosis and Rheumatoid Arthritis (162, 168, 169).

During human immunodeficiency virus (HIV) infection, the number of iNKT cells dramatically decreases in the host (233). In some instances, a complete depletion of iNKT cells was seen (188, 233). A restoration of circulating iNKT cells number has been described after highly active antiretroviral therapy (234). In hepatitis B and hepatitis C infected individuals, the number of iNKT cells

resident in the liver is much higher than those in healthy subjects (235). Interestingly, no increase was observed in the circulating iNKT cells compared to healthy controls (236). The absolute number of iNKT cells in the liver of some Hepatitis C subjects is ten times higher than that in their peripheral blood (236). A single case study reports a girl with iNKT cell deficiency who died of varicella infection following vaccination, with have no other immunodeficiency discernable (174).

Despite these factors, it is still not fully understood what accounts for the variable number of iNKT cells seen across healthy individuals. Outstanding questions also concern the relationship between iNKT cells and conventional T cells. Is there perhaps a signature to define individuals with high iNKT cell numbers compared to those with low iNKT cells? And finally is there a functional consequence of being a high or low iNKT individual?

3.2 Aims of this study

The aims of this study were to

- Develop methods that can be used for accurately enumerating and examining the function of iNKT cell populations in healthy controls and cancer patients.

- Develop an experimental platform to systematically analyse a portfolio of novel glycolipids compounds that have been designed to improve CD1d affinity and skew iNKT responses to a Th1 phenotype.

3.3 Results

3.3.1 Development of an iNKT panel

The FACS panel for identifying iNKT cells was developed by using whole blood sample labeling with six antibodies (Vα24 FITC, Vβ11 PE, CD161 APC, CD3 PerCP, CD4 PE-Cy7 and CD8 APC-Cy7). The iNKT cells were initially identified using a sequential step gating strategy. Figure 3-1 is a representation of this approach for identifying the iNKT cells.

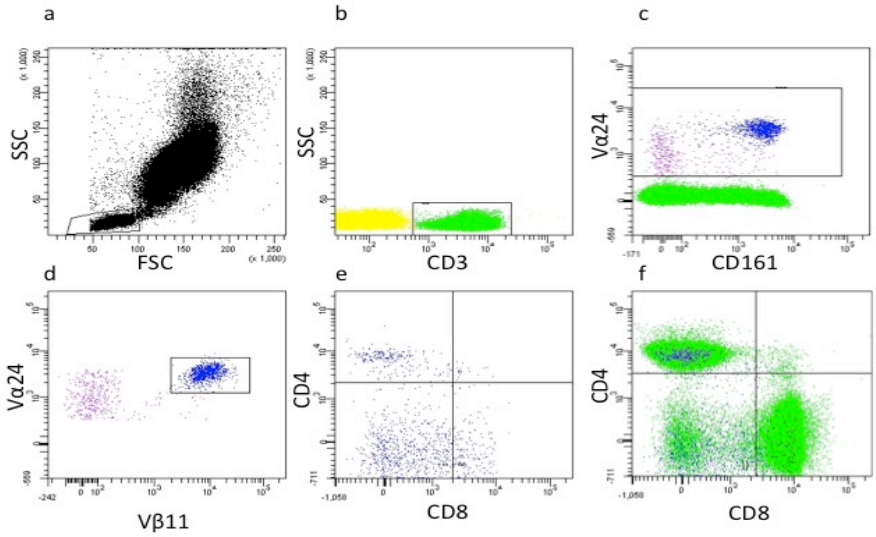


Figure 3.1 Steps in identifying invariant NKT cells. (a) General view of leucocytes in flow cytometry forward scatter/side scatter dot plot. A gate was set for Lymphocytes which represents 18% of the whole population. (b) T cells were identified from the Lymphocyte gate which represented 56% of the Lymphocyte population. (c) Possible invariant NKT cells were identified by using Vα24 and CD161 antibody on the T cell gate. $V\alpha24^{+}CD161^{+}$ cells contribute about 88% of the $V\alpha24^{+}$ T cells. (d) Invariant NKT cells were identified from possible invariant NKT cells by using Vα24 and Vβ11 antibodies. 87% of the possible invariant NKT cells were true invariant NKT cells as defined as $CD3^{+}V\alpha24^{+}V\beta11^{+}$. All of these were CD161 positive. (e) The CD4 and CD8 distribution of invariant NKT cells. (f) The CD4 and CD8 distribution of total T cells.

Examination of Figure 3-1 reveals that iNKT cells represent 1.2% of T cells for this healthy individual. Qualitatively it can be seen that most of the iNKT cells are CD161 positive and express a higher density of Vα24 compared to those Vα24 positive non iNKT cells (MFI of 2773 compared to 1194, $p < 0.002$) The majority of the iNKT cells are double negative (DN) for CD4 and CD8 co-receptors. This qualitative and quantitative assessment provides an accurate identification of iNKT cells in whole blood and prevents some of the non-specific factors that can influence the enumeration of rare populations in whole blood.

3.3.1 Conventional T and invariant NKT cells

From the panel established above, we further analyzed and compared the phenotypic properties of conventional and iNKT cells.

As iNKT cells are a subset of the T cell lineage we compared the CD3 expression between iNKT cells and conventional T cells.

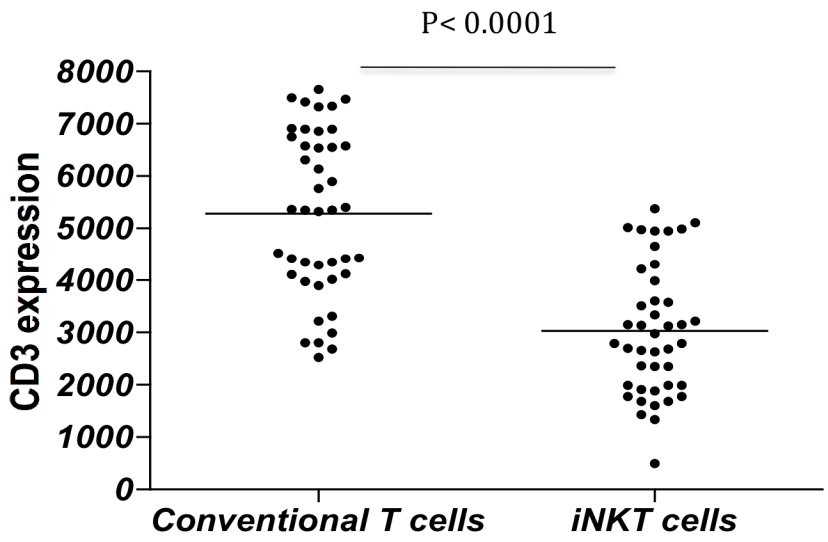


Figure 3.2 CD3 expression on conventional T cells and iNKT cells. MFI of CD3 comparison between conventional T cells and iNKT cells across 43 individual.

From the Figure 3-2, it can be seen that conventional T cells have a much higher expression of CD3 compared to iNKT cells (MFI 5453 compared to 2890, p< 0.0001, n=43). This is intriguing when the previous result of an increased expression of Vα24 is noted in iNKT cells compared to conventional T cells. Populations of iNKT cells and non-iNKT Vα24 T cells were selected for further investigation (Fig 3.3).

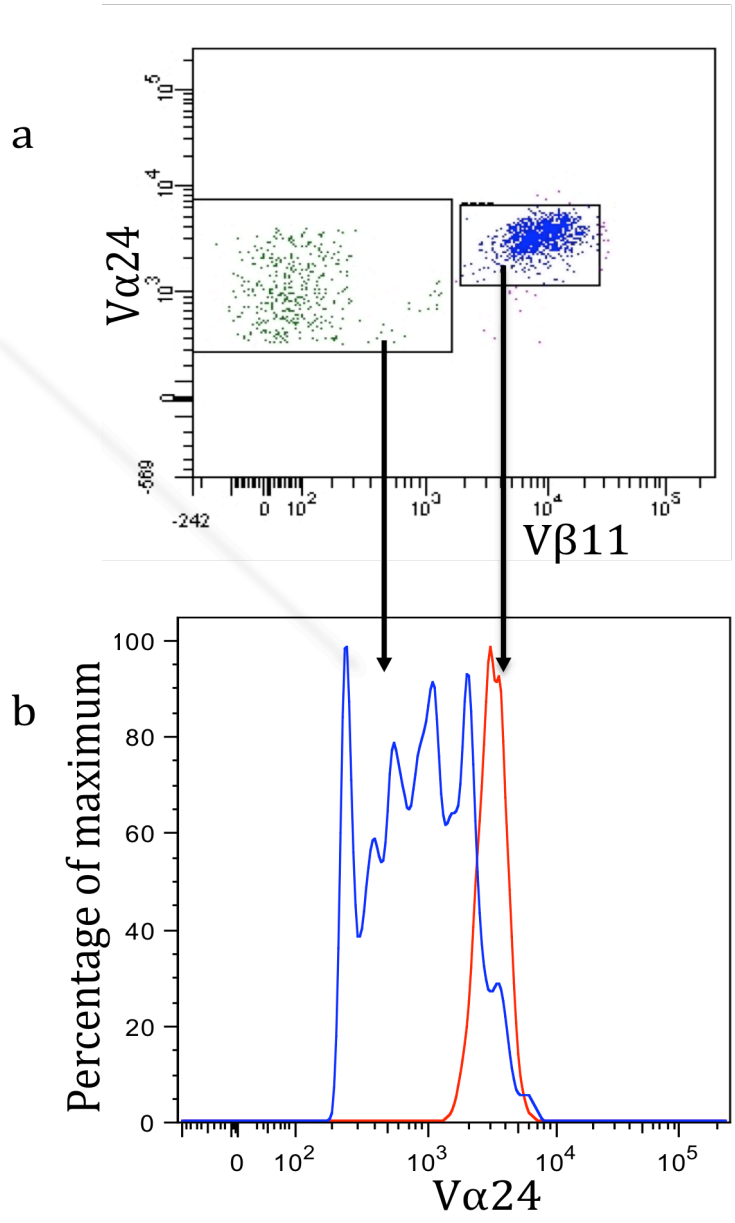


Figure 3.3 Vα24 expression on iNKT cells and non-iNKT Vα24 cells.

A homogenous peak for the density of Vα24 expression is observed in the iNKT cell population. In contrast to this, heterogeneous peaks of Vα24 expression were observed in the non-iNKT Vα24 T cell populations.

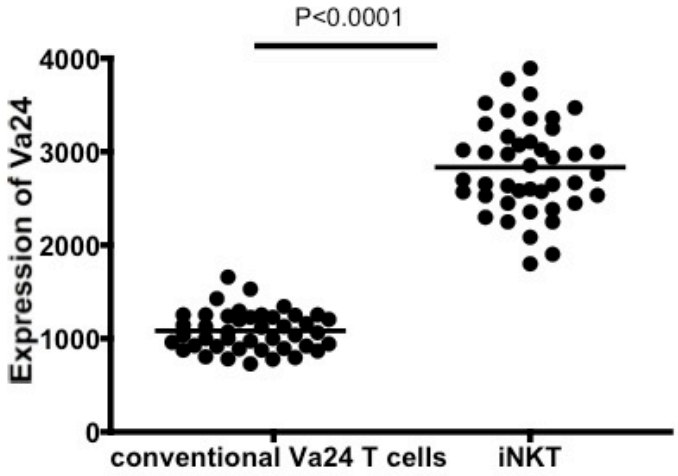


Figure 3.4 Analysis of Vα24 expression on iNKT cells and non-iNKT Vα24 cells across 47 healthy individuals. MFI expression of Vα24 was compared between the conventional Vα24 T cells population and iNKT cells population. The Vα24 MFI is much higher in iNKT cells compared to their conventional counter part.

Figure 3.4 illustrates that across the heterogeneous population, conventional Vα24 T cells showed a much lower receptor expression compare to the iNKT cells despite them having a higher level of CD3 receptor expression.

CD161 is considered as a maturation marker of iNKT cells with only mature and resting iNKT cells expressing CD161. The majority of iNKT cells in adults express CD161 and Figure 3.5 shows that there was no significant difference between the two populations of iNKT cells for CD3 expression.

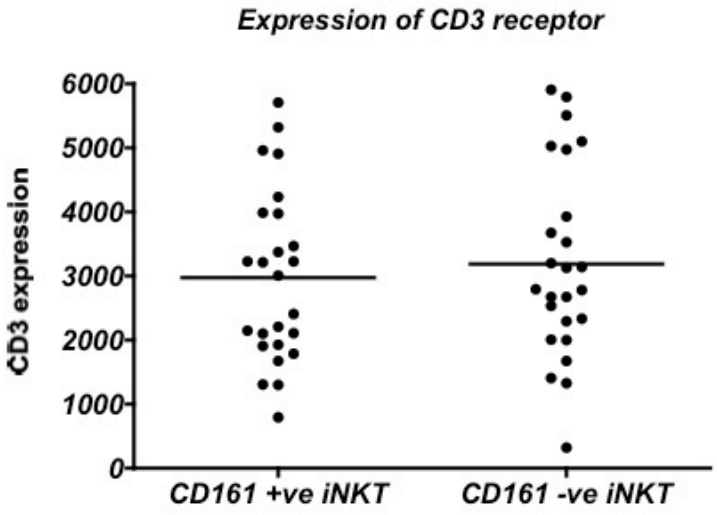


Figure 3.5 CD3 expression in CD161 positive and negative iNKT cells. Comparison of CD3 MFI in the CD161 negative and positive iNKT population. No difference was found in the two populations.

3.3.2 Quantitative analysis of invariant NKT cells

The quantitation and phenotypic evaluation of iNKT cells was then performed on 47 adult controls. We observed that the number of iNKT cells varied considerably from individual to individual as previously reported (Figure 3-6).

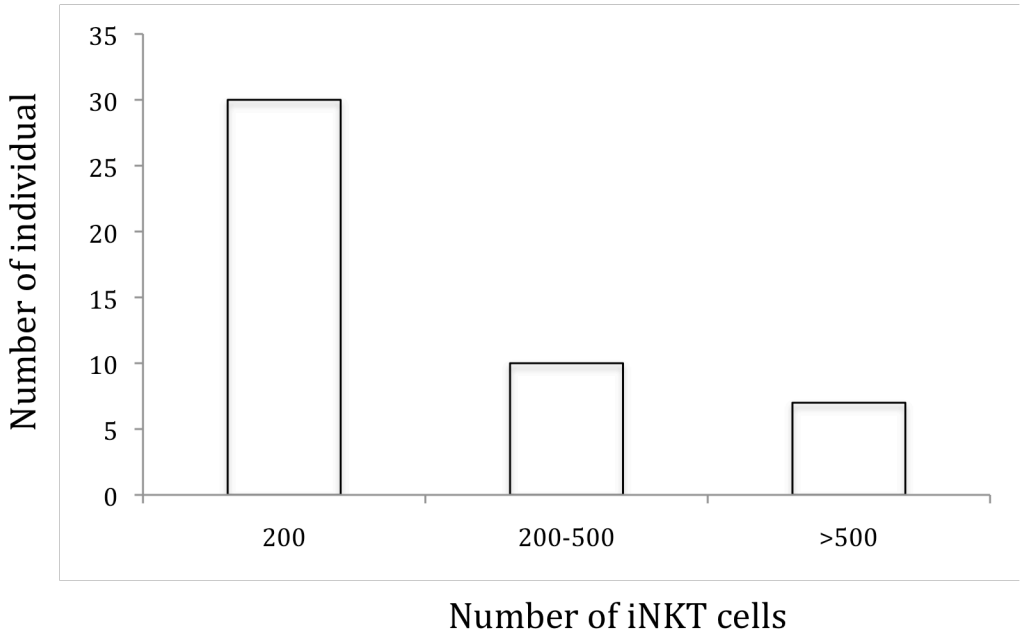


Figure 3.6 The number of variant iNKT cells in the 47 healthy volunteers. Absolute numbers relate to a denominator of 100,000 T cells.

The range of iNKT cells extended over four logs, from 1 to 1100 cells per 100,000 T cells. A skewed distribution was seen in the number of iNKT cells in the control population. Most individuals (30/47) had an iNKT cell number between 0-200, with a mean of around 0.1% of the T cells. Approximately 15% (7/47) of all individuals have more than 500 iNKT cells per 100,000 T cells. We have subsequently dissected the population into individuals with high iNKT cells (>0.5% iNKT cells), medium iNKT cells (0.2-0.5% iNKT cells) and those with low iNKT cells (<0.2% iNKT cells).

We wanted to establish if iNKT cell numbers were stable over a long period of time for an individual. Seven individuals were followed up for up to three years, and no significant changes were identified in each of these healthy controls (Figure 3.7). Our results suggested that the iNKT number was a stable parameter for healthy controls.

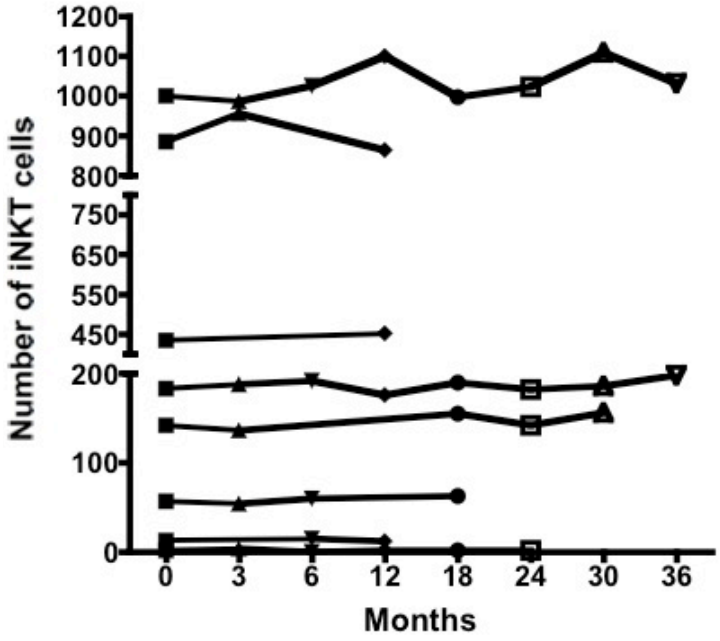


Figure 3.7 Longitudinal changes in iNKT cell number overtime.

3.3.3 Qualitative analysis of invariant NKT cells

As suggested in previous studies, the phenotypic evaluation of iNKT cells identifies functional subsets of these cells. Clonal T cell studies have identified that $CD4^+$ cells mainly promote Th2 like responses whilst $CD4^-$ subsets promote Th1 like responses, through IFN-γ secretion. We extended these analyses by evaluating the CD4 expression **amongst the different groups of individuals** (low compared to medium-high iNKT group) in contrast to previous studies of clonal sub populations from the same individual.

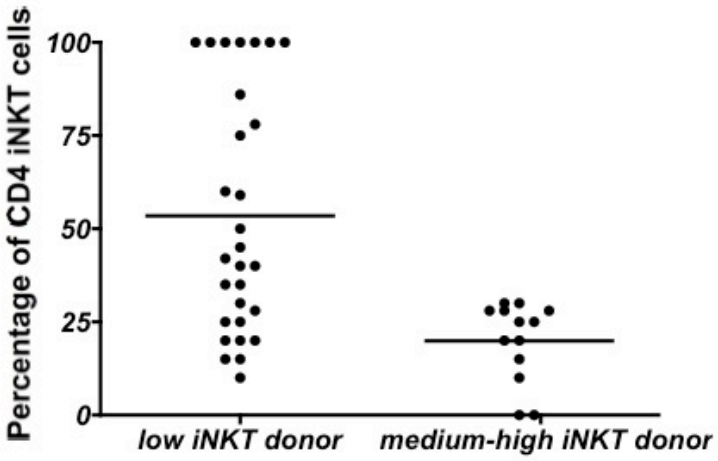


Figure 3.8 CD4 expression on iNKT cells between the low iNKT donor and the medium-high iNKT donor groups. Percentage of CD4 iNKT cells were calculated and analyzed in the low iNKT (<200) and medium-high iNKT population (>200).

As can be seen in Figure 3.7, there is a significant difference in the percentage of iNKT cells expressing CD4 between the 2 groups. The mean percentage of CD4 iNKT cells is notably higher in low iNKT donors (~50% of their iNKT cells) compared to the controls with medium-high levels of iNKT cells (~25% of their iNKT cells) ($p < 0.0074$). This suggested that the number of iNKT cells may correlate with the functional repertoire of these cells for cytokine production in individuals and across healthy populations.

We then further analysed the group to see if the differential CD4 expression seen in the iNKT cells was comparable to that seen in the conventional T cell population.

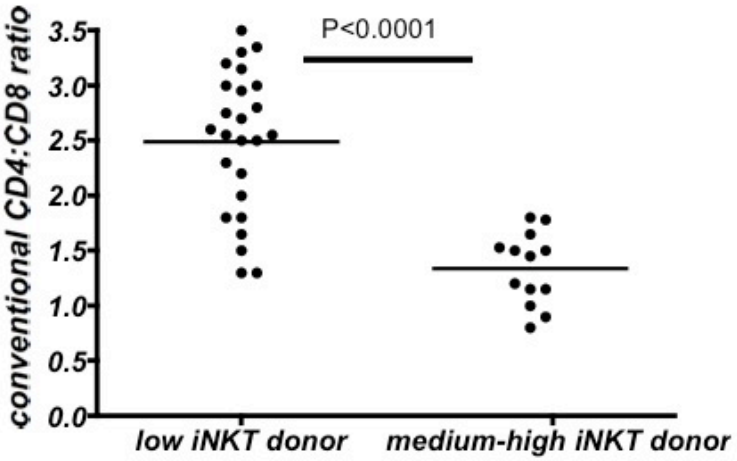


Figure 3.9 Conventional T cell CD4:CD8 ratio in low and high donors

Surprisingly there was also a strong association of iNKT number with the ratio of CD4:CD8 lineage choice in conventional T cells. The control individuals with a lower number of iNKT cells showed a much higher ratio of $CD4^+$ to $CD8^+$ T cells within their conventional T cell lineage compared to the controls with medium-high iNKT cells (CD4: CD8 ratio of 2.5 compared to 1.3 in the conventional lineage, $P<0.001$). To put this another way, those individuals whose T cell selection had resulted in a diverse conventional TCR population with a CD4:CD8 ratio of <1.5 appeared to co-select a higher proportion of iNKT cells. For those individuals who had a low number of iNKT cells (with a CD4 bias), their conventional T cell repertoire is also CD4 biased (as is seen in most normal individuals). We wished to explore this intriguing finding further to assess whether those with low iNKT numbers had a smaller pool of Vα24 or Vβ11 T cells to recruit from.

3.3.4 Immunophenotyping TCR Vα24 and Vβ11 bearing conventional T and iNKT cells

Due to the wide variation in the number of iNKT cells in normal individuals we further analyzed the T cells that expressed the individual Vα24 and Vβ11 receptors (Figure 3.10).

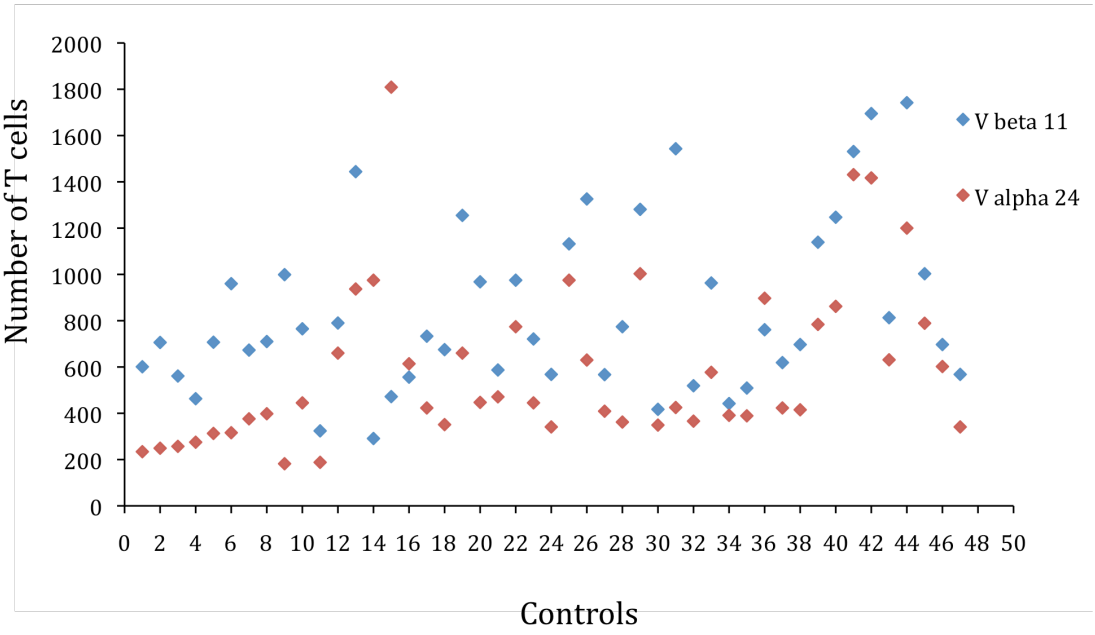


Figure 3.10 The total numbers of Vα24 and Vβ11 in the control population

The results showed that the total number of Vα24 and Vβ11 T cells varied considerably (range 417 to 1695 for Vβ11 and 234 to 1867 for Vα24 per 100,000 T cells) in healthy individuals. The total number of T cells expressing Vβ11 was greater than that for Vα24. Importantly, the mean number of Vα24 cells was no different in those individuals with low NKT cells compared to most of those with medium-high iNKT levels (467 compared to 563 ($P>0.05$)

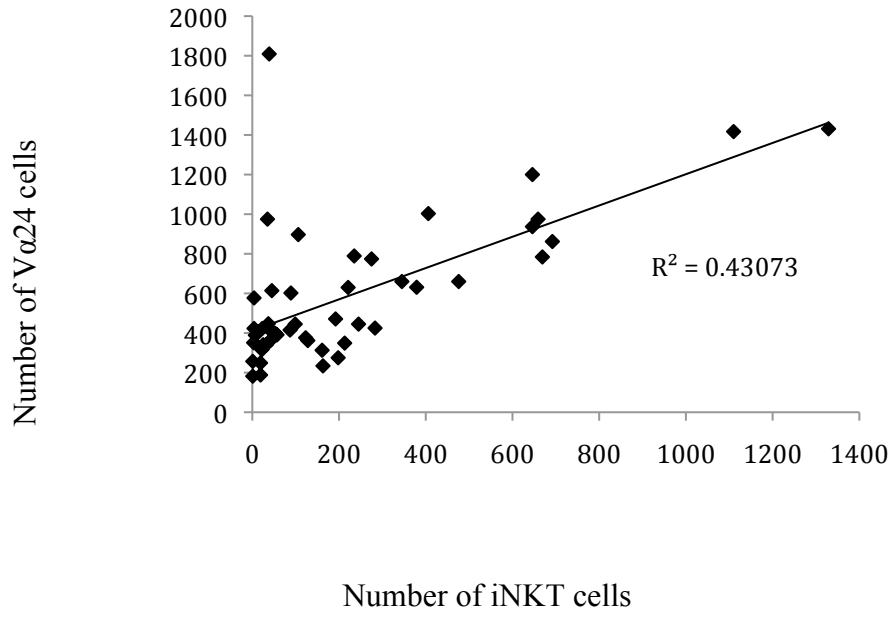


Figure 3.11 Relationship between the total number of Vα24 and invariant NKT cells

To further examine the relationship between iNKT enumeration and the total number of Vα24 and Vβ11 T cells we looked for a correlation between the two.

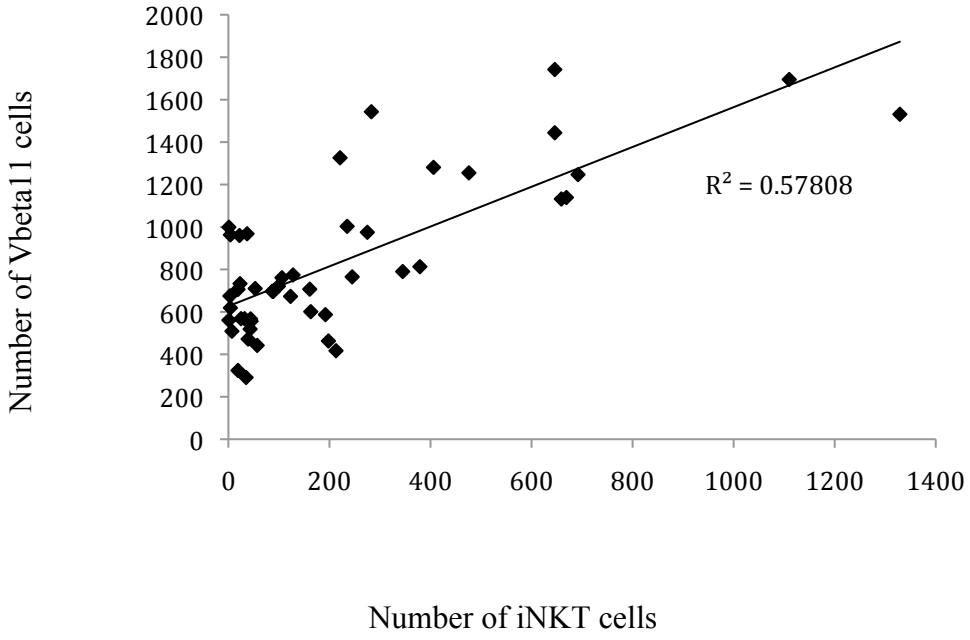


Figure 3.12 Relationship between the total number of Vβ11 and invariant NKT cells

No significant correlations were found between the number of iNKT cells with the total number of Vα24 and Vβ11 T cells (Figure 3-11 and 3-12). This suggests that the number of Vα24 or Vβ11 cells cannot be used to predict the total number of iNKT cells.

Since the number of iNKT cells did not directly correlate with the number of Vα24 and Vβ11 T cells, we examined the percentage usage of these distinct TCR families in the iNKT cells of the controls (Table 3.1).

Sample	Total iNKT cells per 100,000 T cells	Total Vα24 per 100,000 T cells	Percentage of total Vα24 in iNKT cells	Total Vβ11 per 100000 T cells	Percentage of total Vβ11 in iNKT cells
Control1	163	234	69.7	601	27.1
Control2	19	249	7.6	706	2.7
Control3	1	257	0.39	561	0.2
Control4	198	275	72	463	42.8
Control5	161	313	51.4	707	22.8
Control6	22	316	7.0	960	2.3
Control7	123	376	32.7	673	18.3
Control8	53	398	13.3	710	7.5
Control9	1	182	0.55	999	0.1
Control10	245	445	55.1	765	32
Control11	19	188	10.1	324	5.9

Control12	345	660	52.3	790	43.7
Control13	646	937	68.9	1444	44.7
Control14	35	975	3.6	291	12
Control15	39	1809	2.2	472	8.3
Control16	45	614	7.3	556	8.1
Control17	23	423	5.4	733	3.1
Control18	3	351	0.9	675	0.4
Control19	476	660	72.1	1255	37.9
Control20	37	447	8.3	968	3.8
Control21	192	471	40.8	587	32.7
Control22	275	774	35.5	975	28.2
Control23	99	445	22.2	721	13.7
Control24	25	341	7.33	568	4.4
Control25	659	975	67.6	1132	58.2
Control26	221	630	35.1	1326	16.7
Control27	44	409	10.8	567	7.8
Control28	128	362	35.4	774	16.5
Control29	406	1003	40.5	1281	31.7
Control30	213	349	61	417	51.1
Control31	283	425	66.6	1543	18.3
Control32	43	366	11.7	519	8.3

Control33	4	577	0.7	963	0.4
Control34	57	391	14.6	442	12.9
Control35	7	389	1.8	509	1.4
Control36	106	897	11.8	761	13.9
Control37	4	423	1.0	619	0.7
Control38	87	415	20.9	697	12.5
Control39	669	784	85.3	1139	58.7
Control40	692	862	80.3	1247	55.5
Control41	1329	1431	92.9	1531	86.8
Control42	1110	1417	78.3	1695	65.5
Control43	379	631	60.1	813	46.6
Control44	646	1200	53.8	1742	37.1
Control45	235	789	29.8	1003	23.4
Control46	89	602	14.8	697	12.8
Control47	32	341	9.4	568	5.6

Table 3.1 Enumeration of iNKT, Vα24 and Vβ11 T cells in healthy control cohort

It was observed that most of the samples (13/17) with >200 iNKT cells per 100,000 T cells incorporated more than 50% of their total Vα24 chain usage within the NKT cells in peripheral blood (four exceptions had a percentage of around 30-40%). For some of these individuals over 90% of Vα24 T cells were

iNKT cells. In agreement with this, those individuals who were found to have low iNKT cells (<200 per 100,000 T cells) had less than 25% of their total Vα24 usage within the iNKT cells.

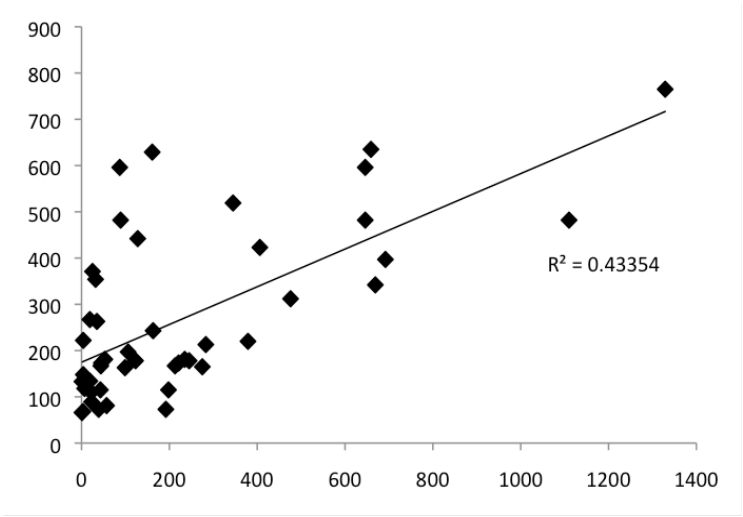
The percentage of total Vβ11 usage in iNKT cells was different to that seen with Vα24. These percentages fluctuated from person to person (0.1-80%), but could still be broadly divided. In those with >200 iNKT cells per 100,000, only 6/17 had more than 50% of their total Vβ11 chain usage within iNKT cells. Those individuals with low iNKT cells (<100 per 100,000 T cells) had less than 15% of their total Vβ11 usage within in iNKT cells. This suggested that the Vα24 restriction element for iNKT selection was more important for iNKT selection choices.

Further evaluations of the immunophenotype of Vα24 and Vβ11 T cells were undertaken. The Vα24 and Vβ11 T cells were divided into $CD4^+$ and non-$CD4^+$ subsets to observe their relationship to the total number of iNKT cells.

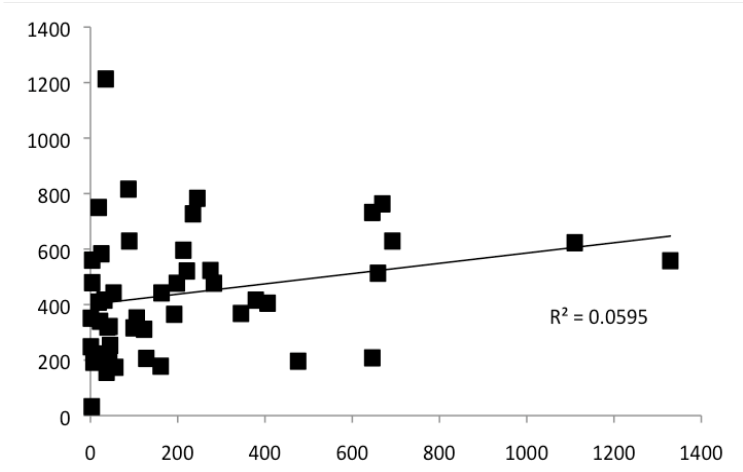
Interestingly, a striking positive correlation was identified between the total numbers of non $CD4^+$ Vα24 positive T cells and iNKT cells within each individual (Figure 3.13b). This association was unique to the non $CD4^+$ Vα24 positive T cells and not shared with $CD4^+$ Vα24 positive T cells, non $CD4^+$ Vβ11 positive T cells or $CD4^+$ Vβ11 positive T cells (Figure 3.12a). This implied that both the Vα24 receptor and CD4 co receptor were influential in determining the selection of iNKT cells.

A

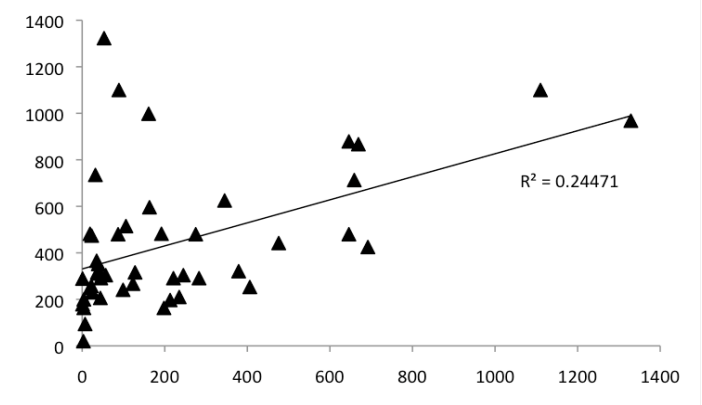
CD4 Vα24 cells vs Number of iNKT cells



CD4 Vβ11 cells vs Number of iNKT cells



nonCD4 Vβ11 cells vs Number of iNKT cells



B

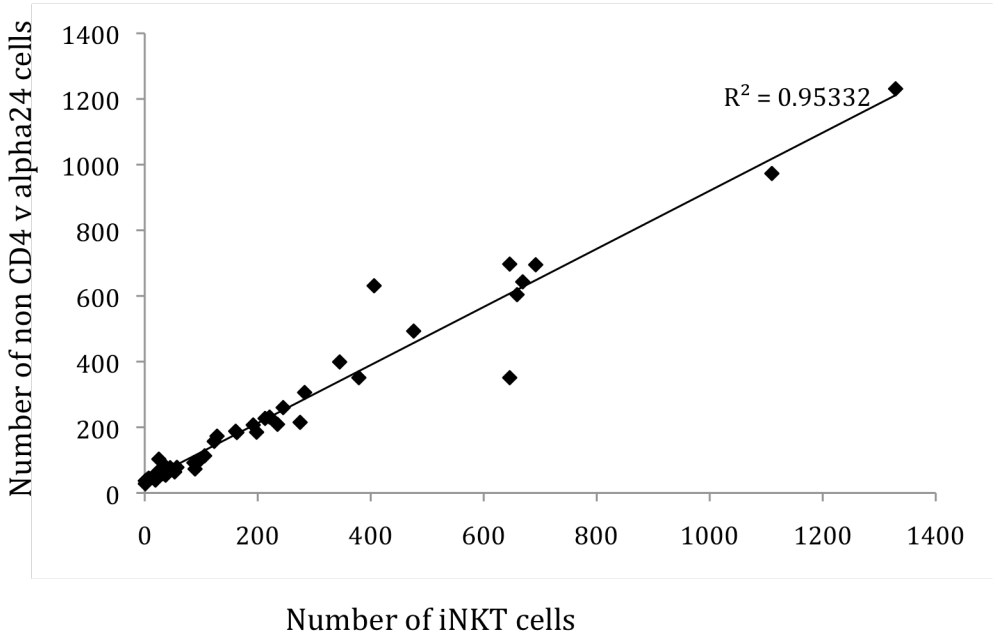


Figure 3.13 (A) Relationship of $CD4^+$ Vα24 positive T cells, non CD4 Vβ11 positive T cells or $CD4^+$ Vβ11 positive T cells with iNKT cell number (B) Relationship of non CD4 Vα24 T cells and number of iNKT cells

In most of the samples with a count of >100 iNKT cells, the Vα24 expression tended to favor non-$CD4^+$ rather than $CD4^+$ cells (i.e. the Vα24 T cells are typically double negative iNKT). In agreement with this, in those samples with <100 iNKT cells count; the immunophenotype of Vα24 favoured $CD4^+$ expression. From Figure 3.13, we can extrapolate that the total number of iNKT cells increases as the total number of non $CD4^+$ Vα24 increases and that the low NKT individuals can be predicted from the $CD4^+$/non $CD4^+$ ratio within their polyclonal Vα24 positive T cells. A ratio of $CD4^+$/non $CD4^+$ Vα24 T cells >2 distinguishes all low NKT individuals from those with >100 NKT per 100,000 T cells in peripheral blood.

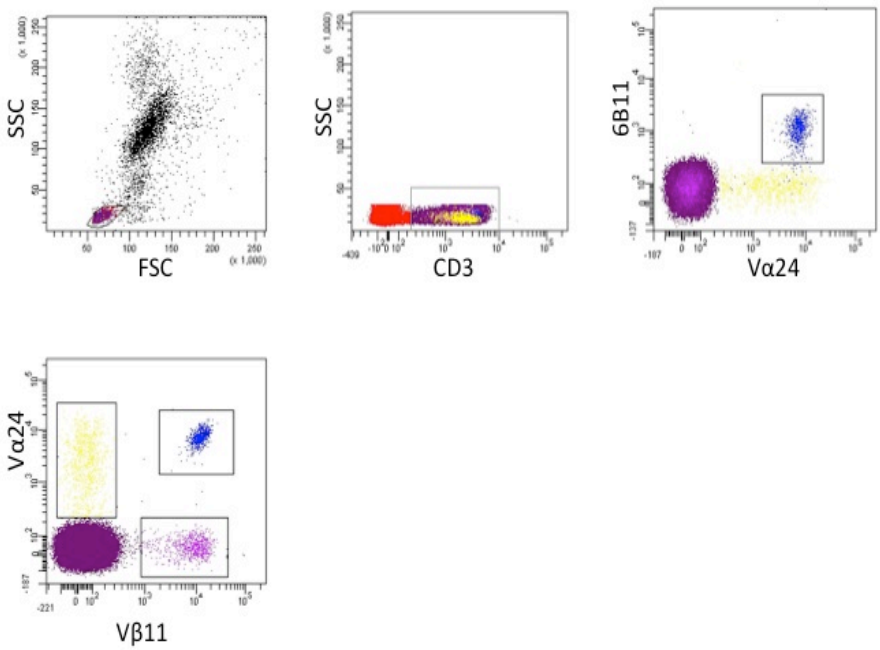
It therefore appears that majority of healthy individuals (63%) have <200 iNKT per 100,000 T cells and that this co associates with such individuals having a

CD4 to CD8 ratio of ~2.5 across their conventional T cell populations. Their Vα24 subsets are composed of a polyclonal Vβx repertoire that are predominantly $CD4^+$ whilst their iNKT cells make up approximately 25% of the subset and are themselves CD4 biased. For those with high iNKT numbers, typically>500 per 100,000, this situation is reversed with a CD4 to CD8 ratio of <1.5 across their T cell populations, iNKT cells accounting for over 85% of Vα24 T cells with a non $CD4^+$ bias. Given this tight association between number and phenotype, and previous linkage to functional outcomes, we further evaluated our population for inherent programming of function.

3.3.5 Functional differences in healthy individuals

We began our functional assessment by first revisiting the phenotyping of our cohort with an antibody that was clonotypic for the invariant Vα24 Jα18 region. We wondered whether the TCR profile of high donors was distinctive from that of the low donors and whether this was influential in shaping the co receptor phenotype of the populations and potential functional attributes (Figure 3.14).

A



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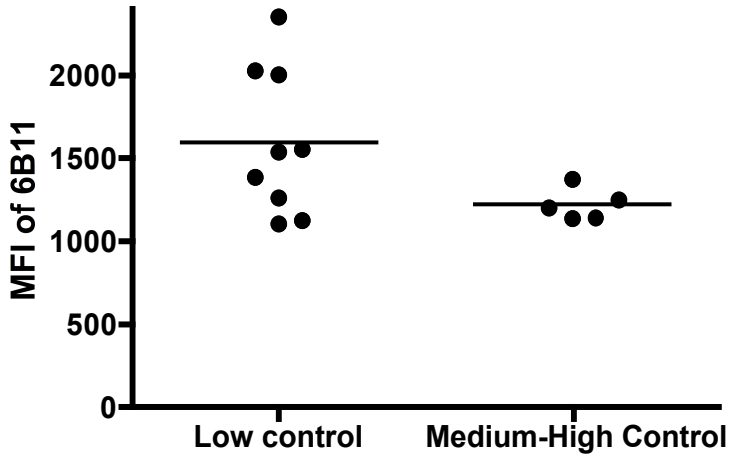


Figure 3-14(a) Analysis of 6B11 expression by FACS. The 6B11 antibody was used in combination with Vα24 and Vβ11 antibodies. (b) Analysis of 6B11 expression on 9 low donors and 5 high donors

This examination suggested that another feature of iNKT cells that was distinctive between low and high donors may operate at the T cell receptor level. The different MFI of the clonotypic antibody for the invariant TCR across the 2

groups was suggestive of a differing average affinity of the TCR between the 2 groups. This observation may be important for both their thymic selection and subsequent antigen responsiveness and function.

To investigate this further we first established whether there was a difference between low and high donors in their capacity to proliferate and sustain an expansion following activation with α-GC. We performed a 7-day proliferation assay using 10^6 PBMC and recorded the result as the fold change in number, per 100,000 T cells compared to day 0 (Figure 3.15).

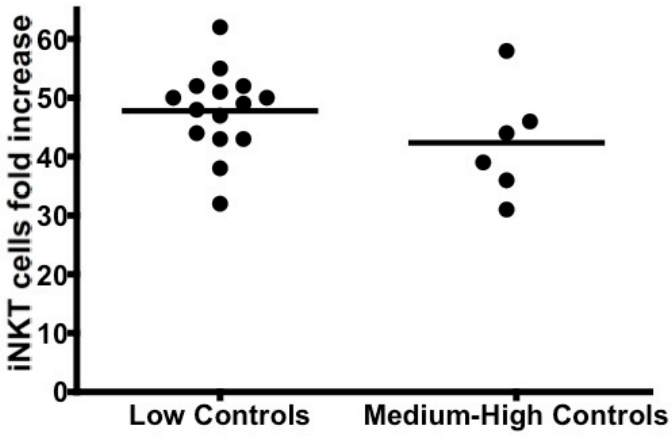


Figure 3.15 Proliferation of iNKT cells in 7-day culture in low and medium-high controls. The number of iNKT cell were enumerate pre-proliferation and enumerate again post-proliferation using flow cytometry.

These experiments showed that both control groups mounted robust proliferative responses with comparable stimulation indices of 40-50 fold.

In light of the previous descriptions of a cytokine bias associated with distinct iNKT subpopulations we evaluated the cytokine signatures of the iNKT populations within our distinctive control group populations. Ex vivo cultured ELISpot assays were set up to measure the Th1, Th2 and Th17 representative cytokines released at day 7, following activation of 1 x10^6 PBMC with 100ng/ml

of α-GC, amongst the 2 groups of healthy individuals. The evaluations from three representatives high and low donors are shown below (Figure 3.16).

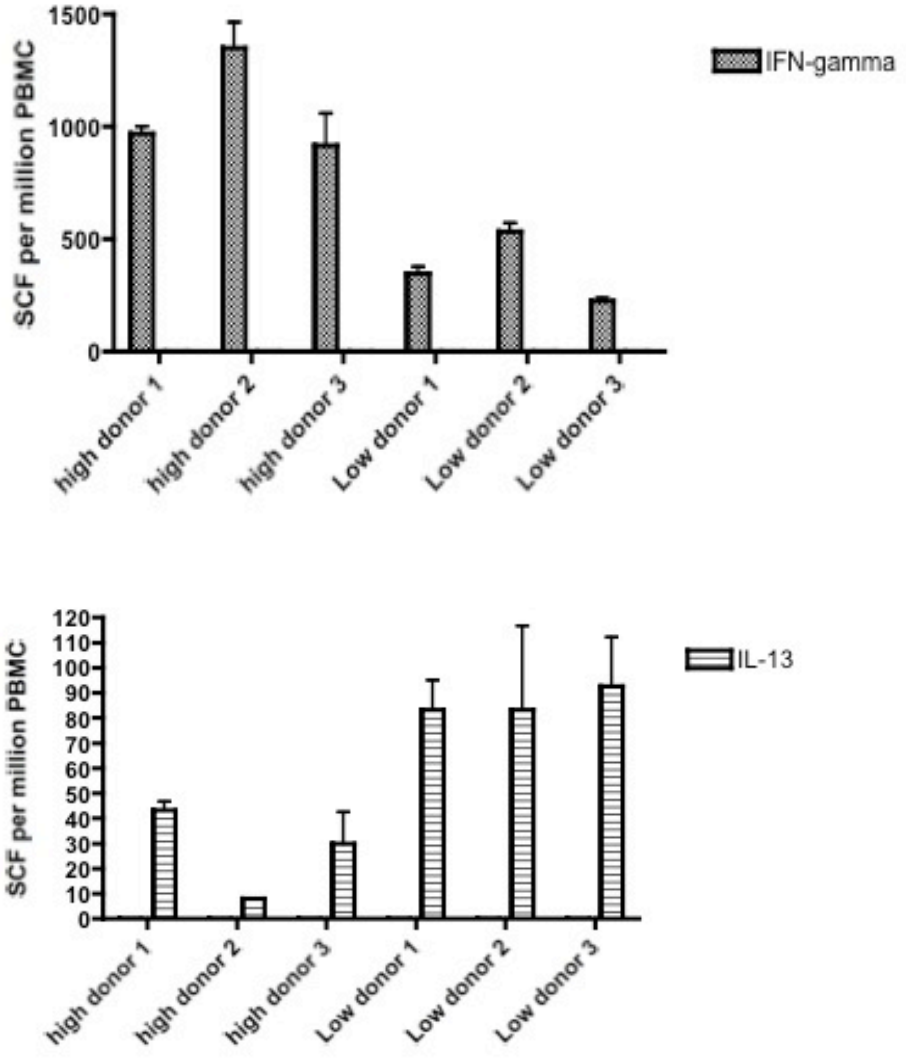
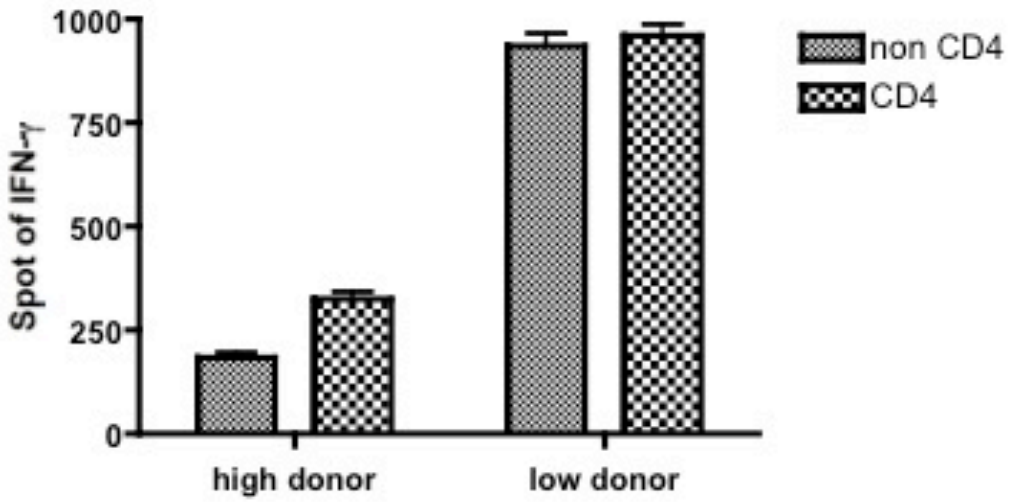


Figure 3.16 Cultured ELISPOT in high and low donors. 4 x 10^5 cultured PBMC were taken to perform the ELISPOT, each pulsed with 1 x 10^5 α-GC pulsed irradiated PBMC.

In the high donor group, the majority of the cytokines produced by iNKT cells were IFN-γ with no or little Th2 cytokines (i.e. IL-13). This marked Th1>Th2 bias in the high donors is likely a representation of the skewed DN iNKT populations present in such individuals In contrast, a significant level of Th2

cytokine production was observed in the low donor group with a less marked Th1>Th2 skew. However, it is worth noting that Th1 cytokine is still the dominant cytokine produced in the low donor group, but is unclear as to which iNKT subpopulation is producing each cytokine in these individuals. Due to the different starting numbers of iNKT between these 2 groups no direct comparison of cytokine production per donor could be assessed. We therefore developed a sorting experiment to control for cell number entry and determine whether there were inherent differences between iNKT cell subpopulations across different control donor groups. To achieve this we FACS sorted 1000 cells of double negative or $CD4^+$ iNKT cells from high and low donors following a 7-day cultured ELISpot (Figure 3.17). These cells were then co-cultured with 10^5 irradiated autologous PBMC pulsed with 100ng/ml of α-GalCer overnight prior to determination of their cytokine production.



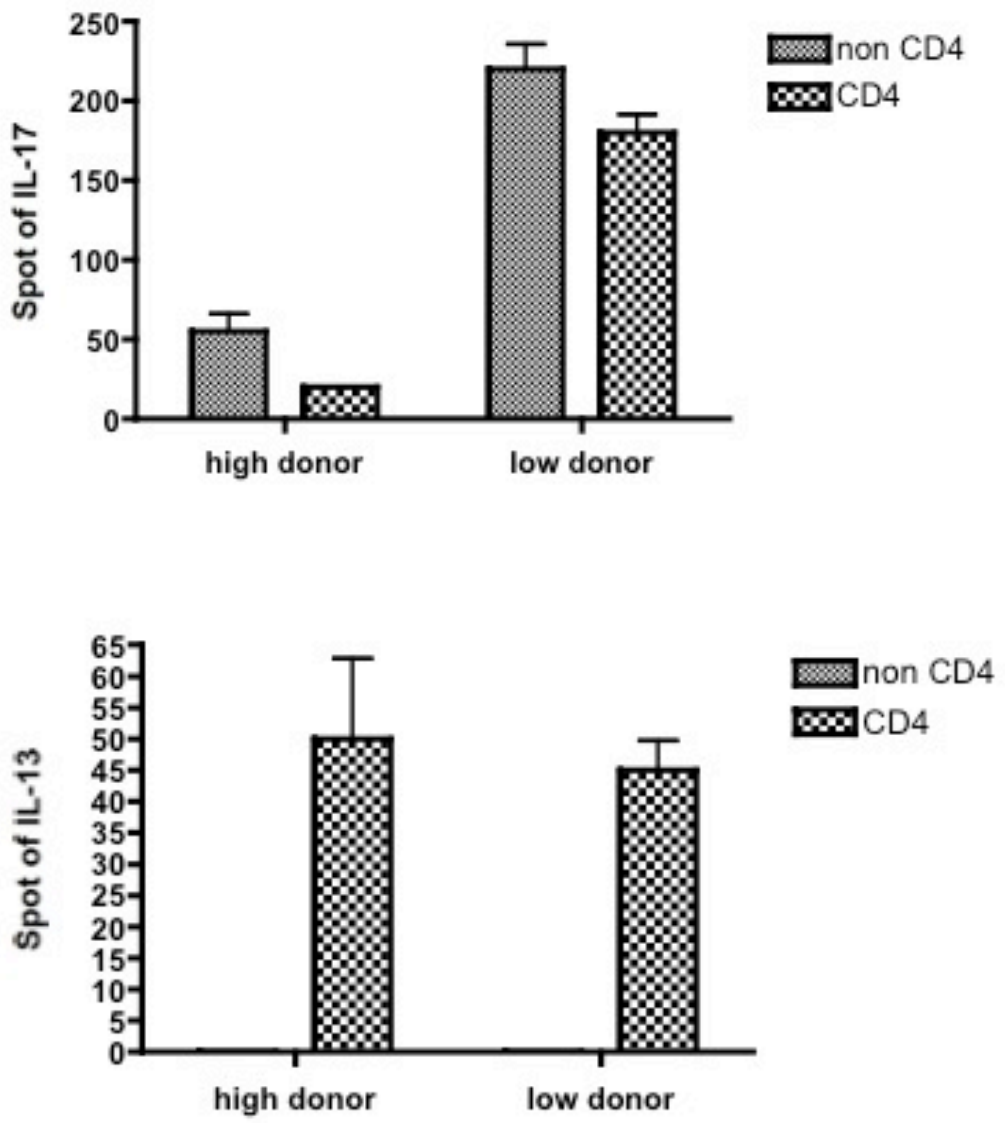


Figure 3.17 1000 sorted iNKT cells both from double negative and CD4 populations of high and low donors were used to perform the cultured ELISPOT, pulsed with 10^5 PBMC with 100ng/ml α-GC.

From Figure 3.17 it can be seen that IFN-γ production is comparable between $CD4^+$ and non-$CD4^+$ iNKT cells in both low and high donors but is approximately 4-5 fold higher from iNKT of the low donors. This inherent behavior of the iNKT cells from the low donors is not predicted from the

proliferation studies. The Th2 type cytokine i.e. IL-13 is only released by the CD4+ iNKT cells, consistent with previous reports on cell lines. The production is surprisingly similar across the high and low donors although it should be noted that some high donors do not possess a CD4 iNKT population and would therefore be deficient for this function. For IL-17, both donors and iNKT cell populations produce this cytokine. The non-CD4 iNKT cell populations predominantly release IL-17 and it is interesting to note that the iNKT cells from low donors were more responsive compared to the high donors.

3.3.6 Summary

• INKT cells in humans are stable and have a distribution that can extend over 4 logs, with all iNKT populations having a distinctively higher expression of Vα24 and lower expression of CD3 compared to conventional T cells

• The CD4 and non CD4 co receptor phenotype of iNKT cells is associated with the number of iNKT cells in controls, with a CD4 dominant profile in low controls and a non CD4 dominant profile in high controls

• The high and low status of iNKT controls is directly or indirectly related to the CD4/CD8 ratio in conventional T cells selection, such that low controls have a ratio of >2.5:1 and high controls ~1.5:1 ($P< 0.0001$).

• The selection of iNKT cells is influenced by the use of CD4 by developing Vα24 T cells such that where there is predominance of conventional Vα24 $CD4^+$ T cells, few iNKT cells are present. Conversely as iNKT numbers increase the DN signature of these cells dominates the Vα24 lineage with few conventional Vα24 $CD4^+$ T cells present.

• iNKT cells from high donors appear to have a different TCR signature to low donor iNKT cells (MFI intensity to a clonotypic (6B11) antibody).

•Functional analyses of iNKT cells in all controls shows restriction of TH2 type cytokines to the CD4 subset, whilst IFNγ is comparable across both CD4 and non-CD4 subsets and IL-17 is predominantly from the non-CD4 subset.

• Whilst iNKT cells in low and high donors have comparable proliferation profiles there appears to be a significant increase in IFNγ and IL-17 production on a cell-cell basis in low donors compared to high donors.

3.4 Discussion and conclusion

Previous studies have shown that the number of iNKT cells differs greatly between different individuals (122). However, little is known about what give rises to these variations. This study has shown that this variability is not just related to the overall usage of Vα24 or Vβ11 but is predictable by the usage of CD4 in Vα24 T cells. Furthermore a relationship exists between the number of iNKT cells and (i) the CD4: non CD4 ratio of invariant Vα24Vβ11 NKT cells, (ii) the partitioning of cytokine function to these subsets across low and high donors and (iii) the CD4:CD8 ratio of all conventional T cells. This complex arrangement is not easily explained and may relate to many factors that shape both the development of iNKT cells and conventional T cells both in the thymus and in the periphery.

Within the TCR instructive model, iNKT cells first undergo TCRβ rearrangement before TCRα (237). The expression of the invariant Vα24Vβ11 TCR and its commitment to the iNKT lineage is not determined until the DP stage. The potential iNKT cells expressing Vα24 (and Vβ11) in the double positive stage may then react with the CD1d expressed by a double positive developing thymocyte within the thymic cortex (66). If the TCR binding is too strong, the pre-iNKT cells will die from negative selection. If the pre-iNKT cells do not bind to any CD1d on double positive thymocytes, they will then die from neglect. Only those that bind with a moderate affinity will survive and mature to become an iNKT cell. This receptor affinity model has been previously proposed to account for the final DN bias of NKT cells where it has been argued that the TCR affinity is sufficient for positive selection whilst co receptor signaling leads to negative selection. Concurrent with this idea are the studies that use mouse strains that ectopically express MHC class II on the CD1d positive thymic cortex, leading to a suppression of iNKT cell development in these mice (229, 238, 239).

Another interesting result in this project is that in those individuals with low iNKT cells, the immunophenotype of Vα24 conventional T cells favoured CD4+

expression. How might this fit with the TCR instructive model? As Vα24 positive T cells are not absent in these individuals (instead associating with other Vβ chains and often utilizing the CD4 co-receptor), this suggests that classical T cell selection may be favoured and CD1d selection disfavoured in these individuals. This could occur if most double positive cells with an expression of Vα24 and Vβ11 die from negative selection upon CD1d and CD4 engagement, with residual Vα24 T cells, perhaps with a lower affinity for CD1d and MHC class II, left for conventional T cell selection with a CD4 coreceptor bias. In recent years, many studies have been performed on Vβ spectratyping, but only very few studies have analysed Vα spectratyping. A study of TCRα spectratyping showed that most of the expressed Vα24 will predominantly be expressed with Vβ11, and that the dominant phenotype for Vα24 T cells is double negative(240). The results in my thesis show that this is not always the case. In fact, 29 out of 47 healthy individuals (61.7%) have shown predominance of CD4 usage in total Vα24 T cells, most often in low iNKT controls. See appendix table 1.

It is tempting to speculate that high iNKT controls, where CD4 dominance is not seen in iNKT cells, may preferentially bypass negative selection perhaps through utilization of a different Vα24:Vβ11 CDR region configuration that would normally be negatively selected in low controls where CD4 may play an additional role. Indeed the iNKT cells of low donors have a heterogeneous 6B11 signature that may be consistent with a wider spectrum of invariant TCRs being representative of low iNKT donors that have passed through strict negative selection in the presence and absence of CD4.

The function of iNKT cells is highly related to their phenotype (163, 224). Previous studies have shown that the double negative subset predominantly releases the Th1 type cytokine and the Th2 type cytokine is secreted by the CD4 subset. I show in my work that there is not only a relationship between the subsets and cytokine release, but that this is influenced by the status of the donor for subset partitioning as well as a quantitative differences between such subsets **across low and high control groups.** At a population level this results in high

donors preferentially producing the Th1 type cytokines, whereas low donors are capable of producing both Th1 and Th2 cytokines. Finally we have established that iNKT cells in low donors are more responsive for cytokine production compared to the iNKT cells in high donors. We would postulate that this functional attribute is linked to the selection process that distinguishes high and low donors at the multiple levels we have described. Further studies should address the affinity of the invariant TCRs of low and high controls to establish whether any differences here may account for the quantitative and qualitative differences that we have identified in the control populations.

Chapter 4 Effect of Environment on iNKT cells

4.1 Introduction

INKT cells are able to produce Th1, Th2 and Th17 types cytokines. They have been recognized as one of the most important cells in bridging innate and adaptive immunity (241). The key to an effective immune response is often the initial cytokine milieu that can subsequently polarise a T cell response (242). The diverse cytokines produced by these cells make them the ideal candidate to operate as important immunoregulatory cells.

As with other immune cells, we postulated that iNKT cells were highly influenced by their local environment, in particular, the types of antigen presenting cells and cytokines present.

4.1.1 Invariant NKT cells and antigen presenting cells

INKT cells respond to glycolipid bound CD1d presented by antigen presenting cells, to enable activation and proliferation. Studies have suggested that the effector cytokines produced by iNKT cells may differ according to the characteristics of the lipids and presentation pathways utilised. The loading of the prototypic ligand α-GC requires endocytosis by a lipid carrier protein, such as ApoE, entering the endosomes for subsequent processing (243). The subsequent processing is highly dependent on the pH and presence of lipid transferring proteins (244). Thus, glycolipids like α-GC will preferentially load to phagocytic CD1d antigen presenting cells such as dendritic cells and macrophages. In contrast, a α-GC analogue OCH, can be directly loaded on the cell surface leading to the preferential production of Th2 type cytokines (245). This glycolipid with a shortened acyl and sphingosine chain can be easily loaded on cell surface CD1d of non-phagocytic antigen presenting cells such as B cells (244). A further notable difference between such phagocytic and non phagocytic

CD1d positive antigen presenting cells is their own cytokine production and level of co-stimulatory molecules (244).

The dendritic cell has been recognized as one of the most potent antigen presenting cells. Studies have shown that both human and mouse dendritic cells can efficiently present α-GC to iNKT in vitro and in vivo (213, 242, 246, 247). In vitro studies of human iNKT cells have shown that both immature and mature dendritic cells were able to induce proliferation and cytokine production of iNKT cells. These studies demonstrated that at a low effector:target cell ratio, mature dendritic cells induced greater proliferation and IFN-γ production. No differences were observed between monocytes, immature and mature dendritic cells at a higher effector:target cell ratio (246). However, a more recent study showed that the cytokine production by the iNKT cells is not always preferential to IFN-γ through dendritic cells. The study by Iwabuchi et al. suggested that the pre-treatment of mature dendritic cells is as important as the antigen presenting cells themselves. They suggested when pre-treating dendritic cells with IL-4, a high level of IFN-γ production was achieved by iNKT cells; in contrast, when pre-treating dendritic cells with IFN-γ, a higher level of IL-4 was produced (242).

A high level of CD1d is expressed on B cells, in particular marginal zone B cells (248). A study by Batista et al. demonstrated how the B cell receptor could recognise a specific CD1d antigen in vitro. They identified that the B cell receptor binds to lipids with a very low affinity that is sufficient for receptor mediated endocytosis (249). They also suggested that after initial recognition by the B cell receptor, iNKT cells are required to further help B cell proliferation and specific antibody production (249).

4.1.2 iNKT cells and cytokines

Early murine studies found that cytokines were extremely important for the development of iNKT cells, several important cytokines/receptor have been

identified in receptor knockout mice. Table 4.1 gives a summary of the key cytokines that influence the development of iNKT cells (250).

	Stage 1 $CD44^-$ $NK1.1^-$	Stage 2 $CD44^+$ $NK1.1^-$	Stage 3 $CD44^+$ $NK1.1^+$
IL-2Rß (251)	Not known	Not known	Reduction
IL-7 (252)	Reduction	Reduction	Reduction
IL-15 (252, 253)	Normal	Normal	Reduction
IL-15Rα (252, 254)	Normal	Normal	Reduction
IFNγR (255)	Normal	Normal	Normal
IL-12Rβ (256)	Not known	Not known	Normal
IL-4Rα (256)	Not known	Not known	Normal
CD45 (257)	Not known	Not known	Reduction
CD137 (258)	Not known	Not known	Reduction
GMCSFRβ (259)	Not known	Not known	Reduction

Table 4.1 Summary of Cytokines effect on iNKT cells

As shown above, most of the cytokines and their receptors do not affect the early development of iNKT cells. The only cytokine is known to significantly affect the early development of iNKT cells is IL-7. Most of the other cytokines have their influence in later stages of iNKT development and maturation. This maturation can be influenced both in the thymic and extrathymic compartments.

To date, three cytokines have been found to play an important role in the function of iNKT cells (260-262). In early studies, IL-7 was the cytokine thought to play a major role in iNKT cell proliferation and cytokine production, both in humans and mice (262). It was suggested that IL-7 drove a Th2 type biased iNKT cytokine profile, whereas IL-12 would drive a Th1 type biased cytokine production. In vitro studies have also found there is significant contribution of IL-2 and IL-15 in the ex-vivo proliferation of iNKT cells (261).

4.2 Aims of this study

- Investigate the effect of different APC's on iNKT cell proliferation and cytokine production

- Investigate the effect of the cytokine milieu on iNKT cell proliferation and cytokine production

4.3 Results

4.3.1. Cytokine and APC effects on iNKT cells of high and low donors

Previous research has suggested that both cytokines and the nature of the antigen presenting cell can influence iNKT cell function. However, the combined effects of both antigen presenting cells and cytokines upon ex vivo polyclonal iNKT cells have not been systematically studied. In this component of my thesis, I have examined this aspect by systematically depleting various CD1d expressing antigen presenting cells including B cells, monocyte and myeloid dendritic cells. During this study, plasmacytoid dendritic cells were not examined because previous studies have suggested the plasmacytoid dendritic cells have no CD1d expression on their cells surface (263). As discussed in the previous chapter, controls may be considered as low or high donors with distinctive characteristics. Therefore, we continue to divide the controls into two groups when investigating iNKT cell proliferation and cytokine production.

As seen in Figure 4.1, the nature of the antigen presenting cell does have an influence on iNKT cell proliferation. For high donors there appears to be ‘APC redundancy’ as all depletions (mDC, B cells or monocytes) retain a proliferation index that is similar to the undepleted PBMC setting. In contrast the low donors (i.e. majority of healthy controls) show comparable levels of proliferation in the absence of B cells or mDC but are absolutely dependent upon monocytes for their activation.

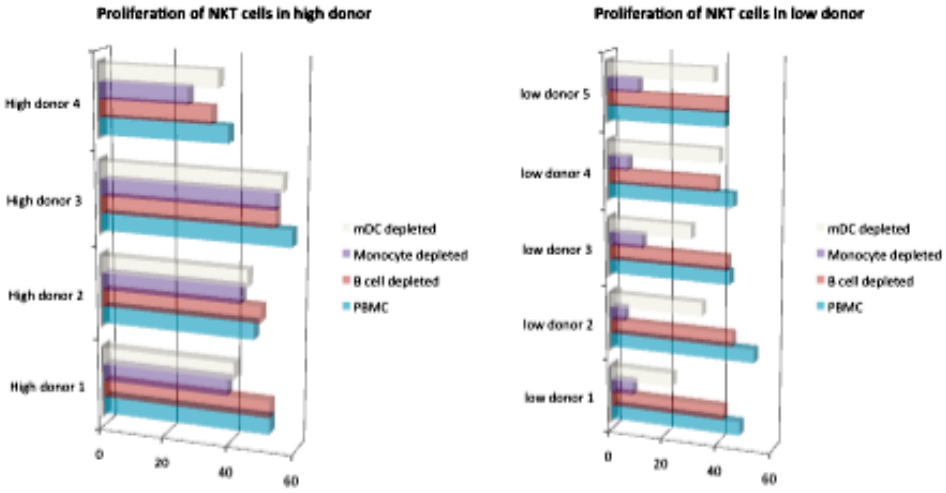
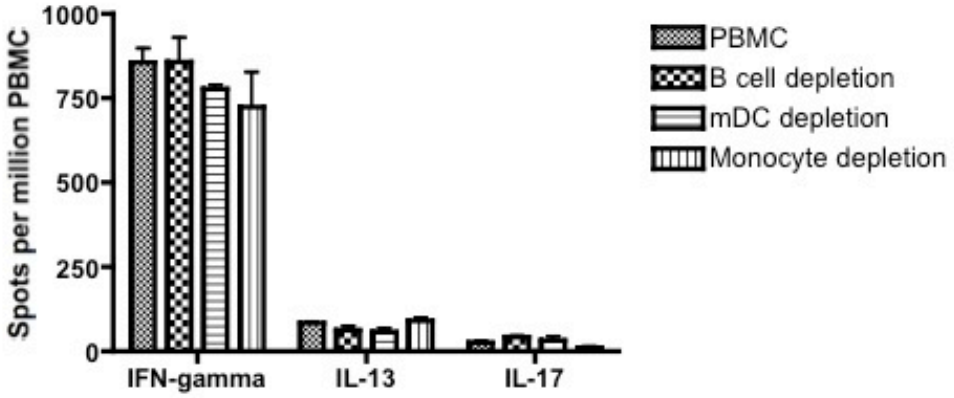


Figure 4.1. Proliferation of iNKT cells in high and low donors with selected antigen presenting cells. The antigen presenting cells were selectively depleted and cultured with iNKT cells for seven days. The proliferation index was calculated by comparing the number of iNKT cells pre and post culture.

This approach was also extended to cytokine production by PBMC under the above conditions (Figure 4.2). These results support the proliferation studies with monocyte depleted cultures having no ability to support cytokine secretion from iNKT cells of low donors. The production of IFN-γ, IL-13 and IL17 was not that different across the other depletion settings in both high and low donors.

A



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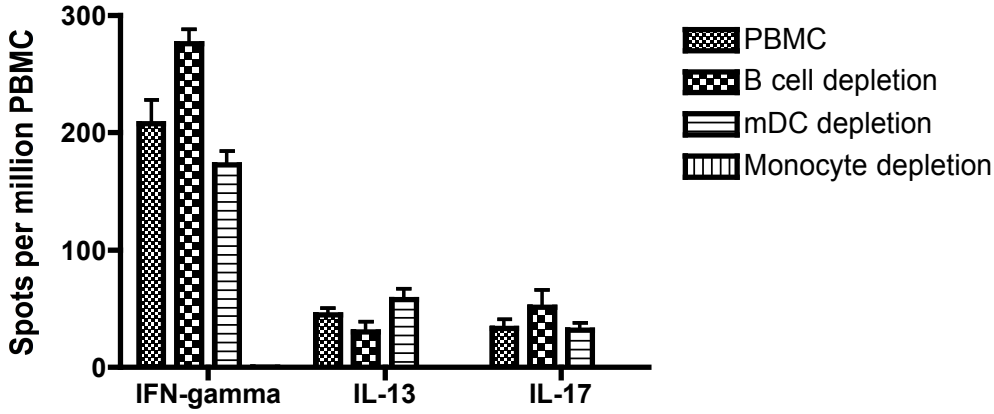


Figure 4.2. Cytokine responses of high and low donors following CD1d +ve subset depletion. (A) Cytokine analysis in high donor. (B) Cytokine analysis in low donor.

To extend these observations the monocyte population was then returned to the monocyte depleted cultures, and the proliferation was reassessed. Figure 4.3 shows that this restored the normal proliferation capacity.

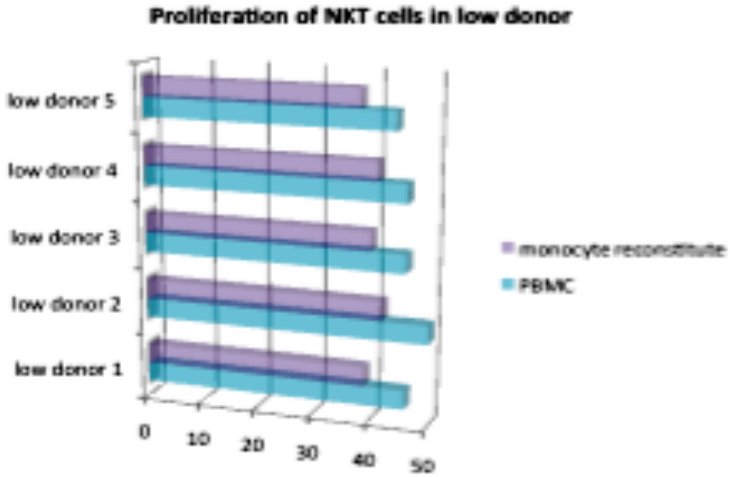


Figure 4.3. Proliferation of iNKT cells in low donors following monocyte reconstitution.

As CD1d is highly expressed in most B cells, and was sufficient to support iNKT cell proliferation in high donors we artificially increased the input number of iNKT cells in the culture of the low donors to see if this restored responsiveness. Figure 4.4 shows that even when the iNKT cell numbers are artificially increased to that of a high donor they remain inactive in the presence of only B cells.

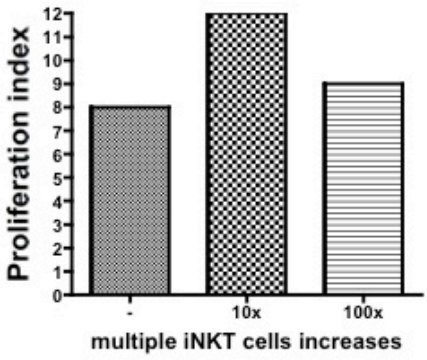


Figure 4.4. iNKT proliferation in low donors following an increase in iNKT cell number input. The first bar represent no artificial increase in iNKT cells (230 cells in culture), second bar represents a ten fold and the last a 100x increase of the normal number.

Previous studies have suggested that IL-2, IL-7 and IL-15 are important for the proliferation of iNKT cells. IL-2 and IL-15 are believed to be more important for the extrathymic maturation and proliferation of iNKT cells. As all the proliferation experiments involve the addition of IL-2, IL-15 was chosen for further examination as an influence on promoting INKT cell proliferation.

Figure 4.5 shows the effect of IL-15 in both high and low iNKT controls with depletion of selected antigen presenting cells. No significant changes were observed in comparison to Figure 4.1, suggesting that the simple production of IL15 from monocytes was not an explanation for the differences in reactivity between the low donor iNKT cells and the type of effective APC.

Of additional note was that the provision of this cytokine did not particularly enhance the proliferation of iNKT cells in the controls with high or low iNKT cells.

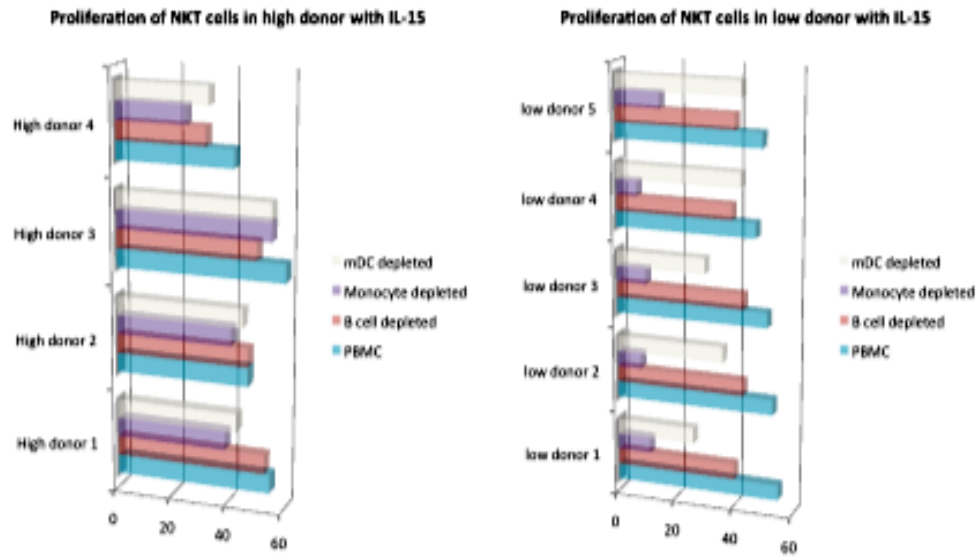
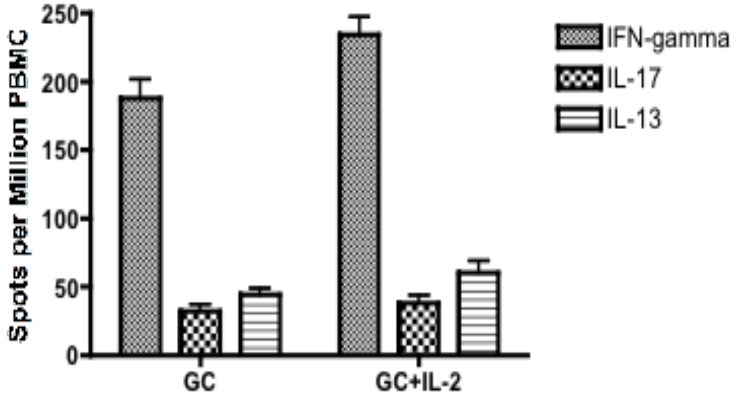


Figure 4.5. Proliferation of iNKT cells with IL-15 in high and low controls.

4.3.2. Effect of cytokines on qualitative iNKT cell responses.

Following on from the observation that IL-15, in the presence of IL-2, did neither promote iNKT proliferation of both donor groups or correct proliferation in the absence of monocytes we looked at the iNKT cytokine responses that might be influenced by these additional cytokines. With the addition of IL-2, a small increase in total cytokine production was observed (Figure 4.6). IFN-γ, IL-17 and IL-13 showed an overall increase but this did not achieve statistical significance.

A



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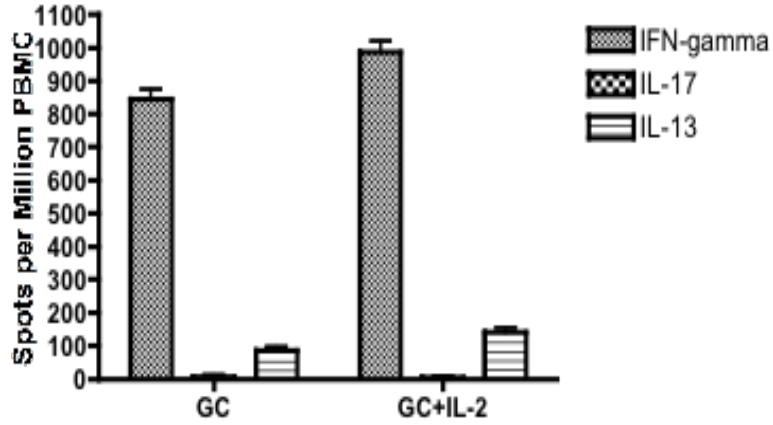
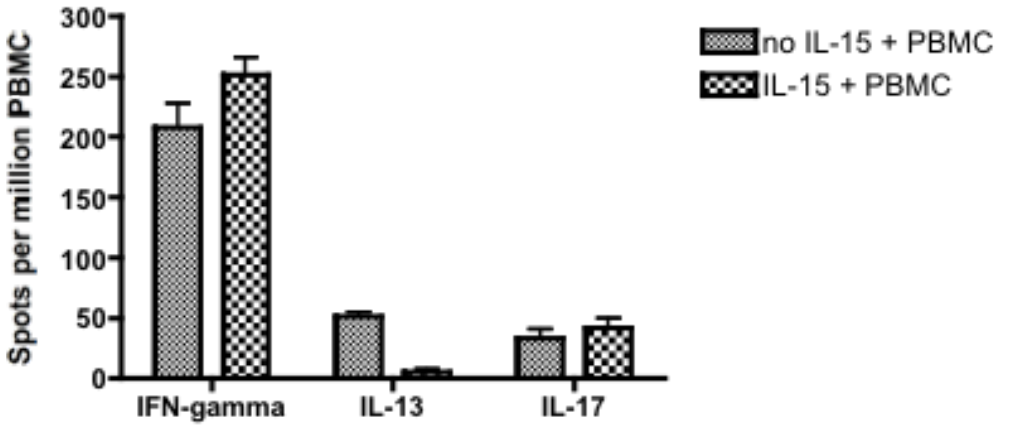


Figure 4.6 Influence of IL-2 on cytokine secretion of low and high donors.

(A) Cytokine analysis in low donors. (B) Cytokine analysis in high donors. Representative of 4 selected donors.

Following addition of IL-15 a notable difference occurred between the cultures of both high and low donors when PBMC where used as the APC (Figure 4.7). A reduction in IL-13 production by the iNKT cells was observed in high and low donor iNKT cell cultures in the presence of IL-15. The sharp decrease of IL-13 production was accompanied with an increase of IFN-γ in both IL-15 cultures. No significant differences were observed in IL-17 production in both high and low donor iNKT cell controls.

A



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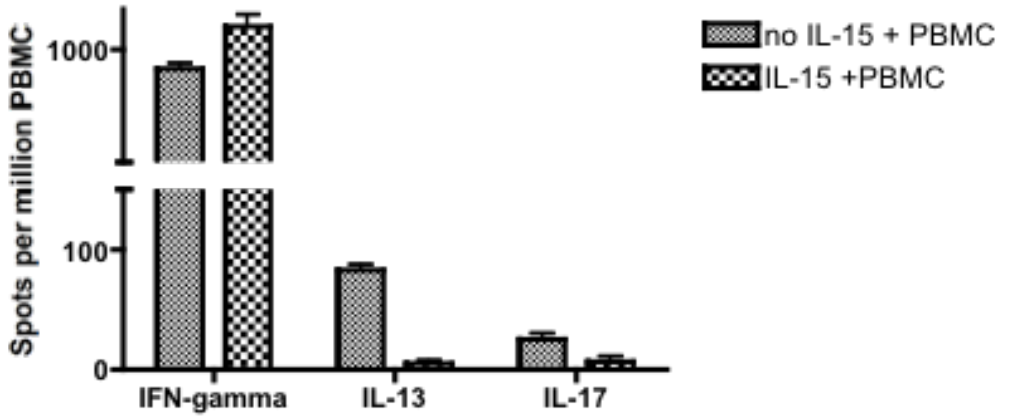


Figure 4.7. Analysis of iNKT cytokine responses in the presence and absence of IL-15. (A) Cytokine analysis in low donors (B) Cytokine analysis in high donors. Representative of 4 selected donors.

We further investigated this observation to assess whether the IL-15 effect was dose dependent (Figure 4.8). A titration series was undertaken on a low donor

and a clear relationship was established between the amount of IL-15 and the degree of IL-13 reduction following antigen specific activation of iNKT cells.

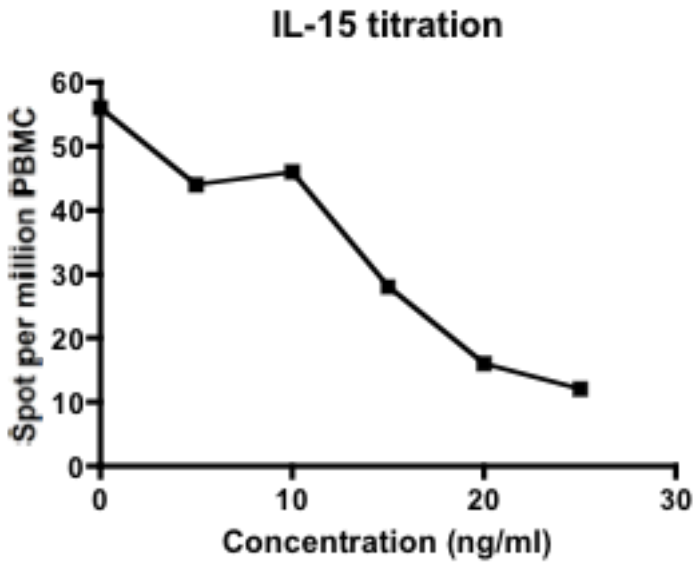


Figure 4.8. Effect of titrated exogenous IL-15 upon IL-13 production from iNKT cells.

IL-15 is able to influence the qualitative response of iNKT cells towards a Th1 profile but is unable to further increase IL-2 supported proliferation or B cell activation of iNKT cells from low donors.

4.3.3. The effect of monocytes and IL-15 on the cytokine production of iNKT cells of high donors.

As described previously, monocytes are critical for the proliferation (and cytokine production) of iNKT cells from low donors, but were not indispensible for the proliferation of iNKT cells from high donors. To extend our evaluation of the influence of monocytes as APC's upon iNKT cell functions, we evaluated

cytokine responses in high iNKT donors in the presence and absence of these cells. We were also interested in how IL-15 influenced the cytokine skewing, reduced IL-13, of iNKT and therefore combined cytokine addition and cell depletion to see if the mechanism of these observations could be established.

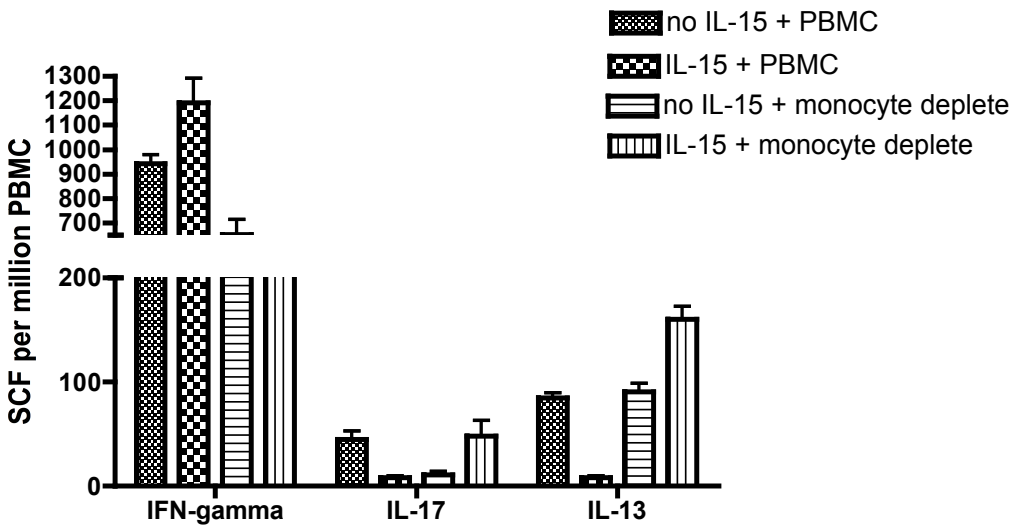


Figure 4.9. The effect of monocyte depletion and IL-15 addition on iNKT cells of high donors

In association with Figure 4.7b it can be seen that a maximal IFN-γ response is seen with PBMC and IL-15 (~1100/10^6 SFC), reducing slightly in the absence of IL-15 (~900 SFC). The depletion of monocytes from PBMC (in the absence of IL-15), whilst not influencing the previous proliferation studies, effects the cytokine responsiveness, reducing the IFN-γ response further (650 SFC). Surprisingly the addition of IL-15 to monocyte depleted PBMC leads to the greatest reduction of IFN-γ, suggesting an inhibitory effect in the absence of monocytes. For IL-13 it can be seen that the previous reduction upon IL-15 addition (Figure 4.7b) is dependent upon the presence of monocytes as it is not replicated in the absence of monocytes. Therefore in high donors where

monocytes are not indispensible for supporting proliferation they are necessary for a maximal IFN-γ response and IL-13 reduction in the presence of IL-15.

It is known that the β-receptor for IL-15 (CD122) is common with that of the high affinity IL-2-R (CD25, CD122, CD132). IL-15 is distinctive in being able to mediate cis and trans presentation, and it is notable that the IL-15 β-receptor is expressed on iNKT cells. The alpha-receptor is unique for IL-15, and it is expressed on certain antigen presenting cells. Thus, a further investigation was performed to establish whether IL-15 receptor α was differentially expressed between B cells and monocytes.

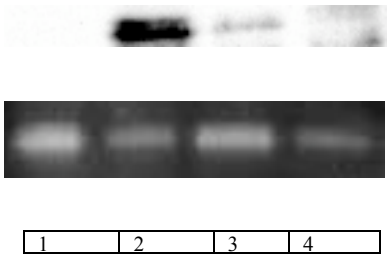


Figure 4.10. Western blot of IL-15R-α expression on monocytes and B cells. Lane 1 monocyte with no IL-15, Lane 2 monocyte with IL-15, Lane 3 B cells with no IL-15, Lane 4 B cells with IL-15

From the western blot (Figure 4.10), it can observed that B cells and monocytes with no addition of IL-15 show no or very low levels of IL-15 Receptor α expression. In contrast, following the addition of IL-15, an upregulation of IL-15Rα was observed in monocytes but not B cells. This further supports the observation that the nature of the CD1d positive APC is crucial in shaping iNKT responses and suggests that the results shown earlier relate to an important interaction between IL-15 acting on monocytes to shape the nature of the iNKT response.

Finally the expression of the individual IL-15R subunits on iNKT cells were examined, to assess if they were both present and/or differentially distributed between the CD4 and DN subsets For CD132, the common γ chain, both CD4 and DN cells demonstrated similar levels of expression (Figure 4.11). Similarly the expression of the CD122 did not differ between the iNKT subsets (Figure 4.12).

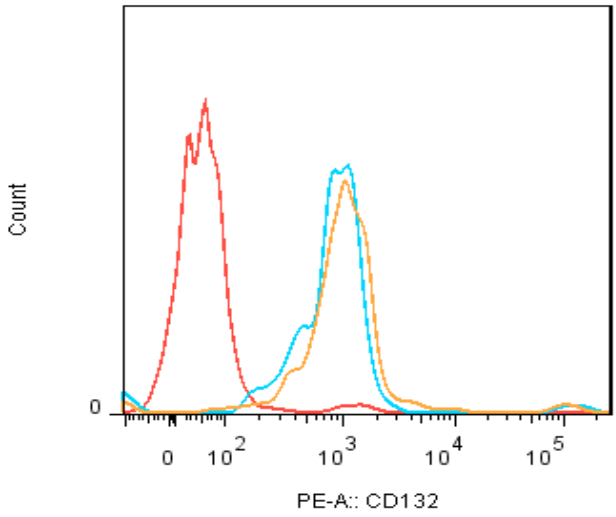


Figure 4.11. CD132 expression on iNKT cell subsets

Red represent CD132 negative control cells, blue represents CD4+ve iNKT cells and yellow represents DN iNKT cells.

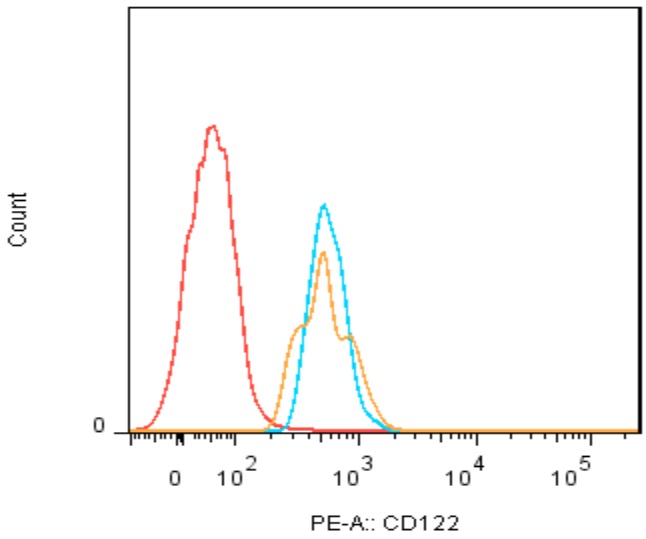


Figure 4.12 CD122 expression on iNKT cell subsets.

Red represents CD122 negative control cells, blue represents CD4+ve iNKT cells and yellow represents DN iNKT cells.

4.3.4 Summary of antigen presenting cells and cytokine effect to iNKT

• Monocytes and B cells are distinct APCs for iNKT responses

• Individuals with low iNKT cells have an absolute dependence of upon monocytes for supporting iNKT proliferation and cytokine production.

• CD1d positive B cells are unable to support activation of iNKT cells from low donors

•Individuals with high iNKT cells have APC redundancy in supporting proliferation but require monocytes for maximal IFN- γ responses

• IL-15 can skew the cytokine diversity of iNKT responses towards a Th1 bias, through the reduction of IL-13. This effect is more notable in low iNKT donors where the Th2 cytokine producing CD4 subset is numerous.

• The IL-15 mediated effects upon the CD4 positive iNKT cells are mediated through monocytes.

• All iNKT cells express the β and γ IL-15R subunits, whilst monocytes and not B cells express the IL-15R α subunit. This may allow IL-15 mediated signalling to occur through this selective APC:iNKT cell interaction.

4.4 Discussion and conclusion

During this study, we have identified that in low donors, monocytes are crucial for the proliferation of iNKT cells. In contrast, B cells and myeloid dendritic cells are redundant APC's for supporting proliferative and cytokine responses. This selective APC usage, despite CD1d is expressing on B cells, was not expected.

Surprisingly, the same effect was not seen on the iNKT cells of high donors. In this setting, depletion of any one subset did not abrogate iNKT proliferative responses, suggesting that for this function all 3 APC populations mediate sufficient presentation. However monocytes were shown to be important in the context of maximal Th1 cytokine production from iNKT cells and in permitting IL-15 induced cytokine skewing of IL-13.

The interesting finding that IL-15 can diminish the Th2 type cytokine production in the presence of monocytes for all donors, implies a mechanism of shaping effector iNKT responses along the lines classically described for signal 3 (cytokine influence upon Th1:Th2 outcome) in conventional T cells. We carefully distinguished the effect of monocyte depletion from myeloid dendritic cell depletion, as it is known that CD14 is also weakly expressed on the myeloid dendritic cells (264). Previous studies have also shown that myeloid dendritic cells are key players in the iNKT cells response and can lead to a bias in cytokine production (203, 265). We therefore compared CD14 depletion with BDCA-1 depletion (depletion of myeloid dendritic cells) to examine the effect of selective mDC depletion. The results suggested that depletion of myeloid dendritic cells leads to a minor effect and that the CD14 depletion was principally operating through monocytes.

Unlike other cytokines such as IL-2 and IL-7, IL-15 can be presented in a cis and trans configuration (266). The trans-presentation is achieved by the high affinity of IL-15 to IL-15Rα and further association with the IL-15Rβ (CD122) and

common γ chain (CD132) subunits on the effector cell (266). This is typically achieved through the binding of intracellular IL-15 to IL-15Rα within an APC. We introduced exogenous IL-15 to our system and it was observed that it was able to stabilize the IL-15Rα binding. A speculative model of exogenous IL-15 activation of iNKT cells through trans-presentation is shown below.

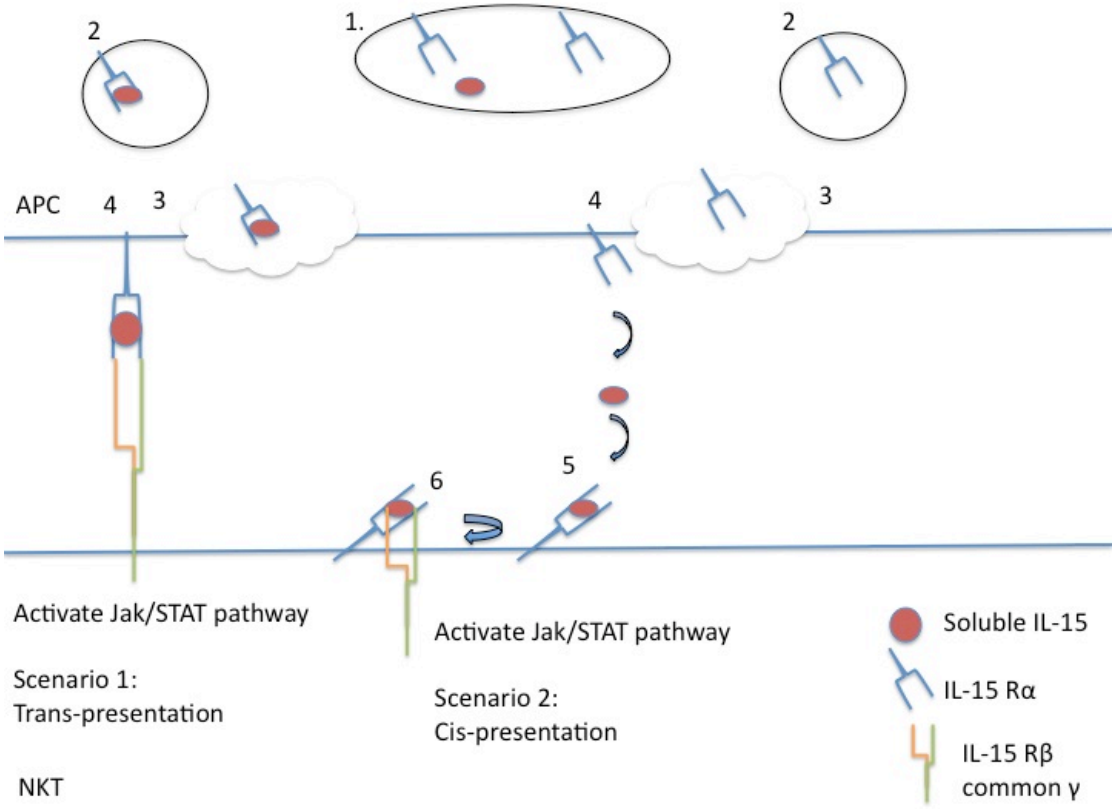


Figure 4.13. A model of IL-15 presentation to iNKT cells

A monocyte may express intracellular IL-15, IL-15Rα or an IL-15:IL-15 Rα complex (Steps 1,2). Cis presentation may be facilitated by stabilisation of IL-15Rα with exogenous IL15, followed by release of this complex (4). This soluble complex may then bind to an effector cell to activate the Jak/Stat pathway (5,6). Trans presentation may be undertaken if the cells expressing the CD122 and CD132 receptors are in approximation to the monocyte expressed IL-15Rα:IL-15. In these scenarios the presentation of IL-15Rα in the resident APC is critical for the trans (direct) or cis (indirect) activation.

In our study, we showed that monocytes but not B cells were capable of stabilizing the IL-15Rα subunit following the addition of IL-15. We propose that for low and high iNKT donors monocytes utilise this mechanism to support the maximal proliferation and Th1 cytokine responses of effector iNKT cells. For low donors this is critical for their particular iNKT repertoire and requires both monocytes (IL-15Rα source) and IL-15 (exogenous or intracellular) to support their proliferation. Through an effect upon the CD4 subset of iNKT cells this can also mediate a reduction in IL-13 production. For high donors there is no absolute dependence upon the monocyte-IL-15 pathway as other cells may substitute. However for maximal Th1 responses both monocytes and IL15 are required to both increase IFN-γ and reduce Il-13 production.

Chapter 5 Development of CD1d ligands with diverse functionality

5.1. Introduction

Upon activation, iNKT cells can secrete Th1, Th2 and Th17 cytokines and this property facilitates their importance in immune-regulation. Importantly, iNKT cells can efficiently produce IFN-γ and promote anti-tumour responses in murine models. For example, some studies have shown that iNKT cells, under α-GC activation, can promote anti-tumour responses to melanoma. This anti-tumour response was mainly due to the enhanced production of IFN-γ in such systems (267). At the same time, iNKT cells may produce Th2 type cytokines, such as IL-4 and IL-13 and promote regulatory responses. IL-13 secreted by iNKT cells was previously found to suppress tumour rejection (268). Therefore the balance between Th1 and Th2 iNKT responses for tumour control may be critical. However in other models such Th2 cytokine secretion may be useful in autoimmunity as the secretion of IL-4 by iNKT cells can protect the NOD mouse from the development of Type I diabetes (269).

How do iNKT cells select the types of cytokines to release? In the previous chapters, I have investigated the significance of antigen presenting cells as well as the conditional cytokines that influence the cytokines produced by iNKT cells. Other studies have focused on the affinity of the glycolipid to CD1d as a major contributing factor that influences the types of cytokine released. These studies have suggested that by altering the length or structure of the α-GC analogue, different cytokines may be selectively induced (270)(57, 59). This has been most readily seen in the OCH variant of α -GC where the acyl and sphingosine chains are reduced in size, leading to a Th2 deviation.

5.1.1 α-GC modification

5.1.1.1 The CD1d-ligand-iNKT cell complex

Although α-GC has been recognized as the "prototypic" ligand for CD1d binding, is α-GC optimal as a clinical translational therapeutic candidate? The cytokine production induced by α-GC is in a relative Th1 and Th2 equilibrium. A good therapeutic candidate for tumour activity would have a predominant Th1 cytokine production; whilst, a good Th2 candidate ligand could show potential in an autoimmune disease setting.

5.1.1.2 The CD1d-ligand structural complex

It has been increasingly recognized that there may be the potential for iNKT cell ligand use in clinical vaccination strategies as adjuvants or as standalone immunomodulators. Currently, most of the glycolipid modifications are based upon influencing the CD1d-ligand-iNKT cell interaction through reference to the crystal structure.

From the crystal structure it is understood that the sphingosine chain of α-GC is bound to the F' pocket in mouse/C' pocket in human whereas the acyl chain is associated with the A' pocket. The galactose head group of α-GC is not bound to the CD1d but emerges from the groove for recognition by the α-chain of the T cell receptor (109, 110, 177, 271, 272). Furthermore, the 3-hydroxy group on the sphingosine chain together with the *O*-glycosidic link further stabilises the lipid-CD1d complex. The 3-hydroxy group on sphingosine chain has also been proven to be essential for mouse iNKT cell recognition, as it forms an important hydrogen bond with Asp80. In addition, the Thr154 position in mouse forms an additional hydrogen bond with the *O*-glycosidic link. The 2'-OH of the galactose head is also crucial, making a hydrogen bond with the Asp151 in human / Asp153 in mouse (109, 110, 177, 271, 272). The importance of this hydrogen

bound network is to stabilise the galactosyl head for a productive CD1d-lipid-iNKT cell interaction (7).

5.1.1.3 Review of the current analogues

In the therapeutic setting, many groups are actively searching for ideal analogues of α-GC to meet different objectives. The different approaches include changes to the length of the lipid as well as alterations to the saturation of the lipid (7). These different approaches have led to differential effects in terms of changing the stability, binding affinity as well as the cellular loading compartment of the complex (245, 270, 273, 274).

One of the earliest approaches to α-GC modification was to shorten both the sphingosine and acyl chains. One such ligand was the α-GC truncated analogue OCH. The OCH analogue has a 2 carbon truncation on the acyl chain and 9 carbon truncation on the sphingosine chain compared to α-GC (270). The truncated α-GC, OCH was shown to release a high level of IL-4, with a marked reduction of IFN-γ compared with α-GC. This finding suggested that by altering the length of α-GC, analogues could be designed to produce desired cytokine outcomes. Indeed, the crystal structure of α-GC bound with CD1d predicted that the carbon chains of α-GC might be the optimal length to stabilise the CD1d-lipid complex (110). Later studies comparing the binding affinity of varying truncations of α-GC further strengthened these predictions (243). Indeed, shortening the chain length of lipid binding to CD1d regardless of the either A' or F' pocket, increases the rate of lipid-CD1d dissociation, and suggests that the optimal length for CD1d-ligand binding is an 18 carbon length sphingosine chain and a 26 carbon length acyl chain (243). These studies showed that incomplete filling of the C' pocket could lower the CD1d stability as well as the binding affinity of TCR to CD1d-lipid complex (243).

Besides the length of lipid chains, the structure of the lipid chains can also influence the binding to CD1d, and hence the cytokine release. In an experiment looking for selective anti-tumour responses by administering α-GC analogues

with removal of the hydroxyl group to mice with melanoma, a greater degree of tumour regression compared to α-GC was shown (34). Introduction of an aromatic group to the fatty acid chain of the α-GC also enhanced the stability of CD1d- lipid complex, and led to a selective Th1 type cytokine profile (275). A more recent study showed that by replacing the amide group with a triazole group, an enhanced Th2 type profile was seen. This suggested that by changing the amide moiety of α-GC, a different immune response could be obtained (276).

Recently, the Cerundolo group has introduced a new group of iNKT-CD1d ligands. This group of lipids do not have a glycosidic link between the hydrophilic head and ceramide and one such compound, threitolceramide, had some interesting functional properties (7). This group showed that this synthetic compound activated iNKT cells efficiently, influencing other cells including DC maturation as well as T cell priming. The threitolceramide-CD1d complex had a relatively lower binding affinity compared to the α-GC-CD1d complex. The group suggested that the new ligand gave the particular advantage of sub maximal activation through by passing target cell killing (i.e. APC), allowong continued NKT activation (277).

5.1.1.4 Problems with current analogues

As discussed above, studies in recent years have found that modification of the α-GC lipid chains could polarize the cytokine secretion of iNKT cells (176, 267). The translational implication of this being that it may be possible to alter α-GC to achieve the ideal cytokine response for the specific treatment of tumours and/or autoimmunity (197). Currently 4 approaches have been adopted in the modification of α-GC: sphingosine core modification, fatty acid modification, amide modification and sugar modification (276). However, current data shows that the clinical effects of these analogues are moderate and the mode of action is not fully understood.

Another criticism of existing analogue identification systems is that most of the functional and cytokine release data are analysed in a murine system. Early

studies suggested that mouse and human CD1d structures are highly conserved. Indeed, human iNKT TCR α and β chains show a high sequence homology to mouse iNKT α and β chains (278). Furthermore, human iNKT cells can recognise and cross-react with mouse CD1d- α-GC complexes with similar affinity and vice versa for mouse iNKT cells (279). However, some studies indicate that the non-conserved region of CD1d can lead to a difference of recognition in iNKT cells. A recent study showed that a modified analogue preferable to Th2 type cytokine production showed a high Th2 type cytokine release in human, but failed to produce detectable amount of cytokines in murine systems. Not only did the analogue not lead to the cytokine production in mouse NKT cells, it also failed to be recognised by NKT cells within the CD1d complex (280). Studies have also identified important differences in the position of the galactose ring (281) which cause inter-species differences in antigenicity (282). Thus, the cytokine profile for a particular analogue in a mouse system is not necessary the same as the cytokine profile in humans.

Furthermore, current analogues that promote a robust Th1 cytokine response often result in maximal activation of iNKT cells. However, studies have suggested that the maximum activation of iNKT cells might not be as useful in therapeutic setting as the overstimulation of iNKT cells can cause activation induced cell death, in particular of the antigen presenting cells (243, 277). It has also been shown that the uncontrolled activation of iNKT cells can lead to an anergic state of iNKT cells (283, 284). Therefore engineered compounds that might dissect some of these features may be advantageous over exciting compound analogues.

5.1.2 Proposed α-GC modification in this project

Within this project, we have investigated alternative α-GC analogues, which may favour a Th1 immune response in humans. The modification of α-GC is based on current understanding of the stimulatory model for cytokine selectivity. Compared to other modified α-GC analogues, the approach in this project

involved modification of different side chains and domains including the polar domain, apolar chain and the galactose unit, which allows more opportunities to identify the ideal ligand for enhanced iNKT activity.

Furthermore, we have additionally investigated the activation threshold of iNKT cells stimulated by these ligands through the use of the postulated anergy marker programme death-1 (PD-1). In naïve T cells, PD1 is express at a very low level. Upregulation of PD1 expression occurs when T cells activated by professional antigen presenting cells. PDL1 is the ligand for PD1, being expressed in B cells, monocytes, dendritic cells[1]. The interaction of PD1 and PD-L1 has been found to an importantly role in the development of central and peripheral tolerance[2, 3]. Recently, PD1 has been considered as an important therapeutic target for many diseases including cancer, autoimmunity and transplant rejection where interference with tolerance is beneficial [4]. PD1 targeting cancer therapy has been the most promising with successful inhibition of metastasis in a myeloma mouse models [5, 6]. Currently, monoclonal antibodies specific to PD1 are undergoing phase I clinical trials in human in combination with cancer vaccine studies [7].

It was first suggested in 2008 by Kang et al. that PD1 is essential for the instruction and persistence of anergy in iNKT cells (285). PD1 is present at minimal levels on iNKT cells pre-activation, with an upregulation upon activation. No down-regulation of PD1 was observed when there was persistence of anergy, but this could be reversed by addition of a PD1L antagonist (285) (286-288).

5.1.2.1 Apolar chain modification

From the crystal structure of CD1d, it was observed that the acyl chain fits the A' pocket in an anticlockwise direction, whilst the sphingosine chain fits into the C' pocket with a straighter conformation. As the most stable confirmation for the hydrocarbon chain is an antiperiplanar structure, which has an angle of 180 degrees, this means that upon binding, the hydrocarbon chain is forced to adopt a less stable conformation. As seen in the CD1d- α-GC crystal structure, there are

many “bends” observed when the two hydrocarbon chains are accommodated into the CD1d cavities. In discussion with our collaborators at the Department of Chemistry, University of Southampton (Dr Bruno Linclau) it was observed that some of these bends are close to a *gauche* conformation (60 degree dihedral angle). *Gauche* conformations are less stable than *anti* conformations because of steric repulsion. In collaboration with our chemists a series of modifications were devised that could stabilise the *gauche* conformation, hypothetically leading to a positive impact in the binding affinity.

Previous studies demonstrated that the *gauche* confirmation could be stabilised by CF_2, S and *anti*-vicinal difluoride modifications (289). This led to the synthesis of **32A** (Figure 5.1).

The second type of analogues studied contains modifications that alter the hydrogen bonding between the GalCer and CD1d (and the TCR). The amide group was modified in compound 31B, whilst in 43B the sphingosine group was modified. Compound 44B is a hybrid of 31B and 43B (subsequently referred to as 31, 32, 43 and 44).

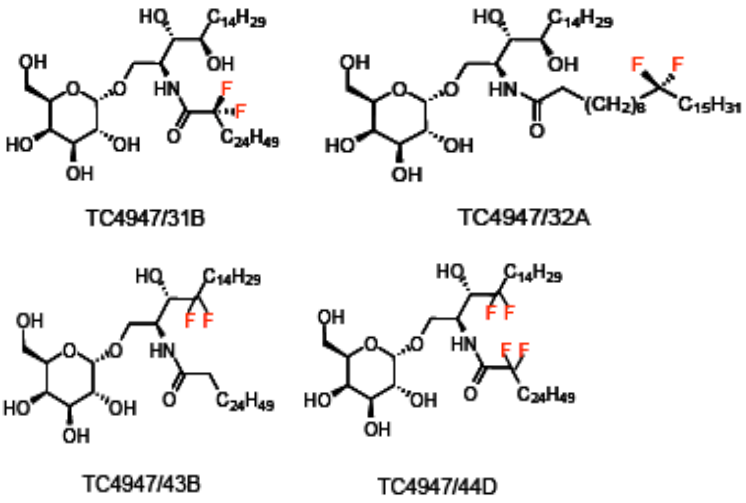


Figure 5.1 Structure of glycolipid analogues with acyl and sphingosine chain modifications

A second collaborative chemist (Dr Serge Van Calenbergh) at the University of Ghent, Belgium also designed a series of analogues principally based upon the galactose head. Few studies had focused on this group at the start of project, as it was not directly involved in CD1d-lipid binding. In contrast, this group is important for the interaction with the iNKT cell TCR. This group of modified compounds (Table 5.1) was also profiled to compare iNKT responses that are relatively independent of CD1d affinity but relates to TCR affinity.

5.2 Aims of this study

- To analyse the functional impact of the different analogues upon ex vivo and cultured human iNKT cells and derivative clones.

- Study the effect of the modified ligands in heterogeneous human population samples, at the level of proliferation, cytokine production, activation and anergy compared to the prototypic Th1 and Th2 ligands α-GC and OCH respectively.

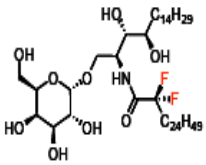
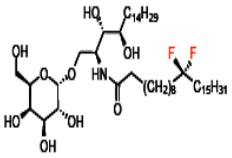
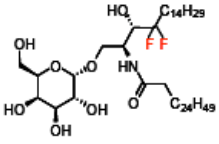
- Compare the functional differences between human and mouse studies and the potential application of these ligands in a therapeutic setting.

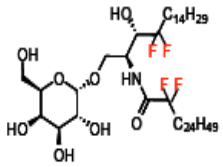
5.3 Compound evaluation results

To analyse the effects of different analogues upon iNKT cells, several parameters were evaluated. Studies of iNKT cells included proliferation comparisons for each analogue as well as the ability of analogues to produce cytokines as assessed by ELISPOT, intracellular cytokine analysis and multiplex approaches. Furthermore, the killing ability of the analogues was also analysed.

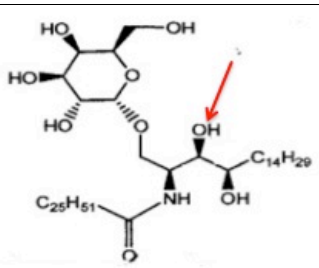
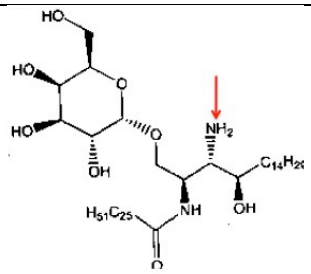
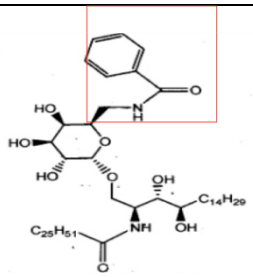
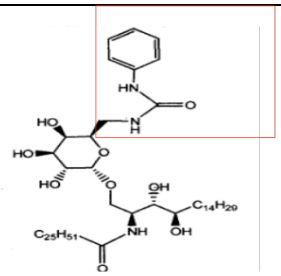
Table 1 summarises the structural modification of analogues used in this chapter:

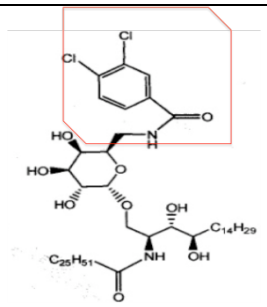
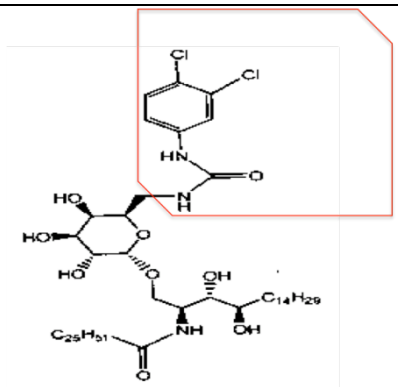
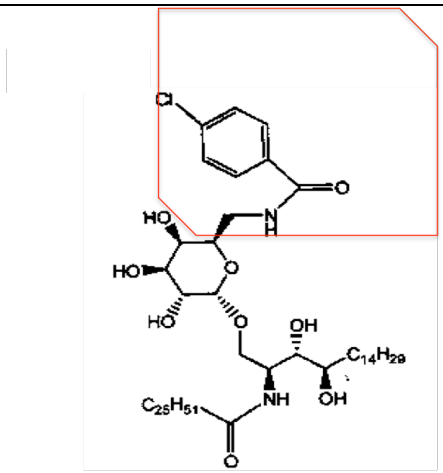
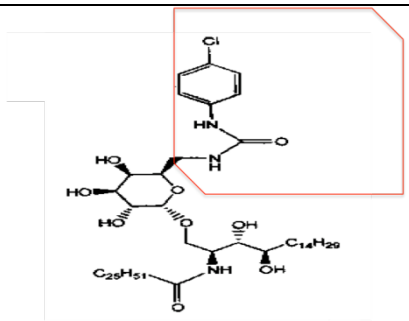
a) University of Southampton modification modification (Dr Bruno Linclau):

Analogues	Structure	Modification	Contact
31		**Amide group (Acyl chain)**	**CD1d**
32		**Acyl chain**	**CD1d**
43		**Sphingosine chain**	**CD1d**

44		**Sphingosine and amide group (acyl chain)**	**CD1d**

b) University of Ghent modifications (Dr Serge Van Calenbergh)

MT315		**Sphingosine Chain**	**CD1d**
MT323		**Sphingosine Chain**	**CD1d**
MT422		**C'6 (Amide substitution)**	**TCR**
MT424		**C'6 (Urea substitution)**	**TCR**

MT430		**C'6 (Amide substitution)**	**TCR**
MT440		**C'6 (Urea substitution)**	**TCR**
MT432		**C'6 (Amide substitution)**	**TCR**
MT438		**C'6 (Urea substitution)**	**TCR**

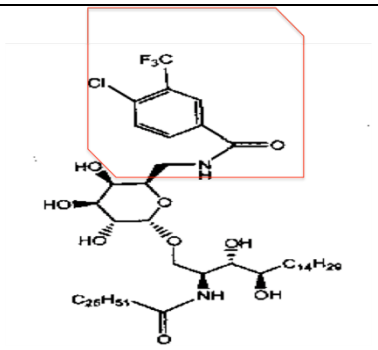
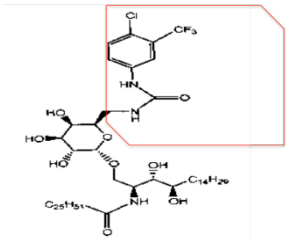
MT434		**C'6 (Amide substitution)**	**TCR**
MT442		**C'6 (Urea substitution)**	**TCR**

Table 5.1 – Summary of modified ligands used during this study

Those principally modified for CD1d binding from University of Southampton (A) and mainly iNKT TCR interaction from University of Ghent (B) are shown.

5.3.1. iNKT proliferation with analogues

The target 'ideal' analogue was initially defined as that which could increase IFN-γ and be able to expand iNKT cells compared to α-GC. Figure 5.2 shows the proliferation profile of some of the analogues, including the two prototypic analogues (α-GC and OCH) compared with those possessing acyl and sphingosine chain modifications.

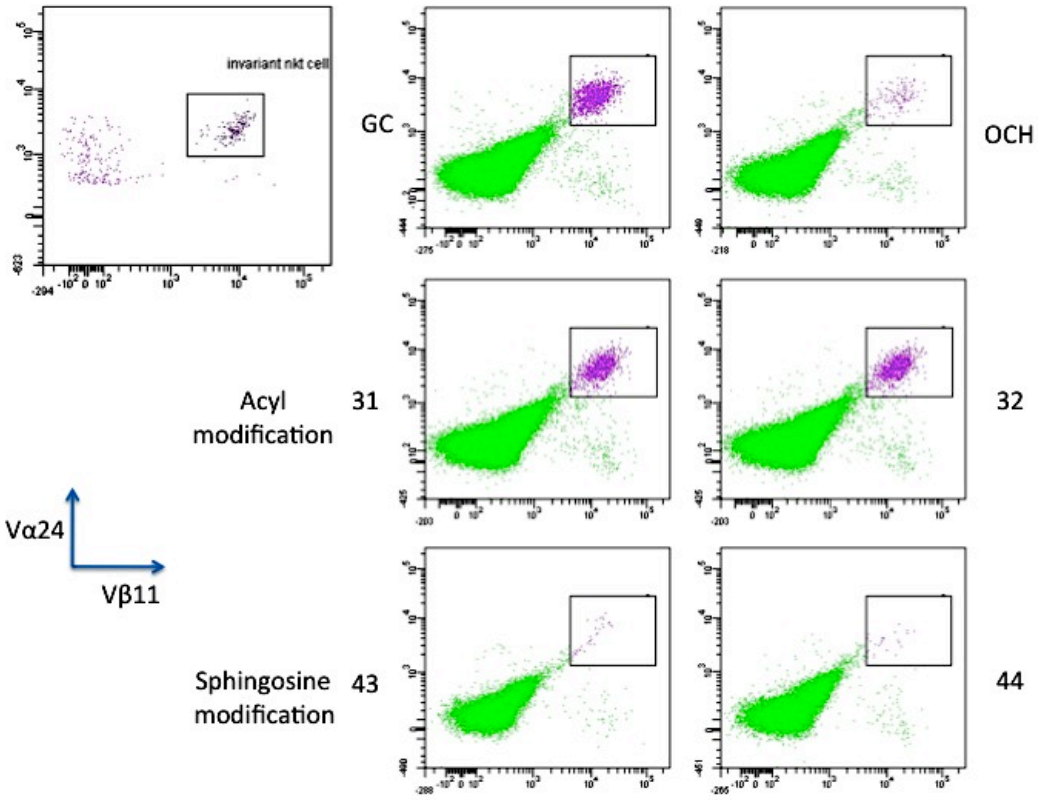


Figure 5.2. Proliferation profile pre and post activation with α-GC, OCH and acyl/sphingosine chain analogues.

Figure 5.2 shows that the inducible proliferation of iNKT cells with the compounds 43 and 44 is reduced compared to the proliferation potential from the acyl chain modified compounds. As introduced from the previous chapter, we asked if the ligands act differentially upon the iNKT cells of high and low control groups.

Figure 5.3 shows that the proliferation of iNKT cells in a selection of high donor (>500 iNKT cells per 10^5T cells) and low donors (<100 iNKT cells per 10^5T cells).

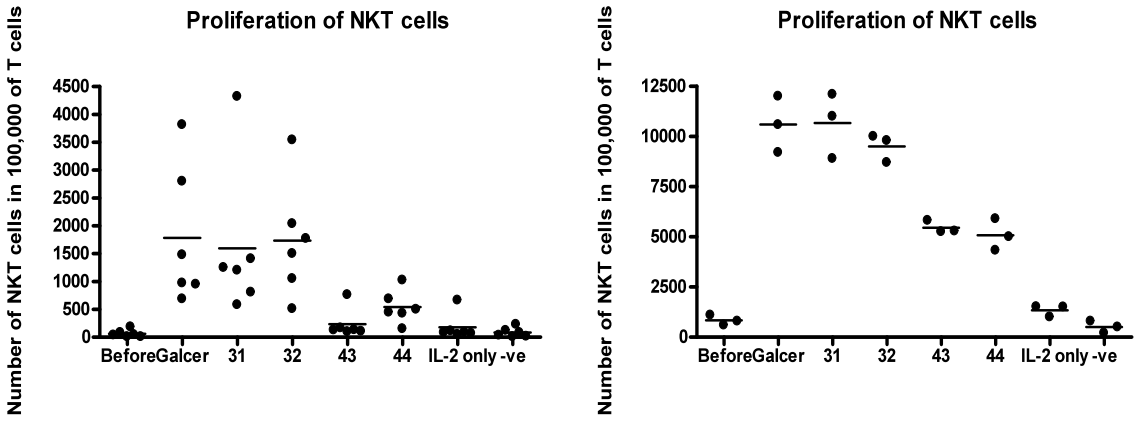
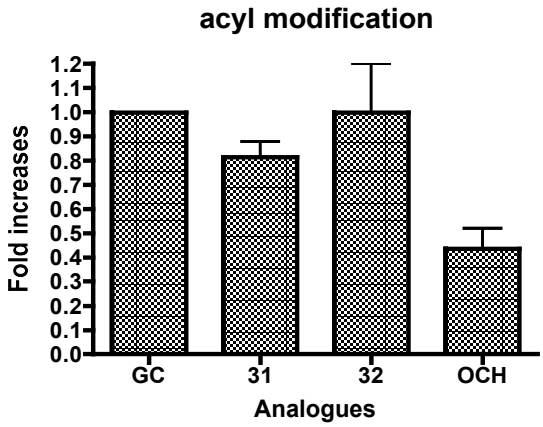


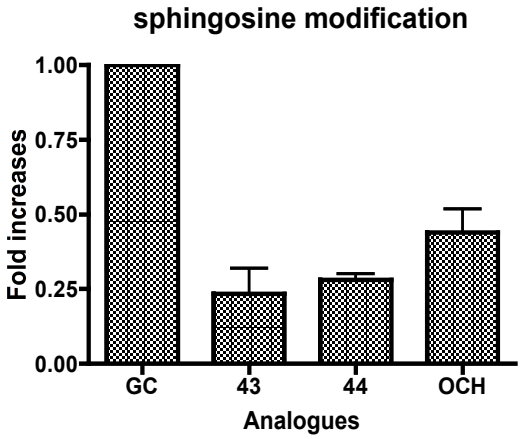
Figure 5.3 Proliferation of iNKT cells in different donors (left: low donor, right: high donor) with different analogues. Pre proliferation number compared to post proliferation number.

Surprisingly, compounds 43 and 44 were able to induce proliferation in the high donors but not the low donors, suggesting that the analogue displayed specificity between these two groups of healthy controls. This suggested that the ligands were able to bind to CD1d but that this was insufficient to activate the iNKT cell repertoire of low donors. Due to the predominance of low donors amongst the population we undertook most of our subsequent experiments on low donors to allow the results to represent a homogeneous population. However it should be noted that the differences in initial iNKT portfolio may impact upon such ligand screening activities, as suggested by the data in Figure 5.2.

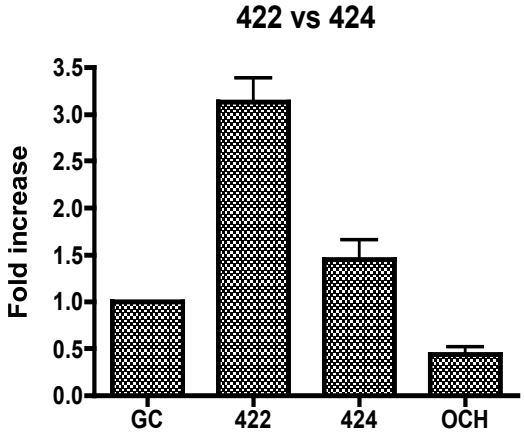
The analogues were subsequently grouped according to their closely related structure for further comparison to α-GC and OCH. The groups included; (i) alterations in the interaction with CD1d (Acyl chain modification (31 and 32), Sphingosine modification (43 and 44)); (2) alterations predominantly in C3 position (315 and 322); galacosyl head alterations with amine or urea substitutions. The proliferation responses for the complete set of analogues is shown in Figure 5.4, each run with α-GC (gold standard for strong ligand) and OCH (gold standard for weak ligand) for comparison.



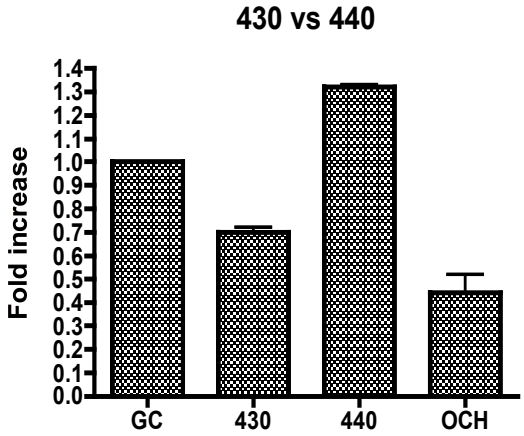
Proliferation index	50	44	50	24
Number of controls	15	9	9	5



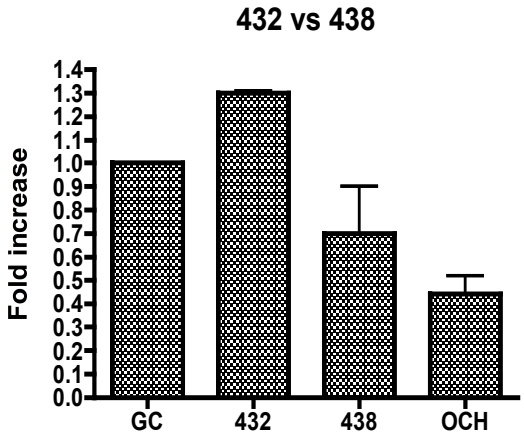
Proliferation index	50	10	14	24
Number of controls	15	8	7	5



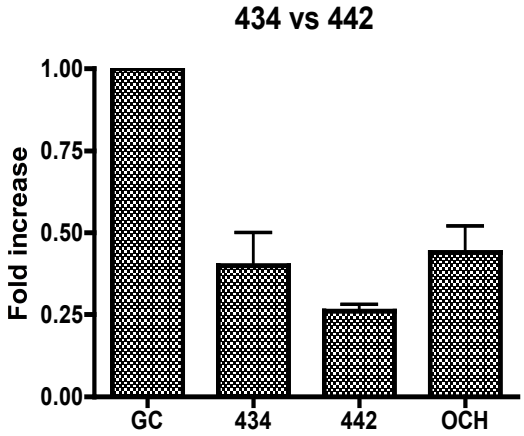
Proliferation index	50	154	75	24
Number of controls	15	5	5	5



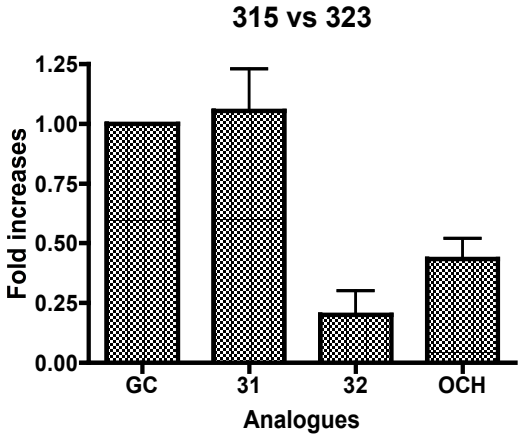
Proliferation index	50	35	65	24
Number of controls	15	5	5	5



Proliferation index	50	65	38	24
Number of controls	15	5	5	5



Proliferation index	50	20	13	24
Number of controls	15	5	5	5



Proliferation index	50	48	14	24
Number of controls	15	5	5	5

Figure 5.4 Proliferation index of Acyl, Sphingosine and 6' Galactose modified compounds. The compounds 422, 430, 432 and 434 are all amide substitution (next to α-GC) whilst 424, 440, 432 and 442 are urea modified (next to OCH).

No significant differences were found between the acyl chain modified compounds and α-GC. In contrast, mark reductions of iNKT cell proliferation were observed with the sphingosine modifications, falling below that seen with OCH. Interestingly the compounds 422 and 424, which had an amide (422) or urea (424) substitution at the C'6 position, showed contrasting results with the 422 showing the greatest proliferation ability of all ligands tested. For most of the other ligands tested the amide modification had a comparatively higher proliferation profile compared to the urea modifications (exception for 430 and 440).

5.3.2 Cytokine production by iNKT cells following analogue activation

To extend the study of the different analogues on iNKT cell function, we analysed the cytokine production from iNKTs following a 7 day expansion and overnight activation. We analysed IFN-γ, IL-13 and IL-17 production from a minimum of 4 healthy controls for each analogue.

Figure 5.5 shows that analogue 31 has a similar IFN-γ and IL-13 profile compared to α-GC. Whilst unremarkable in the proliferation assays, compound 32 shows a distinctive cytokine profile to both α-GC and OCH with an increased IL-13 signature. Interestingly, both analogues showed good IL-17 productivity compared to the two prototypic analogues, with a three-fold increase in IL-17.

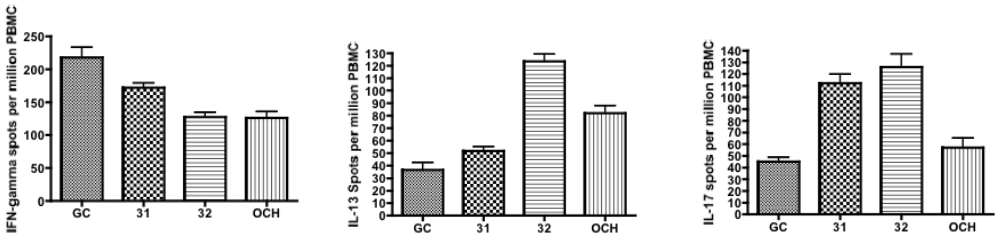


Figure 5.5. Cytokine production by acyl chain modified compounds ELISPOT of IFN-gamma, IL-13 and IL-17 cytokine production by PBMC with specific ligand.

Figure 5.6 is the multi-cytokine profile of the sphingosine chain modified compounds. When it is remembered that both analogues, 43 and 44, showed a very poor proliferation response, the excellent cytokine production observed is all the more unexpected. Both analogues were able to increase the Th1, Th2 and Th17 cytokines compared to their respective gold standard. This suggests that these ligands can bind to CD1d but are able to dissociate proliferation from cytokine responsiveness when activating iNKT cells of low donors.

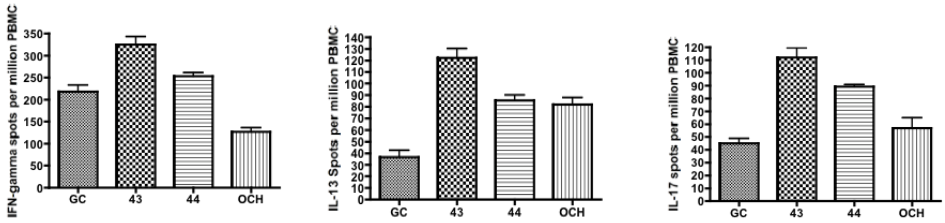
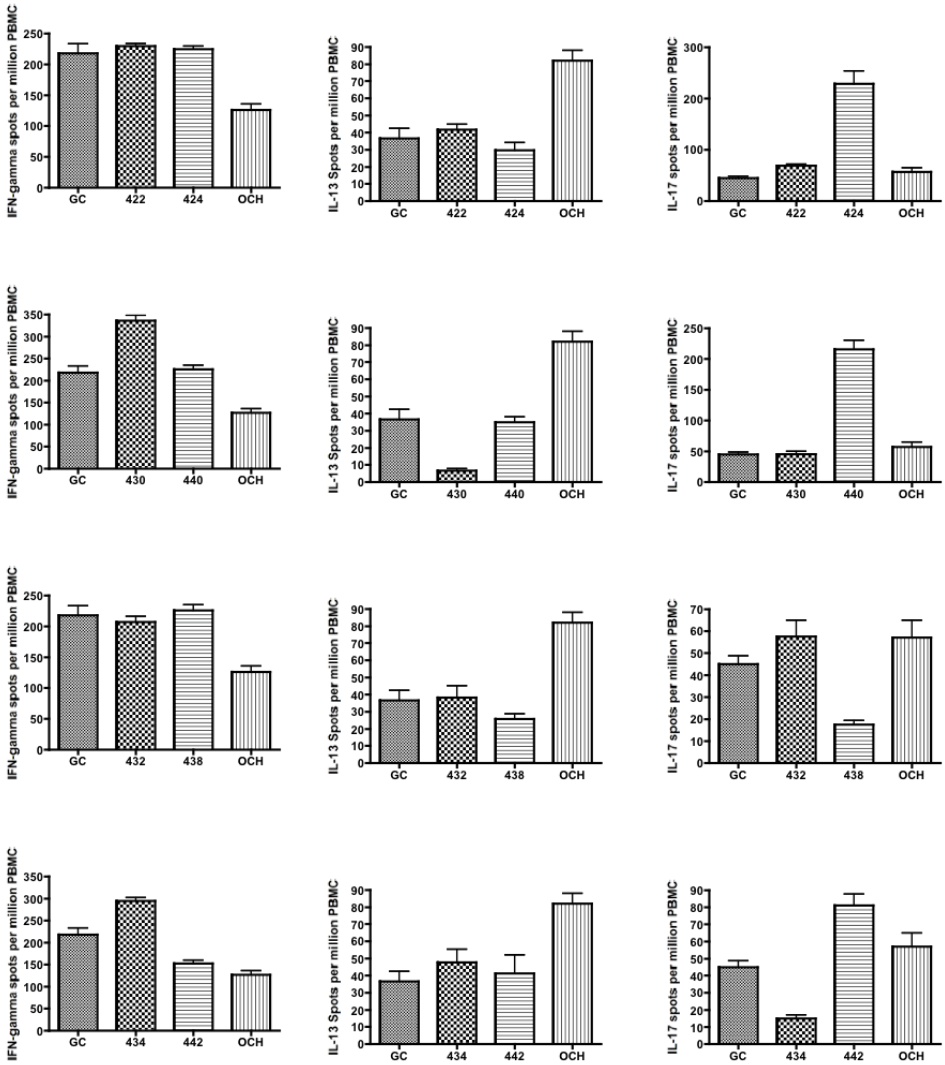


Figure 5.6. Cytokine production by sphingosine chain modified compounds. ELISPOT of IFN-gamma, IL-13 and IL-17 cytokine production by PBMC with specific ligand.

Figure 7 shows the composite results for the galactosyl group modified compounds.



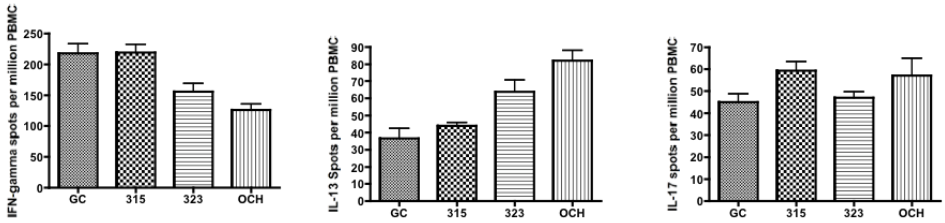


Figure 5.7 Cytokine production by TCR modification analogues. ELISPOT of IFN-gamma, IL-13 and IL-17 cytokine production by PBMC with specific ligand.

It is first interesting to note that the compounds that gave a poor proliferative response, 434,442 and 323, were all able to support cytokine secretion that was similar to the prototypic ligands. Indeed despite the wide array of substitutions in the galactosyl head it is clear that all still permit a productive iNKT TCR interaction at the level of cytokine production. When all the compounds are reviewed, most are similar to α-GC apart from 424 and 430, which demonstrate a significant increase in IL-17 production.

Most modifications did not extensively alter the IFN-γ production by iNKT cells. Surprisingly, the sphingosine modifications (43 and 44) enhanced IFN-γ production despite showing poor proliferative capacity. Besides the ligands 43 and 44, compound 430 was the only other modified compound that also significantly increased IFN-γ production.

One compound, 32, showed a comparable IFN-γ production to OCH and an enhanced IL-13 response. This might suggest that this is an enhanced compound for Th2 type responses that have been proposed to be potentially useful in autoimmune settings.

Interestingly, most of the compounds were shown to be good IL-17 producers compared to the prototypic ligands. Only one compound was found to have lower IL-17 compared to α-GC, analogue 438. The greatest IL-17 producing compounds were found in acyl and sphingosine modified compounds, as well as ligands 424 and 440.

In terms of IL-13 production, most of the analogues have a very similar pattern compare to α-GC, and produce a much lower level of IL-13 compared to the Th2 gold standard ligand OCH. Three compounds showed a comparable level of IL-13 production compared to OCH, compound 32, mentioned previously, together with 43 and 44. A summary of these results in seen in Table 5.2.

A)

	Proliferation	IFN- γ	IL-13	IL-17	Comment
31	++	++	+	+++	Similar to α-GC, with an increased IL-17 production
32	++	+	++	+++	Similar to OCH, with an increased IL-17 production
43	+	+++	++	+++	Poor proliferator, good all round cytokine producer
44	+	++	++	+++	Poor proliferator and all round cytokine producer
α-GC	++	++	+	++	Gold standard Th1 ligand
OCH	+	+	++	++	Gold standard Th2 ligand

Comment	No high proliferator			Minimum two fold increase in IL-17 production by new ligands	

B)

	Proliferation	IFN-γ	IL-13	IL-17	Comment
422	+++	++	+	++	422- Increase in Proliferation
424	++	++	+	+++	424- Increase in IL-17 production
430	++	+++	-	++	430- Change in Th1/Th2 ratio
440	++	++	+	+++	440- Increase in IL-17 production
432	+	++	+	++	
438	+	++	+	–	438- Poor IL-17 production
434	+	+++	+	–	434- Poor IL-17 production
442	+	++	+	++	
315	++	++	+	++	315-Similar to α -GC
323	+	++	+	++	323-

					Similar to α -GC, but with reduced proliferation
Comment	No ligand increase proliferation	No ligand reduces IFN-γ	No ligand enhances IL-13	Maximum 4 fold increase in IL-17	

Table 5.2 – Summary of ligands modified for CD1d binding (A) and predominantly TCR interaction (B)

Although the ELISPOT analysis gave a good indication of the cytokine profile for each analogue, it did not indicate which cytokines were directly produced by iNKT cells or whether the same cell could produce combinations of these cytokines. To accomplish this, a further analysis was carried out utilising intracellular analysis. This analysis gives the results for the cytokines released specifically by the iNKT cells as well as identifying single/multiple cytokine producing iNKT cells subsets. Figure 5.8 demonstrates the approach taken to this using the clonotypic antibody to identify the iNKT cells post activation followed by triple staining for IL-4, IL-17 and IFN- γ. This clearly shows that iNKT cells gated in the first plot are able to produce the various cytokines, and that they appear to be distinct to one cell each in this representative example.

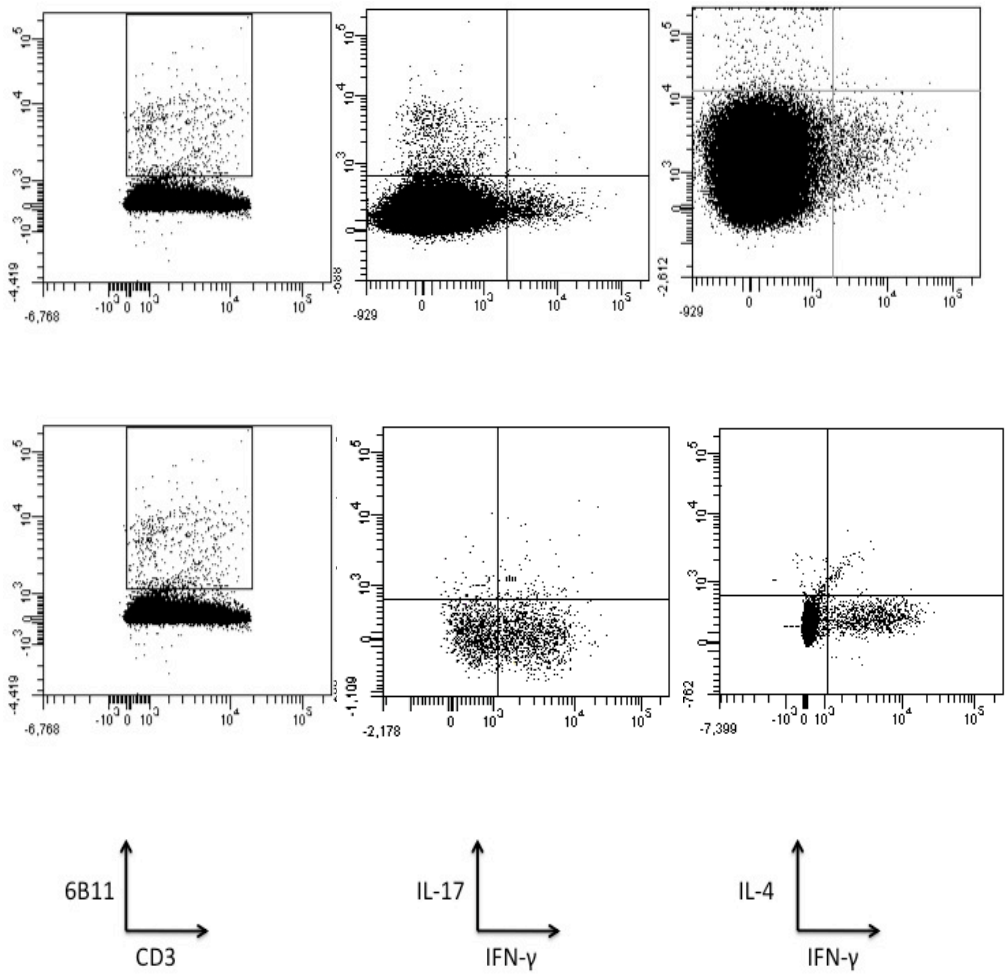


Figure 5.8. FACS analysis of intracellular IL-17, IL-4 and IFN-γ production in iNKT cells post analogue activation. The upper panel shows the cytokine production by total T cells, and the lower panel shows the cytokine production by iNKT cells.

Utilising this set of data we derived the ratio of Th1/Th2 (Figure 5.9) and Th1/Th17 (Figure 5.10) responses that were specific to the activated iNKT cells.

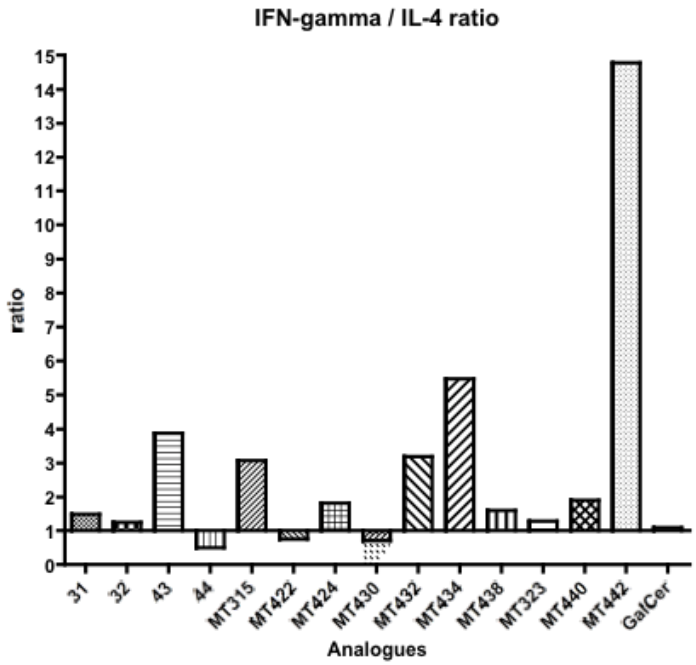


Figure 5.9 Th1/Th2 ratio for intracellular cytokines for all analogues

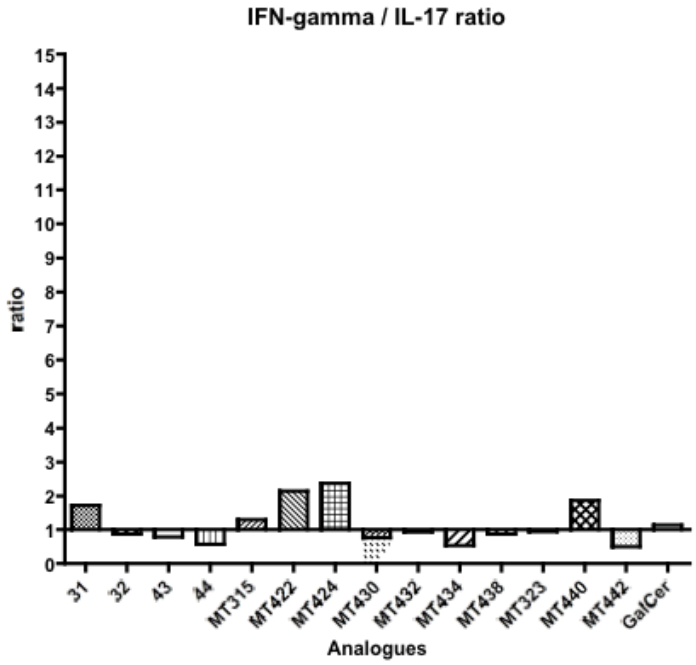
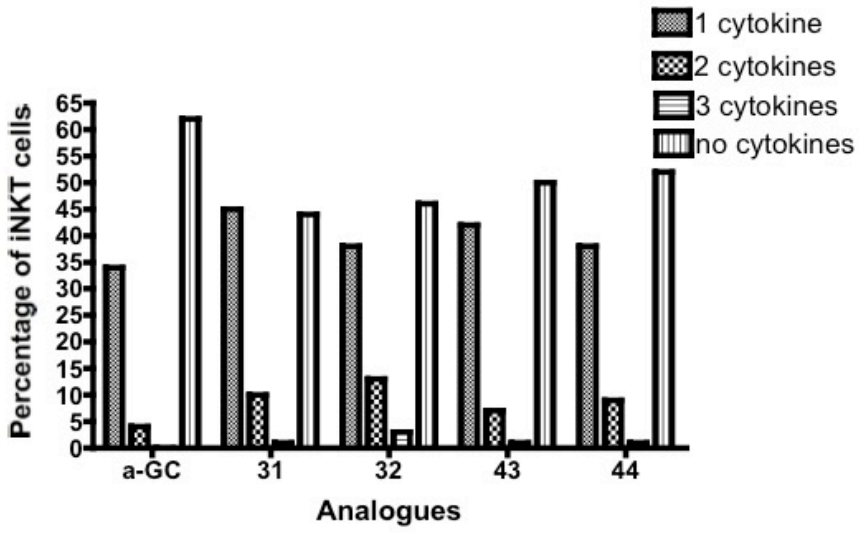


Figure 5.10 Th1/Th17 ratio for intracellular cytokines for all analogues.

Comparing the IFN- γ and IL-4 production by the iNKT cells, it is easily observed that at least 10 compounds achieve a similar or improved IFN- γ production comparing to α-GC. Only three compounds showed an IL-4>IFN- γ profile, suggesting that most compounds are directed to Th1 bias from iNKT cells.

The IFN- γ /IL-17 ratio gave a more dynamic distribution. Roughly, around half of the compounds showed an IFN- γ > IL-17 profile; and the other half showed the reverse ratio but with a smaller effect.

We then extended these analyses to identify whether individual iNKT cells were able to produce single/double or multiple cytokines utilising Boolean gating. This is based on the choosing a defined population and using a combination of commands including “and”, “or” and “not” to define the respective populations. Figure 5.11 displays this data for each analogue illustrating the percentage of cells that produced any one cytokine, no cytokine or a combination of different cytokines.



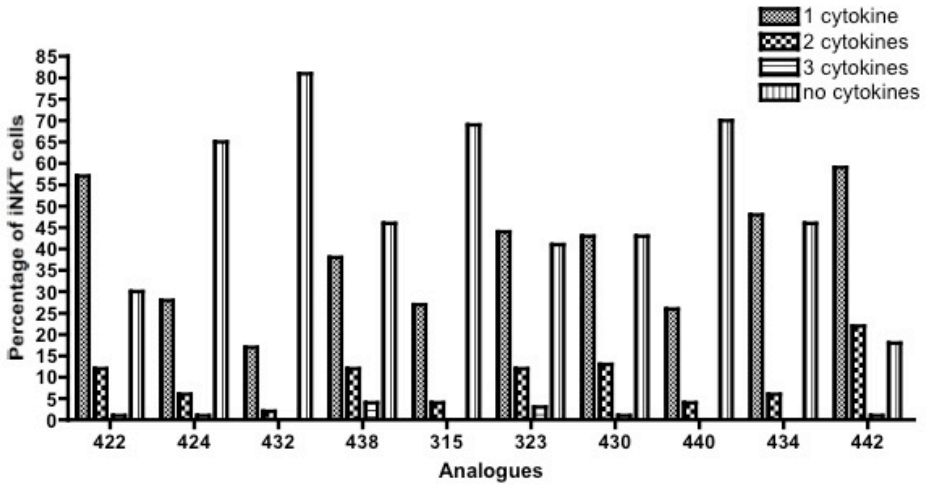


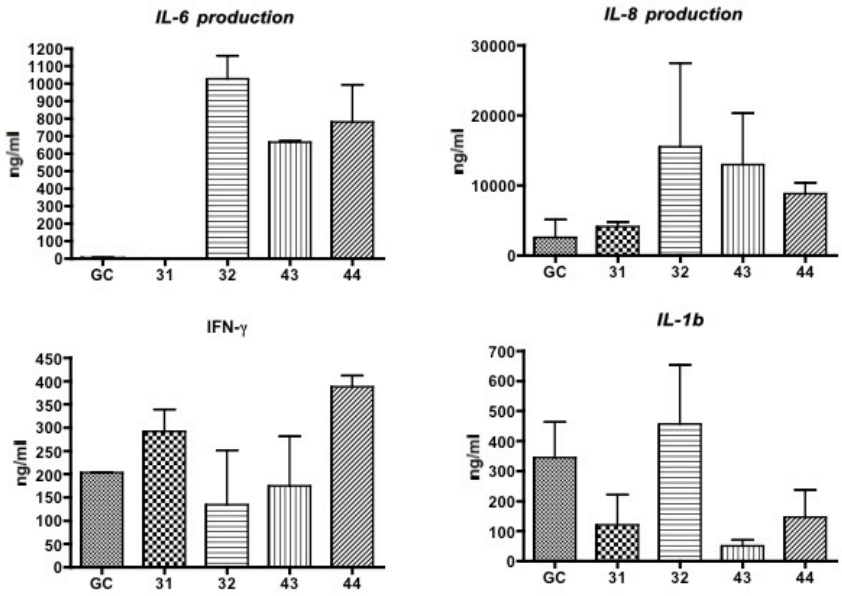
Figure 5.11 Boolean gating analysis of iNKT intracellular cytokine responses following analogue activation.

From the intracellular cytokine analysis, it can be easily seen that most of the iNKT cells (more than half of the iNKT population) are activated by most of the ligands. The acyl and sphingosine analogues were comparable to α-GC in both the proportion of activated cells and the relative distribution of cells producing single or combined cytokines. The second group of compounds were more variable in their responses. Analogues 432 and 442 were notable for having a substantially reduced or increased cytokine signature compared to α-GC. Compound 442 was also distinctive in that a larger percentage of cells were producing 2 cytokines, predominantly IFN- γ and IL-17 when looked at in detail. This was consistent with the ELISPOT results summarised in Table 5.2. It should be noted that the intracellular analysis was done after a seven day culture, and the potential of some ligands to induce anergy is a potential variable in evaluation of this dataset.

Finally, we analysed the cytokines with multiplex analysis. Compared to ELISPOT and intracellular analyses, the multiplex analysis was undertaken after an 18 hours activation of ex vivo cells, compared to the other two approaches which are undertaken after a seven days culture. The 18 hour ex vivo multiplex

assay was only undertaken with the acyl chain and sphingosine chain modified compounds.

We initially undertook the assay to select the optimal time for the measurement of our key effector Th1/Th2 cytokines. These preliminary studies over 2, 4, 6 and 16 hours established that the cytokines could be defined into 2 different groups. Cytokines primarily produced by iNKT cells (including IFN-γ, IL-2, IL-4, IL-10 and TNF-α) which were maximal at 6 hours and cytokines that may be produced by other cell types (including IL-5, IL-6, IL-8, IL-1ß and IL-12) which were maximal at 16 hours. Figure 12 summaries these multiplex cytokine results.



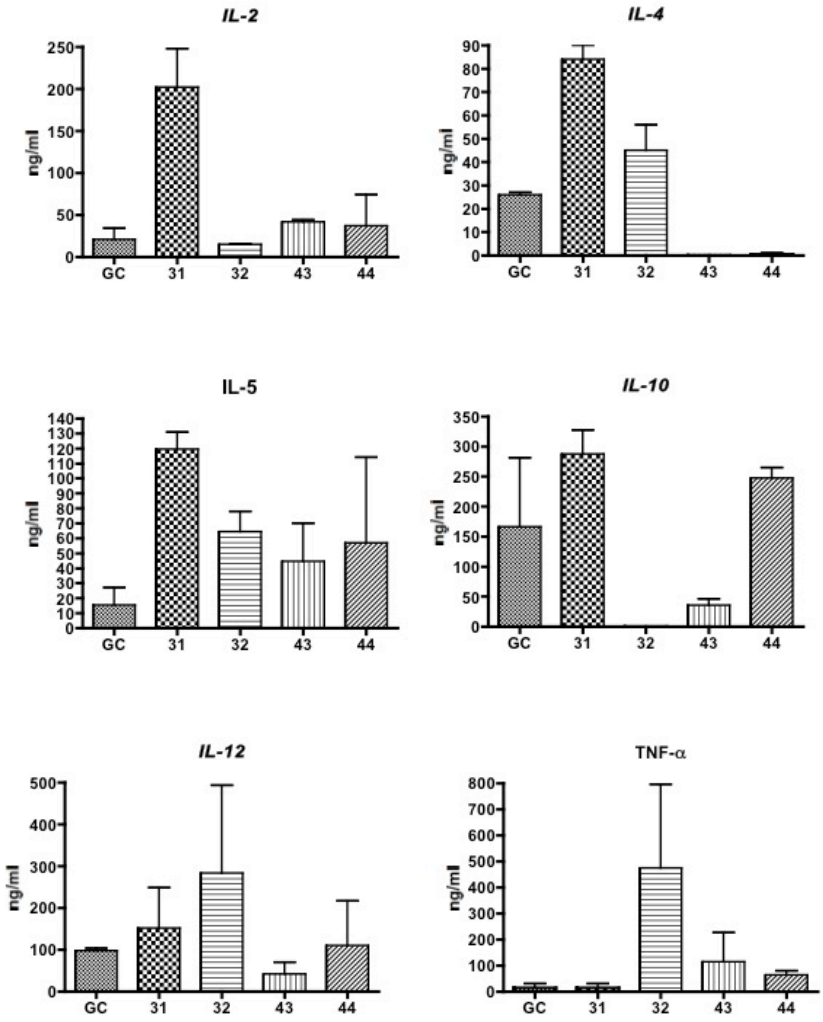


Figure 5.12 Acyl chain and sphingosine chain modified compounds for multi-cytokine analysis after short term culture.

From the multiplex cytokine analysis, all the four analogues produced comparable levels of IFN- γ. Compound 31 showed a higher IL-4 and IL-2 level compared the other ligands at 6 hours. No IL-4 was detected from compounds 43 and 44 at this early time point, in contrast to that seen after a cultured ELISPOT. In terms of the breadth of cytokines produced, compound 32 showed the broadest range. This led to high levels of IL-6, IL-8, IL-1b and IL-12 production at the 16 hour time point as well as IFN-γ, IL-2, IL-4 and TNF-α at 6 hours.

5.3.3 Ligands and iNKT cells – Anergy or not?

From the previous extensive cytokine study, it is suggested that some of the iNKT cells do not produce cytokines. This may be due to a variety of reasons including factors relating to CD1d binding, TCR activation or possibly the effects of the previous 7 day culture and the induction of anergy in the expanded iNKT cells. Figure 5.13 shows the percentage of unresponsive cells after culturing with the ligands.

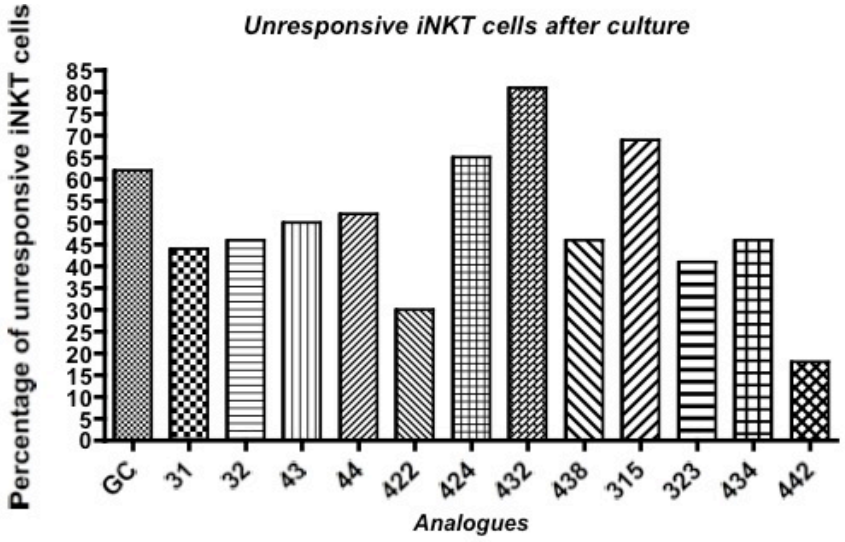


Figure 5.13 Percentage of unresponsive iNKT cells for cytokine production after seven days culture.

As discussed previously, PD1 is thought to be a marker of iNKT cells that have undergone activation induced anergy. We investigated the PD1 expression on iNKT cells after 7 days culture for each of the ligands to establish if this was related to their subsequent cytokine responsiveness. Figure 5.14 is a representative example of the difference between PD-1 expression following activation with α-GC (red) and the ligand 442 (blue).

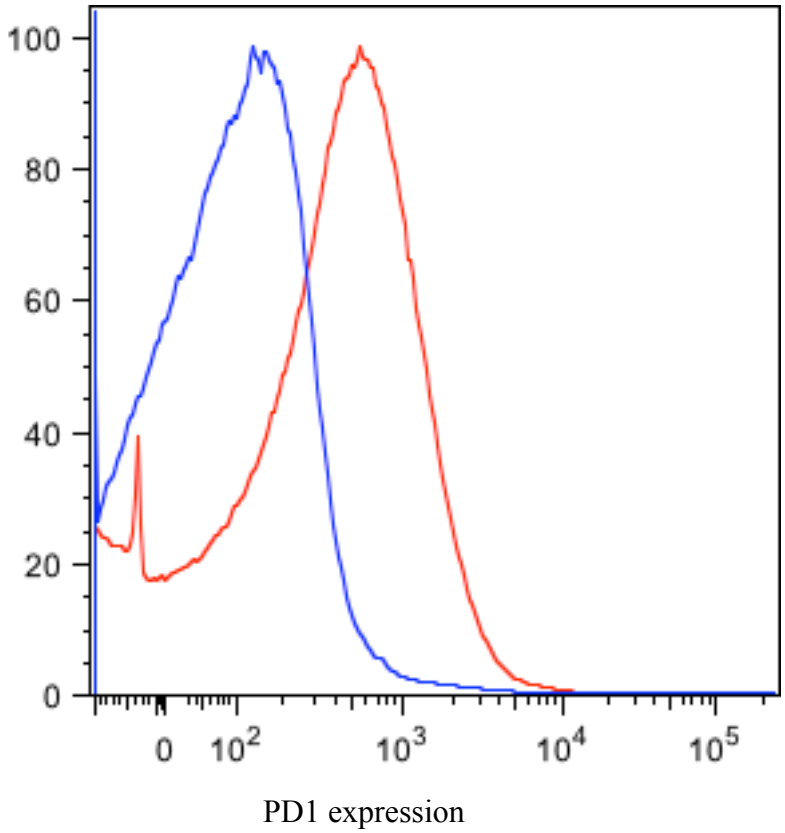


Figure 5.14.Representative PD1 expression of iNKT cells after 7 days culture with α-GC or 442

The PD1 expression of iNKT cells after 7 days culture was quantified using the mean flourcense intensity of PD1 for the whole iNKT cell population. Figure 5.15 shows the different PD1 MFI of the iNKT cells follwing ligands activation.

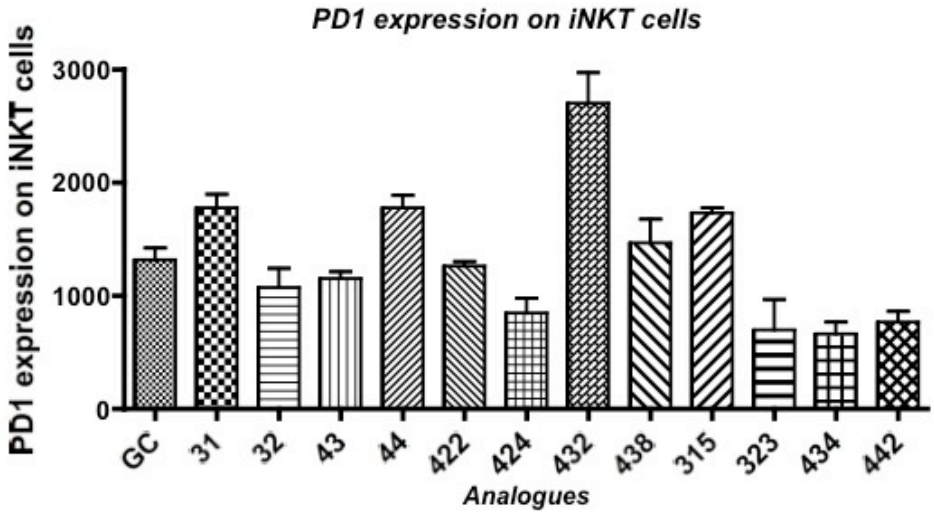


Figure 5.15 Analysis of PD1 expression in all analogues after seven days culture.

The trend for the percentage of unresponsive cells and higher PD1 expressors is generally in keeping with the 7 day culture experiments, especially for those ligands at the extreme end. Analogue 442 which had the lowest number of unresponsive iNKT cells (18%) showed the lowest expression of PD1. Likewise, analogue 432 with the highest number of unresponsive iNKT cells (81%) showed the highest expression level of PD1. These results suggest that the analogues may also differ in the manner in which they induce anergy and that this must also be considered in the interpretation of functional data and the design of optimal iNKT ligands.

5.3.4 Summary of ligand properties

The complete functional data of proliferation, cultured Th1/Th2 ELISPOT and intracellular cytokine activation is shown in Table 5.3 below.

	Proliferation	Th1/Th2 (ELISPOT)	Th1/Th2 Intracellular	Activation (%)
GC	1	1	1	38
OCH	<50%	<1	ND	ND
31	✓	1	1.49	55
32	✓	1	1.24	54
43	<25%	+	3.88	50
44	<25%	+	0.5	48
315	✓	1	3.07	31
323	<25%	1	1.28	22
422	✓	+	0.75	70
424	✓	+	1.82	30
430	✓	+	0.71	57
440	✓	+	1.91	30
432	✓	+	3.19	19
438	✓	1	1.59	64
434	<25%	1	5.47	54
442	<25%	1	14.79	82

Table 5.3 – Summary of modified iNKT ligands for proliferation, activation and Th1/Th2 responses.✓ = similar or higher proliferation index compared to α-GC, + = higher Th1/Th2 cytokine production in ELISPOT assay. Those ligands with an equal or greater response to α-GC are highlighted in gold.

5.4 Discussion and conclusion

Several compounds including 43, 424, 440 and 432 seemed to have a reproducible difference in Th1 responsiveness compared to α-GC, in both ELISPOT and intracellular cytokine assays. Compound 442 showed the highest Th1/Th2 ratio through intracellular responsiveness, but only an average Th1/Th2 ratio by ELISPOT. These differences may relate to the technical approaches used as the amount of cytokine produced may fall above or below the sensitivity of the assay employed i.e. many cells producing a small amount of cytokine may not form a discernable spot. The two compounds (31 and 32) with acyl chain modifications showed very similar activities compared to α-GC in terms of proliferation. By ELISPOT analysis compound 32 displayed a Th2 bias which was similar to that observed in previous mouse experiments (290). Additional in vivo murine studies, though our collaborators at the University of Ghent, have also revealed that some of the 6' -derived galactose modified compounds, including 430 and 424, have a Th1 biased cytokine profile (291-293). This was also the case for analogue 43 in the murine in vivo systems (33). These comparative assessments highlight the need to have testing systems that can correlate animal studies across to man, even when using relatively conserved immune systems such as CD1d and iNKT cells.

The potential of iNKT cells to become anergised by overstimulation is an important area that may limit the potential current ligands to become therapeutic options. We have addressed this as part of our screening programme to assess the differential ligand effects upon both the percentage of activated, cytokine producing cells and the expression of PD1 after 7 days of activation prestimulation to restimulation. α-GC which has been described as inducing anergy through its potent activation activated 48% of cells and had a mean PD-1 MFI of ~1200. Only a few compounds activated more than 60% of the iNKT cells, including 422, 438, 442. Concordant with this, ligand 442 induced the lowest level of PD-1 expression, having the highest number of responsive cells. Compound 432 has the least percentage of responsive iNKT cells and the highest

PD-1 expression. Although this association was not seen across all compounds it may be appropriate to consider low PD1 staining post activation as a marker of anergy resistance, which would be important if iNKT ligands are to be repeatedly administered in a therapeutic setting.

Previous studies have also suggested that other than the cytokines produced by iNKT cells, iNKT cells also highly influence other cell types in particular antigen presenting cells, i.e. dendritic cells and B cells (294-297) through cytotoxicity. It has been suggested that overstimulation of iNKT cells by high affinity ligands may lead to inappropriate APC cell death (271). If this could be selectively modified a longer period of antigen presentation may be permitted which could be beneficial in adjuvant settings. Some of the ligands tested in our screen appear to possess different functional properties compared to α-GC and this aspect of a screening programme would be a useful addition to future studies.

In conclusion, the screening programme has established a range of enhanced, reduced, dissociated functions on human iNKT cells that suggest that the modification of α-GC for specific iNKT manipulation may be a useful undertaking. We have identified compounds that meet the original intention of enhancing the Th1 response of these cells as well as establishing others markers associated with activation, IL17 responsiveness and proliferation/cytokine dissociation. It is clear that a range of different approaches need to be undertaken in the evaluation of new compounds that are intended to manipulate to human immune system and that these must be profiled in animal models and ex vivo human settings to fully explore their potential functions. Future studies may now be directed at examining structural features that may underlie the functional divergences seen with these compounds as well as mechanistic work that begins to better understand how different effector responses may be generated following iNKT activation by different ligands.

Chapter 6 An evaluation of iNKT ligand affinity and specificity

6.1 INTRODUCTION

As discussed previously, CD1 molecules represent of the most conserved family of molecules across mammalian species. Among the five family members in humans, CD1d has received the most attention for a number of reasons. Firstly, compared to other CD1 molecules, the ligands bound to CD1d have been well defined (189, 245, 267, 270, 298). Secondly, the unique population of iNKT cells is conserved in both humans and mice, both reacting to a conserved ligand-CD1d complex (128). Thirdly, iNKT cells have a specific TCR α-chain (Vα24 in humans; Vα14 in mice) and conserved Vβ chain across the species, which allows comparative structural studies to be undertaken. Lastly, the discovery of this cell type's multi functional properties has encouraged the development of CD1d ligands that may have a therapeutic impact in both tumour and autoimmune disease settings (299, 300).

Although the significant contribution of CD1d to immune responses is well recognised, the number of ligands that been identified for iNKT activation remains limited. Indeed, most of the ligands currently used for studying the biological properties of iNKT cells are synthetic ligands (300, 301). Very few ligands have been identified from pathological tissues and during disease settings (302). With regard to iNKT self-ligands, iGb3 was proposed to be the natural ligand for iNKT cells in mouse (303), but this was not thought to be relevant for humans (304).

A better understanding of CD1d and iNKT cells has been achieved through the development of tools such as CD1d tetramers, clonotypic antibodies and TCR antibodies that may robustly identify these rare subsets (305).

At the level of functional cellular studies there has been a particular reliance upon clonal iNKT cell evaluations. In murine studies many paradigms have been established using iNKT hybridomas and then applied to broader in vivo systems of mice and man. As seen in the previous chapter, different ligands were shown to mediate different iNKT functions and we wished to evaluate whether this

related to the original affinity model that led to their design. Therefore we began to develop additional tools to study of these behaviors including a recombinant CD1d dimer for glycolipid_CD1d affinity measurements and a series of ligand specific iNKT clones to examine CD1d ligand cross reactivity.

6.1.1 Current tools for the study of Lipid – CD1d interactions

A few examples of the current tools used to study CD1d-lipid binding include: fluorescent lipid probes (306), Isoelectric-Focusing Electrophoresis (IFE) (307), and Surface Plasmon Resonance (SRP) (308).

The fluorescent lipid probes utilise a system that directly measures the binding of a fluorescently labeled lipid directly to a CD1d molecule. These studies use single chain soluble CD1 proteins and bespoke glycolipids with a NBD group, which provides the fluorescence detection. Such studies suggest that binding is influenced by the construction of each individual probe and pH sensitivity of the lipid probes (306).

Isoelectric-focusing electrophoresis utilises an approach where different shifts in gel position reflect loaded and empty complexes. The enumeration of the density of these recovered complexes representing the binding strength of the different CD1d-lipid complexes (307).

Surface Plasmon Resonance is one of the earliest techniques to analyse and measure the binding of CD1d and lipid. In this system soluble CD1d complexes are fixed to a sensor chip through a streptavidin biotin approach. To measure on and off rates, different lipid ligands can be passed over the complexes and the conformational changes associated with ligand association and dissociation can be measured. This has become the commonest way to measure the on/off rates of different ligands and their associated affinities (308).

Most of these assays involves tagging the lipids or CD1d with an indicator i.e. individual lipid probe or immobilizer i.e. biotin. This allows robust readouts but

does not provide convenience for high throughput screening. Also by modifying each index lipid, unexpected effects may partition to that compound subsequently influencing its affinity (305).

Recently, a new tool has been commercially developed (BD Biosciences) for the investigation of CD1d-lipid interactions. This is a CD1d dimer, consisting of a recombinant soluble CD1d molecule that is fused to an IgG1 backbone. This IgG1-CD1d fusion protein can then be expressed in a mouse plasmacytoma cell line that is heavy chain deleted to produce a bivalent IgG1-CD1d fusion protein that in the presence of β2m forms a functional CD1d structure for glycolipid binding Index lipids can then loaded to the dimer and used for further studies (309).

Shiratsuchi et al. have recently developed this concept and modified the original construct to produce a single chain soluble IgG1-CD1d dimer where β2m has been incorporated into the heavy chain fusion protein through a flexible linker. The latter tool was shown to provide an enhanced stability compared to the former. We choose to develop such a reagent to examine the affinity of some of our ligands for CD1d.

6.1.1.1 Approaches for generating CD1d dimmers/tetramers

Previous studies have produced the CD1d monomer in a *Drosophila* system; where contamination of foreign lipid may occur leading to a very stable complex that does allow the efficient exchange and loading of lipids of interest (108). This has led to most recombinant approaches to CD1d dimers and tetramers now being undertaken in bacterial systems when the individual heavy and light chains of CD1d are prepared prior to loading of the index lipid.

The first tetramer generation techniques were developed about a decade ago, and these have contributed significantly to our understanding of iNKT biology. However, the current approaches to the generation of CD1d tetramer are still

difficult. The process is complex involving numerous stages such as transient gene expression by bacteria, stable gene expression by bacteria (310), expression in insect cells (311), expression in mammalian cells (163), and protein refolding (312, 313) all of which pose their own difficulties. However most of these approaches yield a successful generation of CD1d complexes. Innovations to these processes continue to occur, with recent protocols producing CD1d in lentiviral systems where the yield was shown to be much higher (314).

Traditionally, dimer production was performed in the original *Drosophila* system (308, 310, 315). The dimer was produced by stable co-transfection of both the CD1d and β2m containing plasmids. Currently, most dimer production is undertaken in bacterial expression systems with an IgG1-Cd1d fusion protein.

We decided to adopt the methodology developed by Shiratsuchi et al. with minor changes to develop our single chain β2m-CD1d-Ig dimer (316).

6.2 RESULTS

6.2.1 Constructing the CD1d dimer

We initially introduced a few modifications during the construction of our CD1d dimer compared to that developed by the Shiratsuchi group. Table 6.1 below shows the main differences between the Shiratsuchi dimer and our in-house dimer

Shiratsuchi dimer		***In house dimer***	
Initial Cloned into pcDNA3	Final product in pXIg	Initial Cloned into pGEMT	Final product in pcDNA3
Plasmid containing Ig region		Plasmid without Ig region	
Cloned signaling peptide, β2m and CD1d into plasmid with two steps PCR		Cloned signaling peptide, β2m, CD1d and IgG1 heavy chain with multiple restriction enzyme approach	
Linker present in the plasmid for CD1d and Ig region		Add on linker to the plasmid for the CD1d and Ig region	

Table 6.1 – Approaches to CD1d dimer construction

Following the completion of the above approaches the new inserts were examined by a combination of restriction enzyme digests (Figure 6.1).

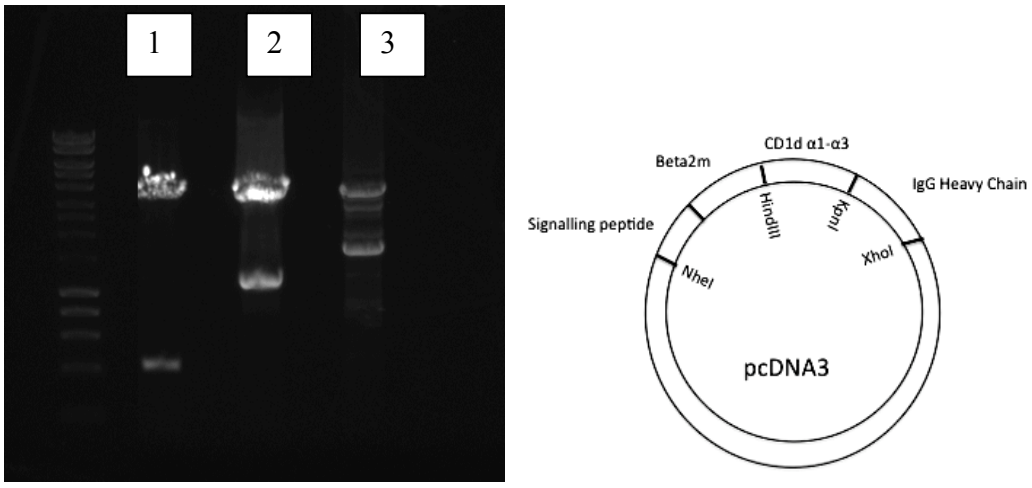


Figure 6.1 Digestion of final construct product and dimer mapz.

Lane 1 is digested with Nhe1 and HindIII for β2m release, lane 2 is with KpnI and HindIII for CD1d release and lane 3 with XhoI and Kpn for heavy chain release

The CD1d single chain construct was sequenced and this confirmed that the signaling peptide (red), β2m (purple), linker (green), CD1d heavy chain (orange) IgG heavy chain variable region and constant regions (blue) were all correct (Figure 6.2).

GCT AGC ATG GAA TGG AGT TGG ATA TTT CTC TTT CTC CTG TCA GGA ACT GCA GGT ATG ATC CAG CGT ACT CCA AAG ATT CAG GTT TAC TCA CGT CAT CCA GCA GAG AAT GGA AAG TCA AAT TTC CTG AAT TGC TAT GTG TCT GGG TTT CAT CCA TCC GAC ATT GAA GTT GAC TTA CTG AAG AAT GGA GAG AGA ATT GAA AAA GTG GAG CAT TCA GAC TTG TCT TTC AGC AAG GAC TGG TCT TTC TAT CTC TTG TAC TAC ACT GAA TTC ACC CCC ACT GAA AAA GAT GAG TAT GCC TGC CGT GTG AAC CAT GTG ACT TTG TCA CAG CCC AAG ATA GTT AAG TGG GAT CGA GAC ATG GGA GGA GGT TCT GGA GGT TCA GGT TCT GGA GGA GGT GCT AAG CTT GTC CCG CAA AGG CTT TTC CCC CTC CGC TGC CTC CAG ATC TCG TCC TTC GCC AAT AGC AGC TGG

ACG CGC ACC GAC GGC TTG GCG TGG CTG GGG GAG CTG CAG ACG
CAC AGC TGG AGC AAC GAC TCG GAC ACC GTC CGC TCT CTG AAG
CCT TGG TCC CAG GGC ACG TTC AGC GAC CAG CAG TGG GAG ACG
CTG CAG CAT ATA TTT CGG GTT TAT CGA AGC AGC TTC ACC AGG
GAC GTG AAG GAA TTC GCC AAA ATG CTA CGC TTA TCC TAT CCC
TTG GAG CTC CAG GTG TCC GCT GGC TGT GAG GTG CAC CCT GGG
AAC GCC TCA AAT AAC TTC TTC CAT GTA GCA TTT CAA GGA AAA
GAT ATC CTG AGT TTC CAA GGA ACT TCT TGG GAG CCA ACC CAA
GAG GCC CCA CTT TGG GTA AAC TTG GCC ATT CAA GTG CTC AAC
CAG GAC AAG TGG ACG AGG GAA ACA GTG CAG TGG CTC CTT AAT
GGC ACC TGC CCC CAA TTT GTC AGT GGC CTC CTT GAG TCA GGG
AAG TCG GAA CTG AAG AAG CAA GTG AAG CCC AAG GCC TGG CTG
TCC CGT GGC CCC AGT CCT GGC CCT GGC CGT CTG CTG CTG GTG
TGC CAT GTC TCA GGA TTC TAC CCA AAG CCT GTA TGG GTG AAG
TGG ATG CGG GGT GAG CAG GAG CAG CAG GGC ACT CAG CCA GGG
GAC ATC CTG CCC AAT GCT GAC GAG ACA TGG TAT CTC CGA GCA
ACC CTG GAT GTG GTG GCT GGG GAG GCA GCT GGC CTG TCC TGT
CGG GTG AAG CAC AGC AGT CTA GAG GGC CAG GAC ATC GTC CTC
TAC TGG GGT GGG AGC TAC ACC TCC ATG GGC TTG ATT GCC TTG
GCA GTC CTG GCG TGC TTG CTG TTC CTC CTC ATT GTG GGC TTT
ACC TCC CGG TTT AAG AGG CAA ACT TCC TAT CAG GGC GTC CTG
GGT ACC ACT AGT GTG CCC AGG GAT TGT GGT TGT AAG CCT TGC
ATA TGT ACA GTC CCA GAA GTA TCA TCT GTC TTC ATC TTC CCC
CCA AAG CCC AAG GAT GTG CTC ACC ATT ACT CTG ACT CCT AAG
GTC ACG TGT GTT GTG GTA GAC ATC AGC AAG GAT GAT CCC GAG
GTC CAG TTC AGC TGG TTT GTA GAT GAT GTG GAG GTG CAC ACA
GCT CAG ACG CAA CCC CGG GAG GAG CAG TTC AAC AGC ACT TTC
CGC TCA GTC AGT GAA CTT CCC ATC ATG CAC CAG GAC TGG CTC
AAT GGC AAG GAG TTC AAA TGC AGG GTC AAC AGT GCA GCT TTC
CCT GCC CCC ATC GAG AAA ACC ATC TCC AAA ACC AAA GGC AGA
CCG AAG GCT CCA CAG GTG TAC ACC ATT CCA CCT CCC AAG GAG
CAG ATG GCC AAG GAT AAA GTC AGT CTG ACC TGC ATG ATA ACA

GAC TTC TTC CCT GAA GAC ATT ACT GTG GAG TGG CAG TGG AAT GGG CAG CCA GCG GAG AAC TAC AAG AAC ACT CAG CCC ATC ATG GAC ACA GAT GGC TCT TAC TTC GTC TAC AGC AAG CTC AAT GTG CAG AAG AGC AAC TGG GAG GCA GGA AAT ACT TTC ACC TGC TCT GTG TTA CAT GAG GGC CTG CAC AAC CAC CAT ACT GAG AAG AGC CTC TCC CAC TCT CCT GGT AAA TGA CTC GAG

Figure 6.2. Sequence of the final single chain CD1d dimer construct

To furnish the single chain construct with a light chain for correct folding the construct was transfected into the J558L plasmacytoma cell line, with a GFP vector to check for the efficiency of transfection. Figure 6.3 shows the overlay of untransfected J558L (Blue) and transfected J558L cells (Red) demonstrating an 85% transfection efficiency, with a viability of the cells of more than 80%.

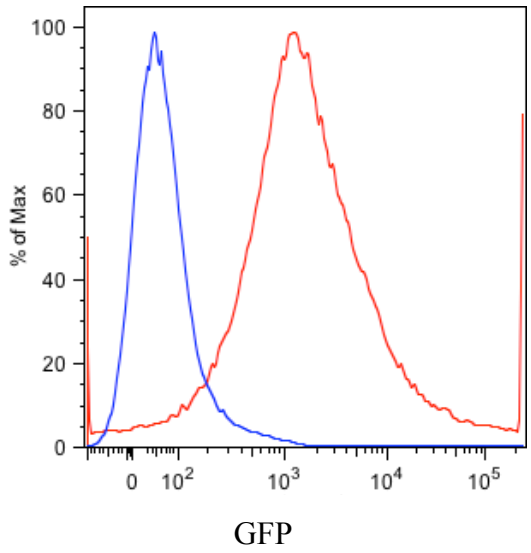


Figure 6.3. Transfection efficiency of GFP and single chain construct following co-transfection

To check the cells for successful CD1d single chain construct expression and folding the cells were permeablised and labeled with an anti-CD1d antibody (Figure 6.4).

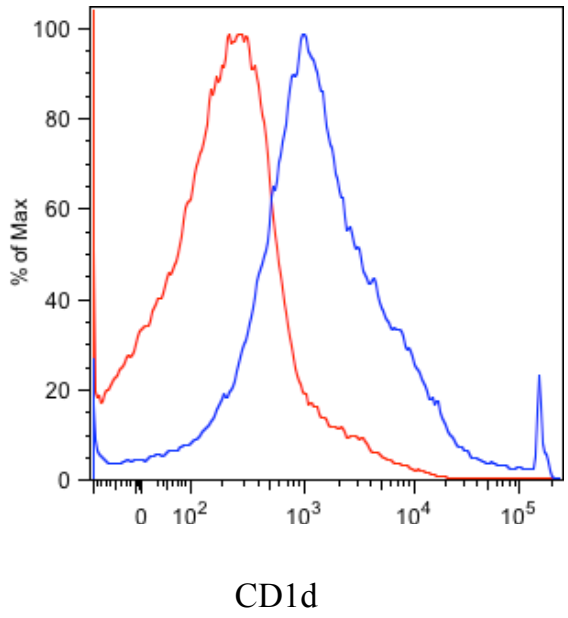


Figure 6.4 CD1d expression in non-transfected and transfected J558L cells

The secreted protein was then purified (IgG affinity column) and an SDS-page gel of the purified protein undertaken with blotting for the linked anti-β2m (Figure 6.5)

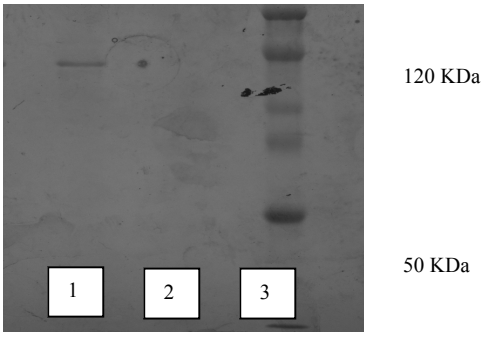


Figure 6.5 β2m western blot of CD1d dimer construct following supernatant purification. Lane 1 - IgG affinity column elution, Lane 2 – affinity column flow through, Lane 3 – size marker lane

The secreted protein was reactive with β2m and of the appropriate molecular weight. A schematic diagram of the single chain dimeric tool is shown in Figure 6.6 and we proceeded to assess whether this was able to identify iNKT cells.

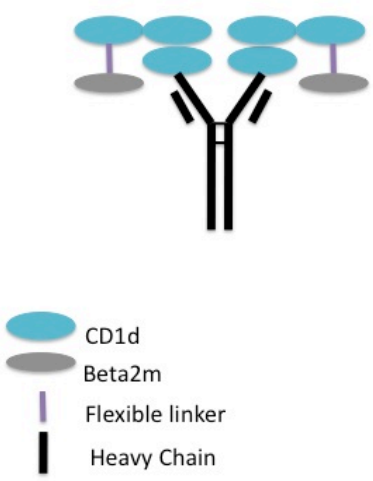


Figure 6.6 Schematic diagram for the single chain CD1d dimer

6.2.2 Recognition of INKT cells

To assess if the CD1d fusion dimer was functional, we first looked at its ability to recognize polyclonal iNKT cells. The dimer was loaded with α-GC overnight and used to stain iNKT cells. Figure 6.7 shows the identification of a small iNKT population with the α-GC loaded CD1d dimer but absent staining with the empty dimer.

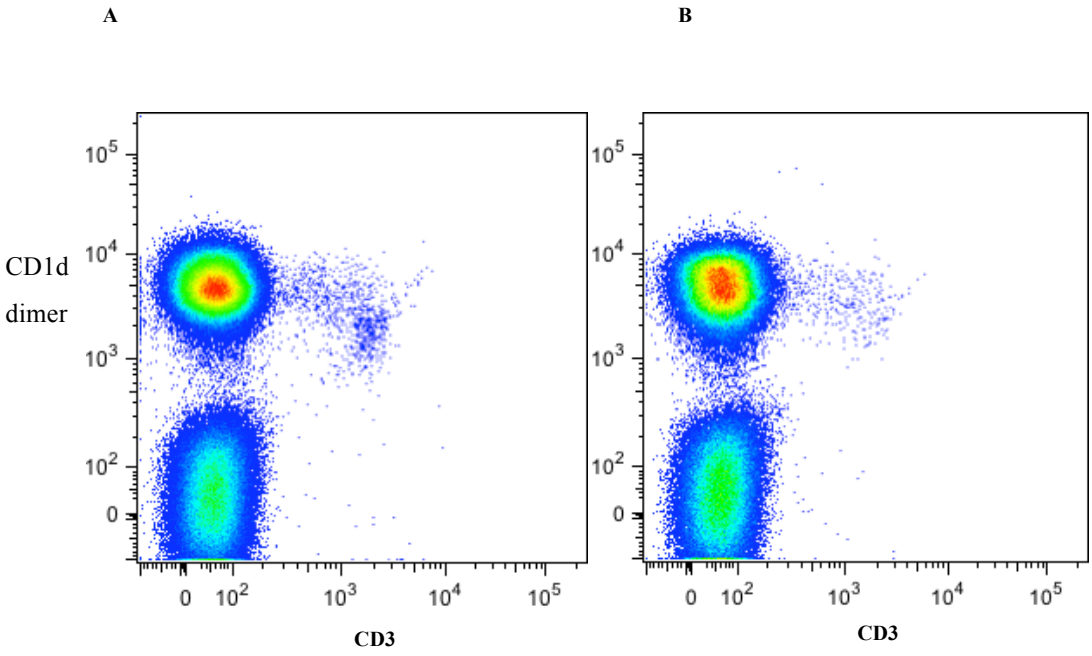


Figure 6.7 FACS staining of human PBMC with a loaded (A) and unloaded (B) CD1d dimer.

6.2.3 CD1d dimer binding to iNKT cells

To assess the binding of different lipids to CD1d, we used the established competition ELISEA assay described by the Shiratsuchi group (305). We set up the competition assay with a fluorescent lipid indicator, namely 18:1 Biotinyl PE (B-PE) (Figure 6.8). The 18:1 Biotinyl PE lipid has been shown previously to bind to CD1b, CD1c and CD1d (317).

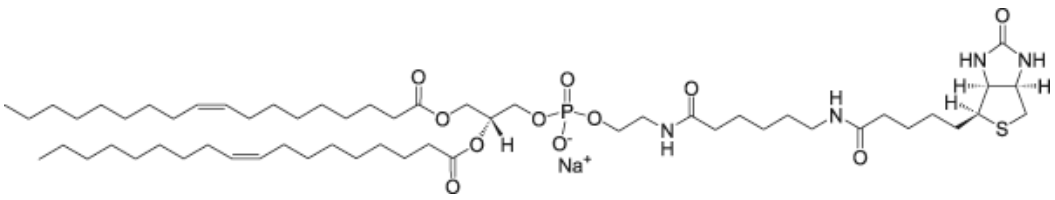


Figure 6.8 Structure of the 18:1Biotinyl-PE indicator lipid used in the affinity experiments.

We established the assay with our acyl and sphingosine chain modified ligands that had been originally designed to influence CD1d binding. Figure 6.9 shows the initial Kd assessment for the reporter 18:1 Biotinyl PE lipid following a titration to saturation binding of the empty dimer. The calculated Kd of 18:1 BiotinylPE from this was 1.54 (0.5 – 2.588) which is similar to that of the original publication (1.68).

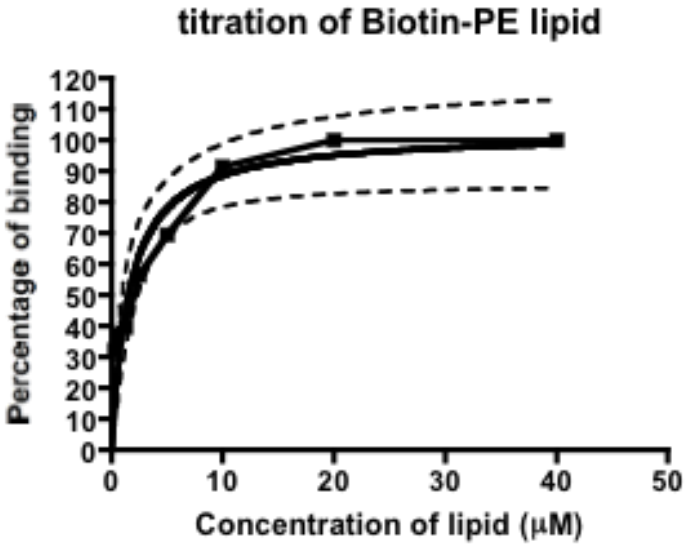


Figure 6.9 Titration of the labeled 18:1Biotinly-PE lipid to evaluate its Kd. The (squares) represent experimental data points, the uninterrupted line represent the best fit curve, and the dotted lines the 95% confidence intervals for the Kd value.

Figure 6.10 shows the ELISA results for the acyl chain and sphingosine chain modification analogues in this heterologous competition assay. Here the 18:1Biotinly PE CD1d complex is initially loaded and an increasing concentration of the index lipid added overnight to compete with the reported ligand. The Ki can then be calculated for the unlabelled ligand using the formula Ki = IC50 / (1 + [B-PE] / Kd). Here the Kd represents that of B-PE calculated previously, the concentration of B-PE used in the preloaded complex and IC50

the concentration of the structurally distinct analogues that reduce B-PE binding by 50%.

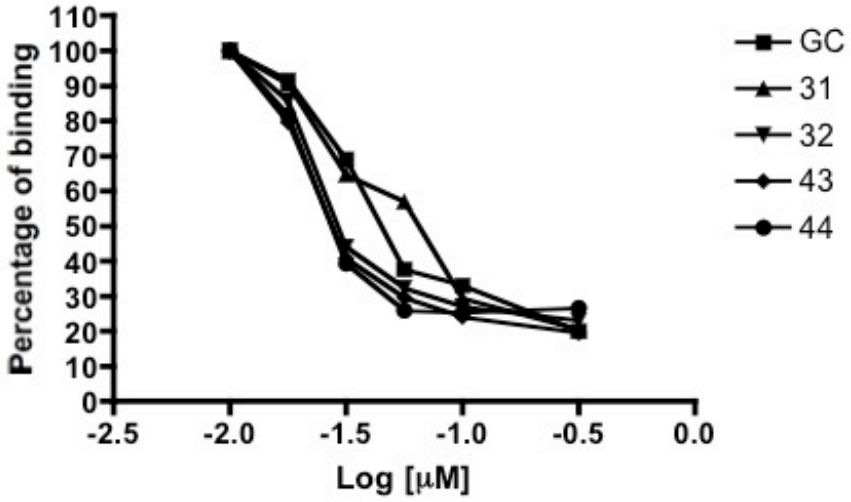


Figure 6.10 Heterologous competition ELISA results for the 4 acyl and sphingosine modified compounds.

The Ki results for the 5 compounds, including α-GC are as follows:

	α-GC	31	32	43	44
Ki Value	0.04 (μM)	0.06(μM)	0.02(μM)	0.016(μM)	0.016(μM)

The Ki value for α-GC obtained from our assay is similar to the value (0.07μM) suggested by Shiratsuchi et al. It is notable that the compounds 31 and 32 are most similar to α-GC, both in these affinity measurements and functional parameters. Compared to α-GC, Compounds 43 and 44 show a slightly improved affinity with an identical Ki of 0.016μM.

6.2.4 Generation of iNKT clones

We followed up our observations relating to the ligand specific differences in proliferative and cytokine responses by generating iNKT clones to the individual analogues and assessing their cytotoxicity and cross reactivity profiles.

Jurkat and U937 cells both express CD1d and were selected as target cells whilst K562 cells, which do not express CD1d, were selected as negative controls (Figure 6.11).

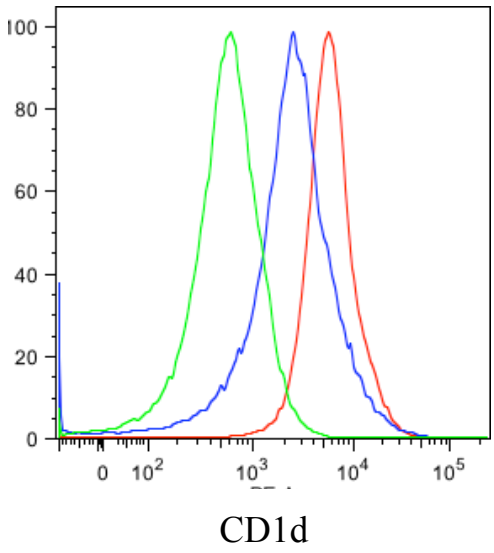


Figure 6.11. CD1d expression of Jurkat (red), U937 (blue) and K562 cells (green).

A flow based killing assay was established using α-GC pulsed target cells, and an NKT cell clone generated with α-GC (gift from Professor Stephan Gadola).

Figure 6.12 is representative of the cytotoxicity observed in the experiments where a cell permeable dye (To-Pro-3) that emits fluoresce in the APC channel is taken up by target cells when they have been recognized by ligand specific iNKT clone. The assay measures the percentage of To-Pro3 positive target cells following co culture at the respective E:T ratios Figure 6.13 shows the killing

ability of NKT cells against Jurkat, U937 and K562 cells in this 'homologous ligand' set up.

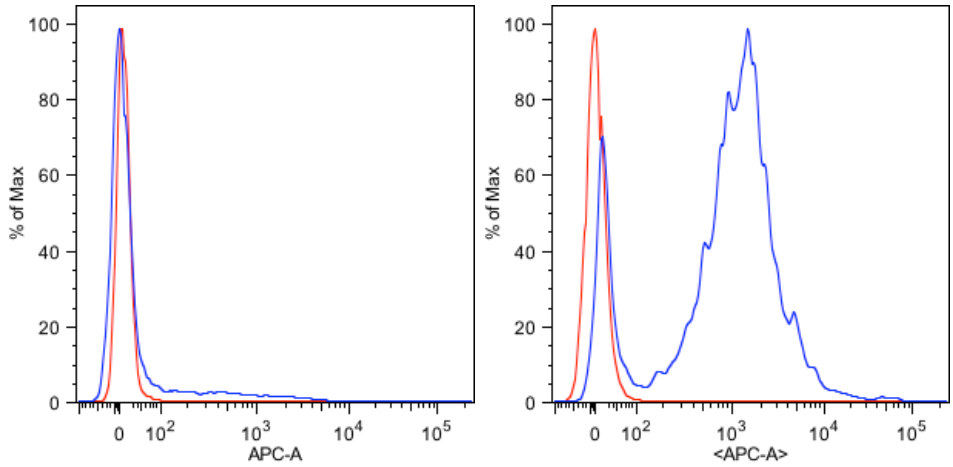


Figure 6.12 Representative example of CD1d mediated cytotoxicity of α-GC pulsed target cells and iNKT cells clones at different E:T ratios (Left 0.1:1 and right 5:1)

The cytotoxicity was proportional to the level of expression of CD1d expressed in the target cells as seen in Figure 13.

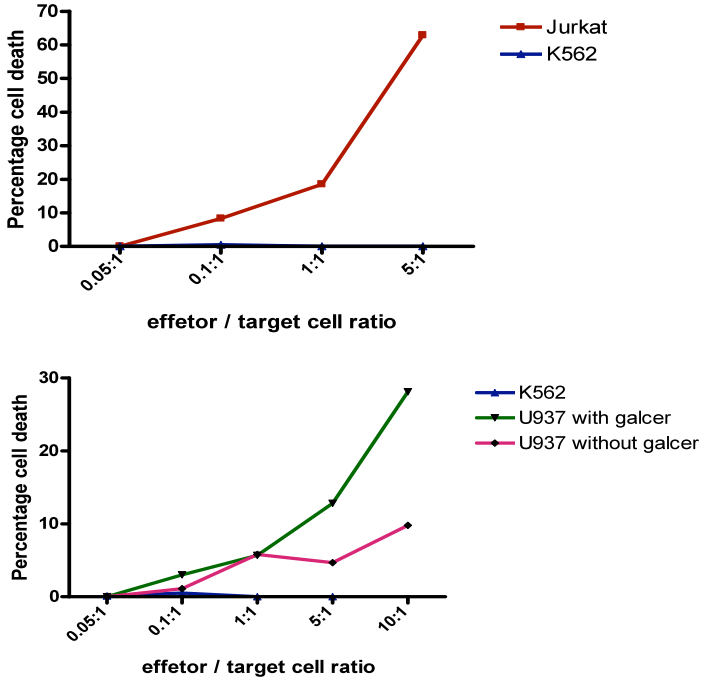


Figure 6.13 iNKT cytotoxicity of α-GC pulsed target cells with differing levels of CD1d expression

As expected, no specific killing was observed with the CD1d negative NK sensitive cell line K562 or in the absence of exogenous lipid ligand.

We then evaluated the ability of the same α-GC iNKT clone to kill compound 31-pulsed U937 target cells. Figure 6.14 shows the unexpected finding of an absence of killing of the pulsed target, despite the previously noted ability of compound 31 to bind to CD1d and initiate functional iNKT cell responses.

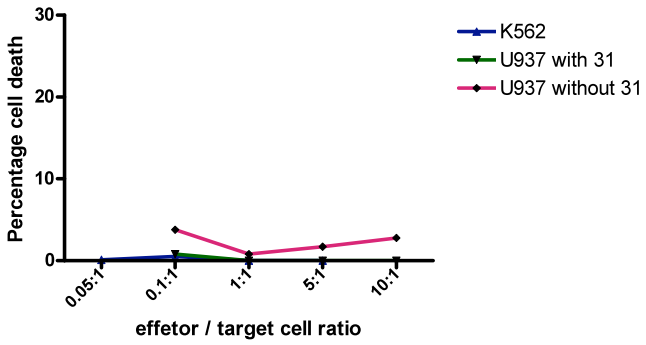


Figure 6.14 α-GC derived iNKT clone killing of a glycoplid analogue (31) pulsed target cell

The absence of killing suggests that the α-GC iNKT line may have glycolipid ligand specificity and that despite the invariant nature of the TCR single antigen recognition may be inducible in iNKT cells. We subsequently generated NKT cell lines against the different analogues being successful for compounds g α-GC, 31, 43 and 44. Figure 6.15 shows two examples (31 and 43) of the iNKT cell line phenotypes. All the iNKT cell clones retained their characteristic Vα24 and Vβ11 expression but in contrast to the previous polyclonal experiment these activated lines lost their expression of CD161.

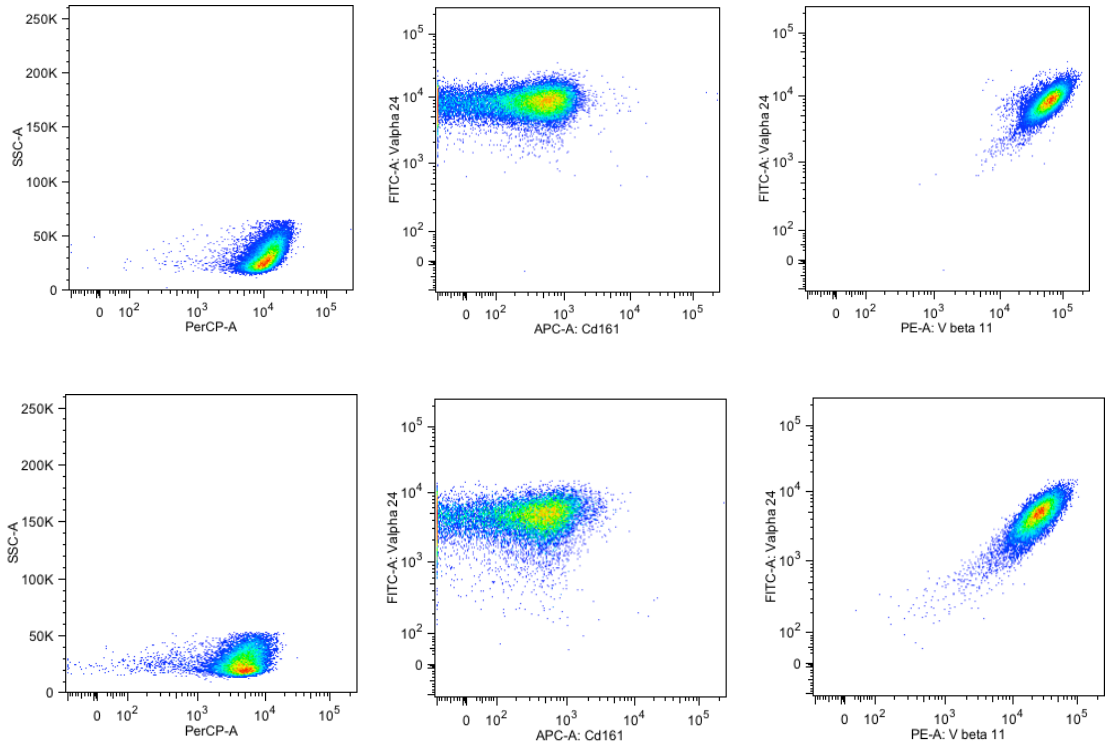


Figure 6.15 Phenotype of the iNKT cells clones

Figure 6.16 shows a 'criss cross' cytotoxicity assay for the first ligand specific clones that were generated against compound 43. A number of observations can be made including the specificity of the clone for its index ligand and the poor cross reaction to the α–GC pulsed target. This implied that there was little heterologous immunity between the iNKT clones for different glycolipid targets.

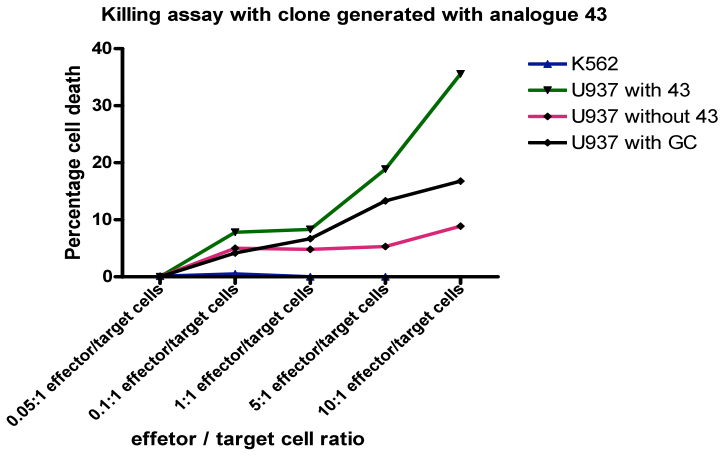


Figure 6.16 Cytotoxicity by compound 43 generated iNKT clones against homologous and heterologous targets.

Similar experiments were then undertaken for the other ligand pairs and these are seen in Figure 6.17.

% Killing — Clones

LIGAND	Alpha-GC	31	43	44
Alpha-GC	28	10	9	6
31	8	34	5	5
43	5	12	39	18
44	7	13	24	43

Figure 6.17 Summary of 'criss cross cytotoxicity assays for ligand specific iNKT clones at a 10:1 E:T ratio.

A similar level of ligand specificity was observed for all of the derived iNKT clones across the different compounds tested. This suggests that antigen specificity may be a property that iNKT cells share with conventional T cells despite their conserved invariant TCR.

6.2.5 SUMMARY

•We have successfully generated a new tool for the assessment of iNKT cells and affinity evaluations of novel CD1d lipids.

•The compounds 31 and 32 have similar Ki to α-GC whilst 43 and 44 showed a slight improved affinity.

•The differences in affinity between the compounds would not appear to explain the many difference observed between these lipids which were all able to induce proliferative, cytokine and cytotoxic iNKT cells responses.

•iNKT cells may be derived to specific glycoplids that do not show cross reactivity to other Cd1d binding lipids. This specificity of ligand recognition is intriguing given the invariant nature of the Vα chain and suggest subtle changes in the Vβ chain may be important for antigen specificity.

6.3 Discussion and Conclusion

During this part of my research, we successfully generated tools to help us evaluate the binding characteristics of our novel compounds. The β2m-CD1d-IgG dimer afforded us the opportunity to compare the Ki of the new compounds with that of α-GC and review whether the original improved affinities were evident and/or related to the diverse functional responses previously reported. The results suggest that the compounds do show some differences compared to the prototypic α-GC but these do not neatly align themselves with the diverse functional responses recorded. This method is an efficient approach for screening the affinity of new compounds for CD1d binding and might be adapted for those altered for TCR interactions in the future.

The dimer did allow recognition of rare iNKT cells in PBMC but the unloaded dimer showed some background staining that would need to be addressed before this is used for robust iNKT enumeration. This staining may represent non specific binding of the fusion protein to Fc receptors on monocytes or B cells and this may be improved with the addition of a Fc blocking antibody for further studies.

In our CD1d binding study, most lipids showed a Ki that was in the same range as α-GC, and similar to that of the original Shiratsuchi group (305, 307). However our binding assay does not truly represent the in vivo loading of glycoplids as these occur in the late endosome which is more acidic (318). Indeed, previous studies have shown that optimal CD1d binding occurs in at around pH 5-6 (319).

In addition to using this tool for evaluating the binding properties of the lipid to CD1d, it may be also used in the context of an artificial antigen presenting cells in a cell free system (305). Such systems are all ready in place for expanding peptide specific cytotoxic T cells for therapeutic indications.

This work has suggested that even though iNKT cells can be considered as innate cells, they can exhibit certain ligand specificities similar to conventional T cells. The degree of cross reactivity between our ligand specific clones was minimal apart from ligands 43 and 44. It is not clear what the underlining reason for this may be; although both 43 and 44 share an alteration in the 4-OH position on the sphingosine chain. A previous study has suggested that even though the 4-OH position is not directly involved does in the binding to CD1d, it might affect the 3-OH recognition (292). The proliferation indices of different analogues also suggested that different ligands might selectively expand iNKT cells when 43/44 were compared to 31/32. The differential effects of compounds 43 and 44 on the iNKT cells of low and high donors also support the previous notion that a distinctive functional difference exists between the iNKT cells of such individuals.

In conclusion in would appear that the original assumption that altering the binding affinity of α-GC analogues would increase the Th1/Th2 cytokine ratio is too simplified. We have shown that the chemical modifications do not significantly alter binding affinities whilst markedly altering functional profiles. These different effects compared to α-GC can be influenced at the level of the type of donor (high vs. low), cytokine environment (IL-2 vs. IL15), effector readout (proliferation vs. cytokines), specificity and murine/human model. Further analogue development programs will need to take a multidisciplinary approach that extends beyond current structure function relationships to aid the rational design of new iNKT modulatory ligands.

Chapter 7 iNKT cells in common variable immunodeficiency (CVID)

7.1 Introduction

Common variable immunodeficiency (CVID) is the commonest primary antibody deficiency, representing a heterogeneous group of primary defects (320). CVID is also commonly known as acquired hypogammaglobulinema, adult-onset hypogammaglobulinemia, or dysgammaglobulinemia (321). Patients generally have a marked reduction in serum immunoglobulin G (IgG) and immunoglobulin A (IgA), sometimes accompanied with a reduction in immunoglobulin M (IgM). They typically present with recurrent sinopulmonary infections and impaired antibody responses to vaccination (322). A bimodal distribution has been described for the age of first presentation of CVID. A few patients present in childhood, but the majority present after the age of 20. The onset of CVID in males is relatively earlier compared to females, with a mean age of 23 compared to 28 years old (323).

One quarter of the CVID patients also present with associated autoimmune disorders, including autoimmune thrombocytopenia, haemolytic anaemia and thyroiditis (320). Lymphadenopathy and organomegaly, particularly splenomegaly, is also noted in a similar proportion.

The clinical features of recurrent infection, antibody deficiency autoimmune events and lymphoproliferation suggest an underlying fundamental immune dysregulation in this disorder. Recent research has established some single gene defects that offer different genotypic explanations for the phenotypic spectrum seen in this disorder.

7.1.1 Genetic defect in CVID

There are four major genetic defects that have been identified in CVID over the last decade. These four are the inducible T-cell costimulator (ICOS) (324, 325), transmembrane activator and calcium modulating cyclophylin ligand interactor

(TACI) (326-328), the B-cell activating factor of the tumour necrosis factor family receptor (BAFFR) (329), and the CD19 (330).

7.1.1.1 ICOS

ICOS was the first genetic defect to be associated with CVID. Nine CVID individuals from four unrelated families were identified to have identical a large genomic deletion of the T cell activation molecule, ICOS (324, 325).

ICOS belongs to a family of Ig-like co stimulatory receptors on T cells, sharing some homology with CD28, CTLA-1 and PD1 (331). In humans, ICOS is located on chromosome 2q33-34, only 300kb away from CTLA-4 and CD28 (332). Unlike CD28, ICOS and the other members of this family are not constitutively expressed on T cells (333). Activation of both the T-cell receptor and CD28 leads to a rapid induction of ICOS expression on the surface of T cells. Cytokines such as IL-12, IL-23 and IL-4 may also influence the expression of ICOS on activated T cells (334, 335). The influence of ICOS is evident for a range of T cell activity including T-cell differentiation, cell survival, cytokine production and the support of antibody production. These multiple activities are secondary to increases in the cytokines IL-4, IL-5, IL-6, IL-10, TNF-α and IFN-γ (333). ICOS mediates this cytokine signalling through its interaction with the growth factor receptor-bound protein 2 (Grb2), which is required for the initial T cell IL-2 production (333, 336).

ICOS is primarily expressed in the thymus and secondary lymphoid tissues where it supports T cell development and CD4 T cell help following initial antigen presentation (337).

Although the initial genetic defect appeared to relate to a geographical region it has been estimated that this autosomal recessive condition may occur in up to 2% of CVID patients (325, 338). The phenotype of such patients includes a lack of circulating CD27+ switched memory B and a susceptibility to neoplastic disease and autoimmunity in the absence of ICOS expression post T cell activation (320).

7.1.1.2 CD19

CD19 deficient CVID patients were first identified in 2006 (330). Four patients from two unrelated families were identified to have homozygous frameshift mutations in the cytoplasmic domain of CD19. A follow up study from another group identified a further 2 patients with a defect in CD19. This was a compound heterozygous disorder a splicing defect on one allele and a deletion on the other (339).

CD19 in an early B cell membrane surface molecule, and it remains on B cells until they differentiate into plasma cells. CD19 forms a signalling complex with CD81, CD21 and CD225 on mature B cells (330) and is essential for stabilising and amplifying the signal derived from BCR ligation (340).

Very few CVID patients have been subsequently identified to have CD19 deficiency. The phenotype of such patients is that of having normal B cells numbers with a decrease in class-switched memory B cells and normal T cell function.

7.1.1.3 Tumour necrosis factor superfamily

Mutations in TNFRSF13B (TACI) and TNFRSF13C (BAFF-R) are found in CVID patients. As both TACI and BAFF-R are tumour necrosis factor superfamily members, they share three common features. They both belong to the Type III transmembrane family, both contain cysteine-rich domains and their expression is restricted to lymphocytes (341). TACI and BAFF-R, together with their ligands BAFF and APRIL, are the key signalling members to regulate the differentiation, homeostasis and function of B cells (341). Both TACI and BAFFR ligands are widely expressed in professional antigen presenting cells such as macrophages, monocytes and dendritic cells (342, 343).

TACI gene is one of the commonest mutations found in CVID patients. About 10 - 15% of the CVID patients were found to have heterozygous TACI mutations. TACI expression is highly related to the maturation of the B cell population. TACI expression is observed on marginal zone B cells and CD27+ memory B

cells, with an upregulation being evident after B cell stimulation (341, 344-346). TACI has a high affinity binding to both APRIL and BAFF (341) and can signal via both NFκB and NFAT/AP1 pathways (341, 347).In TACI knockout mice, a lupus-like autoimmune disorder with lymphocyte infiltration of liver and kidney develops in the absence of hypogammaglobulinaemia (348). In addition, this study has also found that anti-murine TACI antibodies could inhibit the proliferation of B cells (348). Further studies have suggested that the interaction between TACI and APRIL is the key cross talk for IgA induction. They showed that in APRIL deficient mice, there was a deficiency in IgA production (349) and that TACI was a positive regulator for terminal B cell differentiation (348).

CVID patients with TACI mutations normally have single amino acid substitutions, which introduce premature stop codons and frameshifts (350). Such patients typically have low IgG immunoglobulin, but normal range of IgM with autoimmunity and splenomegaly (351).

Very few CVID patients have been identified to have BAFFR mutations. Unlike TACI, BAFFR only binds to one ligand and it is widely expressed on all the peripheral B cells (344). It is understood that the interaction between BAFFR and its ligand is the key for the survival of peripheral B cell subsets, particularly those at a transitional stage. B cell lymphopenia and immunodeficiency are observed in both BAFF and BAFFR deficient mouse model (352, 353).

Up to date, only one individual with CVID has been reported to have BAFF-R deficient; with a 24 base pair deletion of the gene (320). Both class-switched and non-switched memory cells were reduced in the patient with an increase in transitional B cells (320).

7.1.2 CVID and T cells

It is well established that CVID is a disorder characterised by antibody deficiency, but with features that that suggest T cell dysregulation. Indeed effective thymopoiesis of naïve T cells and a lower activation and proliferation

of memory T cells has been demonstrated in CVID leading to a higher rate of apoptosis and abnormal cytokine production (increase in IFN-γ) (354).

One of the major T cells defective in CVID is in the CD4+ naïve T cells and a CVID classification has been proposed relating to this abnormality (355). A correlation was identified between CD4+ naïve T cells levels and the incidence of splenomegaly (356) as well as the amount of IgG and IgA in the serum (355). Most of these patients were subsequently shown to fall into another classification, Warnatz Ia, based upon the presence of memory B cells (357). Furthermore recent studies using TRECs to evaluate thymic output have shown that only the patients in the Ia group have a lower level of TRECs, whereas normal levels of TRECs were found in groups Ib and II (357).

Within the CD8+ T cell subset, further alterations have been identified including an increased number of CTL, a predominant effector-memory phenotype and a high level of serum IL-7 (354). This has led to a Th1 polarisation in many (354) and a higher turnover rate compared to healthy controls (356).

Finally recent studies have indicated that Tregs may also be deficient in CVID patients (358). Tregs from those with autoimmunity showed decreased suppressor function and downregulation Treg proteins including FoxP3, Granzyme A, pSTAT5 and GITR (358). Again this cohort co segregated to that of the Ia patient group (359).

7.1.3 iNKT cells and antibody production

7.1.3.1 iNKT cells and B cells

The classical T cell dependent, B cell activation pathway was described over two decades ago (360). Recently, several studies have established that iNKT cells, just like conventional T cells, can also support B activation and antibody antibody production (249, 361-363).

In such studies an important helper function of iNKT cells was demonstrated in the primary and secondary antibody responses, where increased antibody concentrations were observed when α-GC was co-delivered with the protein antigen (362). A study by Tonti et al. showed that activated iNKT cells, once they express CD40L, can enhance the responses on other B cells that express CD40, and that this is not limited by antigen specific CD1d recognition (363). Other studies have established that iNKT cells can directly interact with B cells (249, 361). In the first study, the interaction of iNKT cells with B cells led to the release of lipid specific antibody via a classical cognate interaction (361). The second study showed that iNKT cells could interact with the CD1d on B cells whilst the lipid was endocytosed by surface IgM. Very high titres of specific of IgM and early class switching were then observed in this model (249). These studies suggest that iNKT cells may play a direct or indirect role in supporting antibody production in mouse models (363).

7.1.3.2 iNKT cells and antibody deficiency

iNKT cell deficiency has been identified in two types of antibody deficiency – severe combined immune deficiency (SCID) and X-linked lymphoproliferative syndrome (XLP) (137, 150).

XLP was the first antibody-deficiency associated disorder shown to manifest a specific iNKT defect. In the first study of XLP patients, no iNKT cells were detected through either CD1d tetramer or the invariant TCR receptor antibody approaches. All these patients were shown to have a defect in SAP, which is the adaptor protein that is critical for SLAM function (150). In XLP patients where the genetic defect is BIRC4 (and not SAP) contrast, the patients displayed a normal range of iNKT cells (ranging from 0.01% - 2%) (364). Following these descriptions a murine knock out of SAP was made which confirmed the absence of iNKT cells and importance of SAP in their development (364).

7.2 Aims

•To investigate the presence of iNKT cells in a large population of CVID patients with no known mutations, controls and a disease control group of patients with a specific B cell defect (X linked agammaglobulinaemia)

•To evaluate the function of iNKT cells in CVID

•To establish if iNKT number or function associates with clinical or phenotypic sub classifications.

7.3 Results

7.3.1 iNKT cell frequency in CVID patients

CVID patients and healthy controls were analysed by six-colour flow cytometer on whole blood. Figure 1 illustrates the raw data plots of the enumeration.

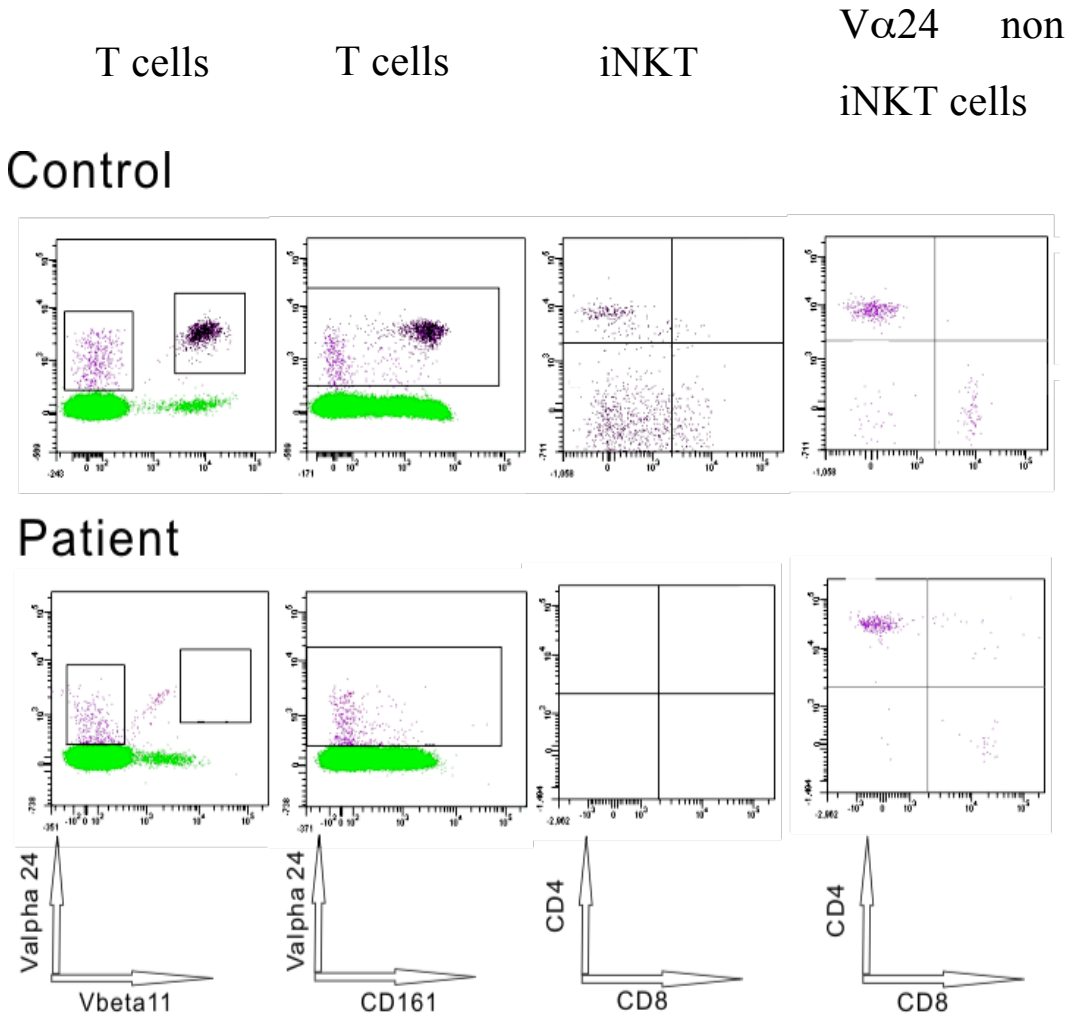


Figure 7.1. Representative iNKT cell enumeration in CVID patient and Healthy control.

A series of 37 CVID patients and 50 healthy controls were analysed over one year period. Compared to the healthy control, no detectable iNKT cells were found in many CVID patients, as seen in Figure 7.1. It is notable that Vα24 cells are present in the CVID patients but not iNKT cells. This lack of iNKT cells was supported by the lack of CD161 positive Vα24 positive T cells.

We then compared the number of iNKT cells in the 37 CVID patients and 50 healthy controls (Figure 7.2).

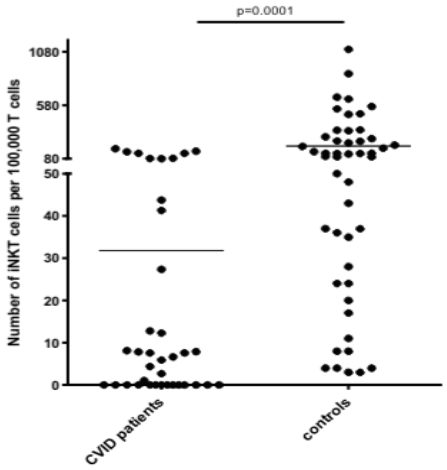


Figure 7.2 Number of iNKT cells in CVID patients and healthy controls

A marked reduction in iNKT cells was observed in the CVID patients compared to the healthy controls, with a mean value of 31 compared to 200 ($p < 0.0002$). However some CVID patients did appear to have a normal number of iNKT cells, suggesting heterogeneity with regard to this T cell subset abnormality.

Two further properties of iNKT cells were reviewed including the expression of the CD4 co-receptor and maturation marker CD161. The ratio of CD4:DN iNKT cells differed between the groups ($P<0.05$) suggesting that CD4 iNKT with a Th2 and Th1 cytokine capacity were present whilst CD161 expression was reduced in the iNKT cells of CVID patients. As mentioned earlier this may be due to

activation or immaturity as both may reduce expression of this CD161.

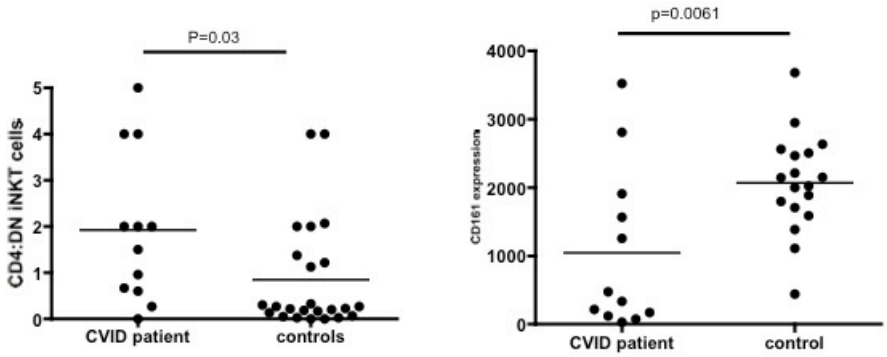
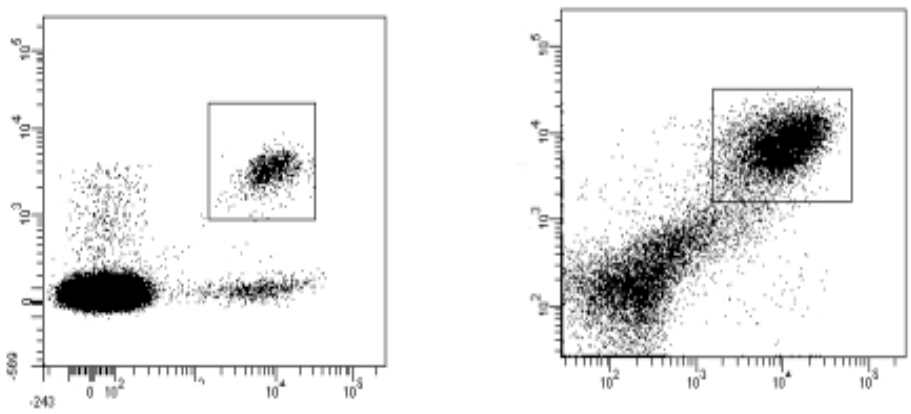


Figure 7.3. iNKT cell phenotype of CVID patients and healthy controls

7.3.2 Function of iNKT cells in CVID patient

iNKT cells were detected in all healthy controls with our approach allowing accurate detection ~5 iNKT cells per 10^5 T cells. (0.0005%). To exclude the presence of iNKT cells below this number in CVID patients we undertook iNKT proliferation studies to assess if such low numbers could be expanded to our detection levels and to calculate the expansion potential of those patients where these cells were detectable. A representative plot of the proliferation experiments of a healthy control is seen in Figure 7.4, following a 7 day culture with αGC.



Pre-proliferation Post-proliferation

Figure 7.4 Proliferation profile of iNKT cells in a representative healthy control.

In the case of CVID patients, only 8 individuals (21%) showed measurable proliferation (Figure 7.5). This was in accordance with the ex vivo enumeration where only 8 individuals had greater than 80 iNKT cells detectable. Of the 16 controls all expanded, with a mean proliferation index of 50. Thus iNKT cells are truly deficient in the majority of CVID patients. From these initial functional studies 2 groups of CVID patients could be discerned. The first group had detectable iNKT cells and these could expand whilst the second group had low or absent iNKT cells that did not proliferate like healthy controls.

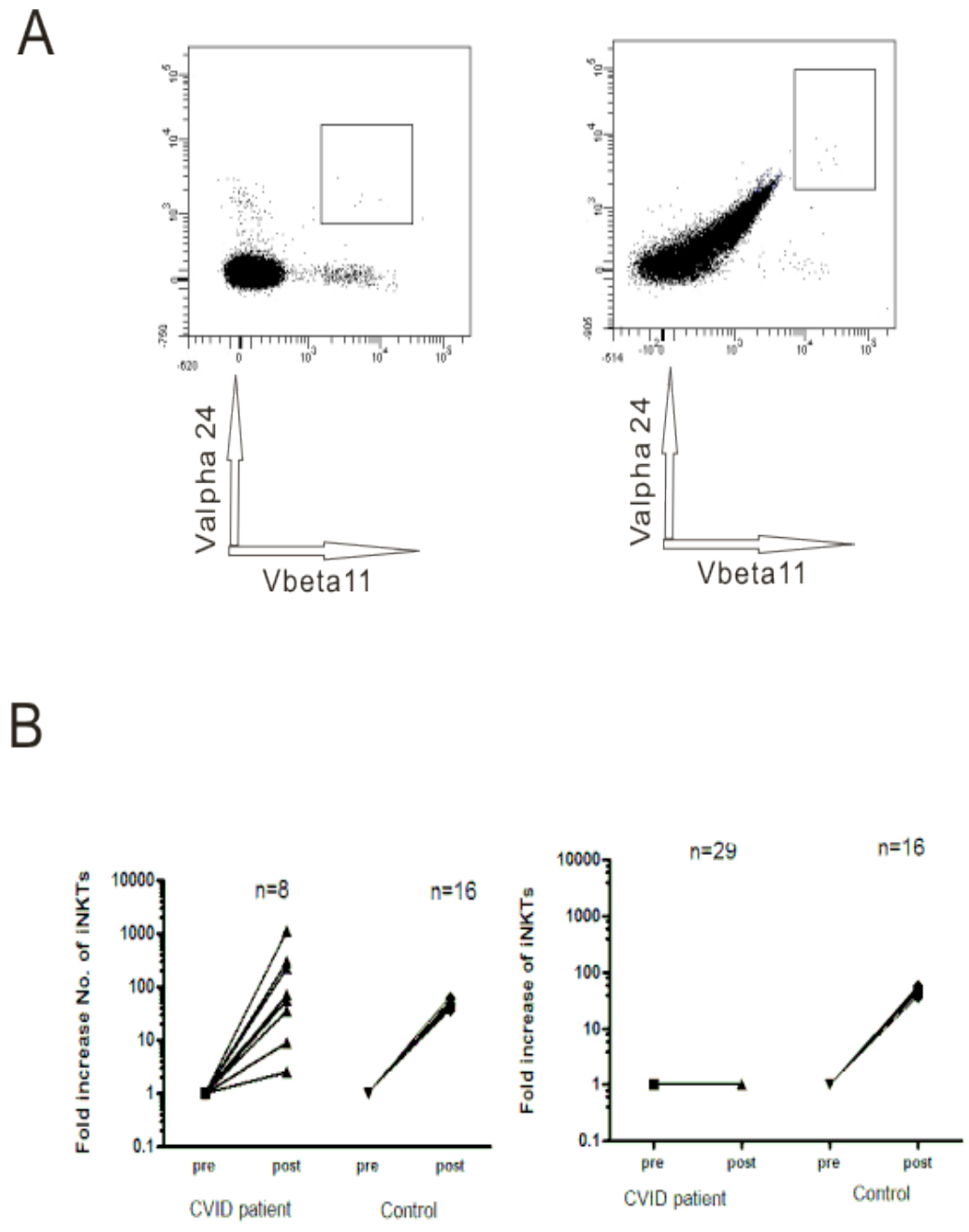


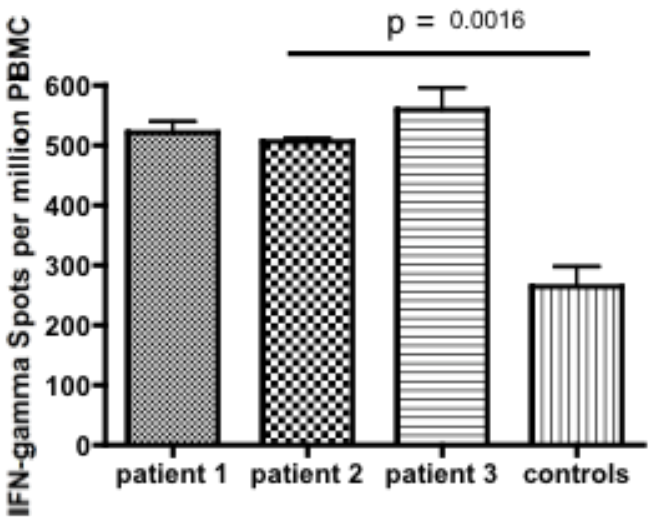
Figure 7.5. Proliferation of iNKT cells

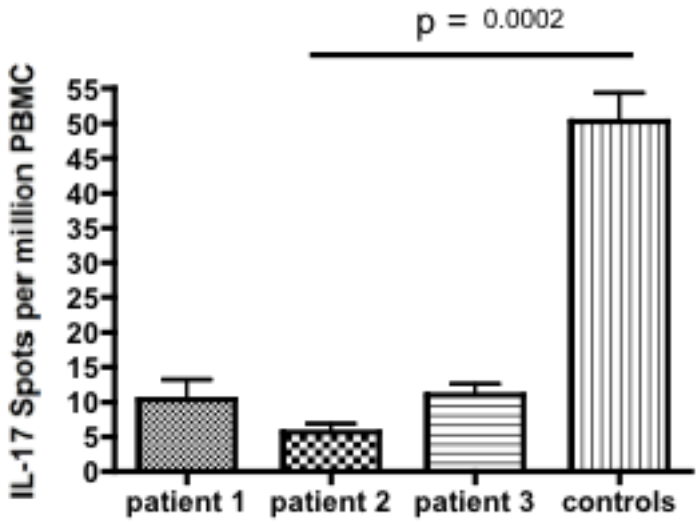
(a) FACS plot for iNKT cells proliferation before and after (b) Two groups of patients were identified based on their proliferation ability

However even those that demonstrated proliferation had a range, 5-1000, that was different to healthy control. Thus CVID patients could be divided into two

groups based on the proliferation essay, namely Group 1 (8/37) who possessed proliferative activity and Group 2 (29/37) that did not.

We then examined those CVID patients who possessed detectable iNKT cells for their cytokine responses following activation. We measured 3 cytokines in 3 representative patients from Group 1 (Figure 7.6).





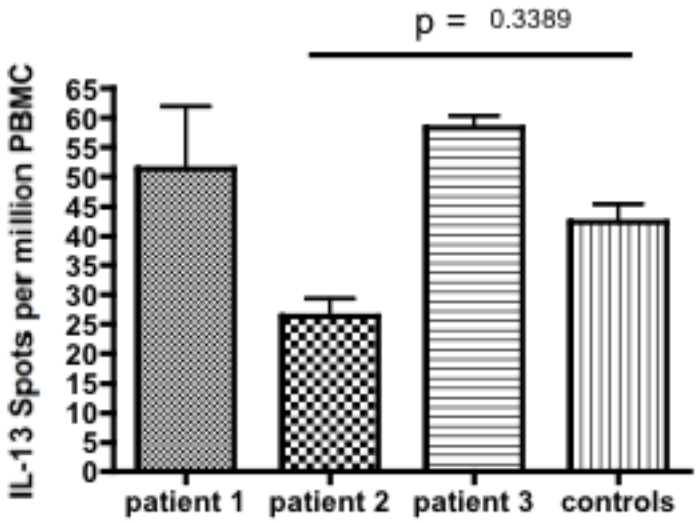


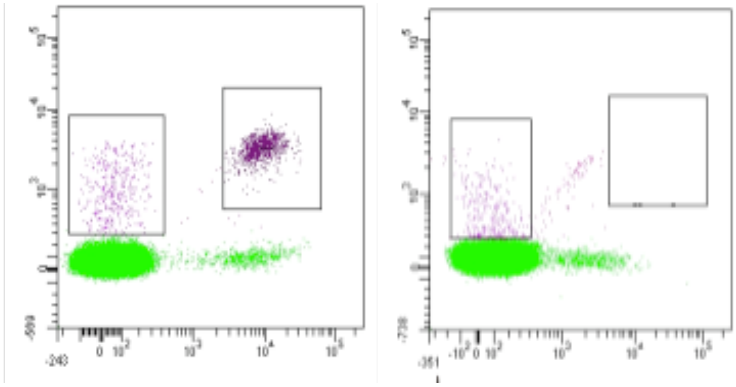
Figure 7.6. Cytokine production by iNKT cells of 3 Group1 CVID patients compared to controls

From Figure 7.6, it can see that the patients did preserve a certain level of multi-cytokine activity and they showed a marked increase in the IFN-γ production compared to healthy controls. It is notable that all the tested patients showed a low level of IL-17 production in whilst IL-13 was not different between the patients and controls.

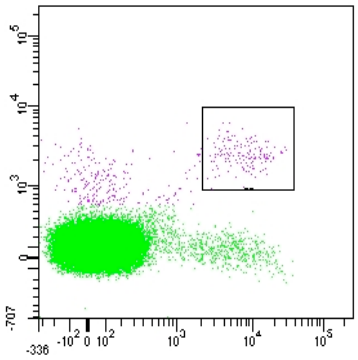
7.3.3 Is the deficiency unique to CVID

To understand and evaluate if the deficiency in iNKT cells is unique to CVID patients, we choose to look at the iNKT cells in another type of immunodeficiency, X-linked agammaglobulinemia (XLA). Both diseases have similar clinical presentations and both patient groups receive the same treatment (replacement immunoglobulin). In contrast to CVID, XLA patients have a well described deficiency of Brutons tyrosine kinase (BTK) which leads to a B cell specific disorder. We first looked at the iNKT cell number in a cohort of 4 patients with this primary immunodeficiency (Figure 7.7).

A



Healthy control CVID patient



XLA patient

B

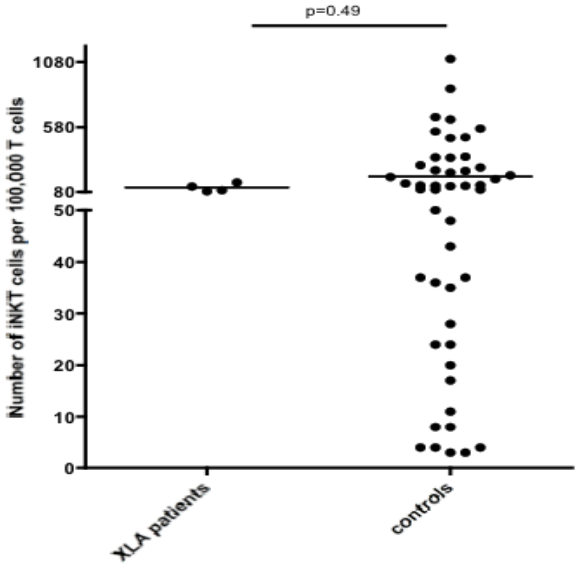


Figure 7.7. Enumeration of INKT cells in XLA patients. (a) FACS comparison of healthy control, CVID patient and XLA patient. (b) number of iNKT cells per 100,000 T cells in XLA patient and healthy control

XLA patients appeared to have a comparable number of iNKT cells compared with the healthy controls. We further evaluated the functionality of these iNKT cells by proliferation assay (Figure 7.8).

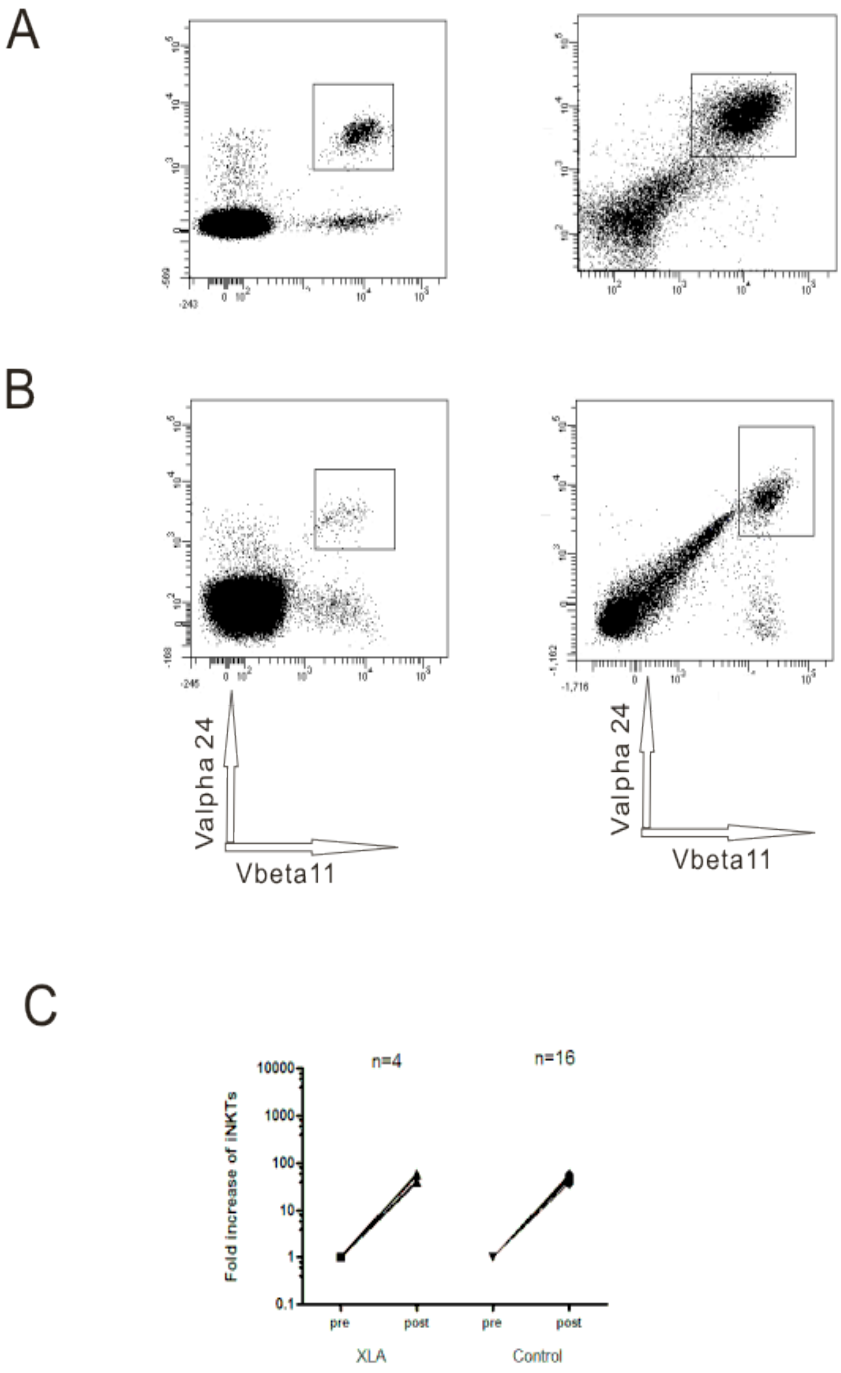


Figure 7.8 Proliferation of iNKT cells.

(A) Representative proliferation of iNKT cells in a healthy control and (B) XLA patient (C) Fold increase of iNKT cells in healthy controls and XLA patients

The proliferation of iNKT cells in this disorder is similar to that of the healthy controls. Therefore the iNKT deficiency of CVID is not related to the replacement therapy and is distinctive to the extended T cell immunodysregulation of this antibody disorder.

7.3.4 Do iNKT cells associate with B cell subgroups in CVID?

Finally the number of iNKT cells was further used to analyse if there is any clinical or phenotypic association with other markers used to subdivide CVID. We reviewed the B cell analysis, which had been undertaken to assess the number of naïve and memory B cells through the markers IgD, CD27, CD10, CD38 and CD21.

No associations were found for non-switched memory B cells, transitional B cells, or plasmablasts. However when the number of class switched B cells were reviewed a correlation was observed between iNKT number and $CD27^{+}IgD^{-}$ status (Figure 7.9).

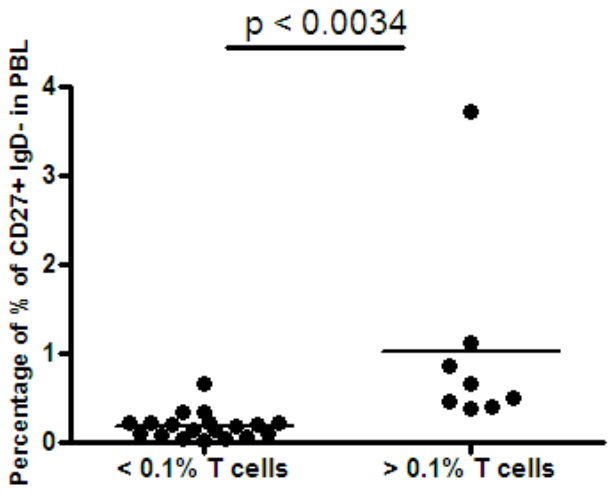


Figure 7.9 Association of iNKT cells with percentage of class-switched memory B cells

There is a significant reduction in the percentage of $CD27^{+}IgD^{-}$ B cells in the CVID patients with less than 0.1% iNKT cells compared to those with more than 0.1% iNKT cells (mean of 0.2% CD27+IgD- B cells compared to 1.2%). The association of class switched B cells with clinical status had previously been proposed to identify those at greater risk of autoimmune and lymphoproliferative complications in CVID. To assess if differences in iNKT number associate with class switched B cells in healthy controls we reviewed the number of class switched B cells in 5 donors with >200 iNKT/10^5 T cells and 5 with <200/10^5 T cells (Figure 7.10).

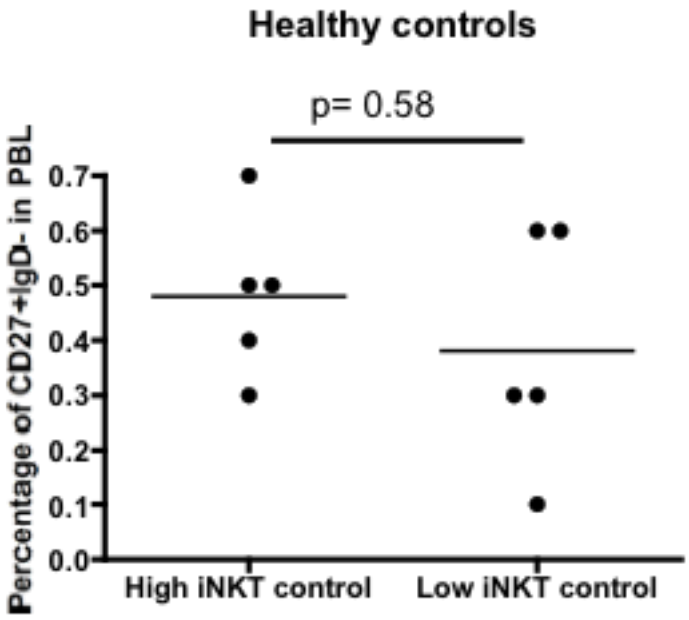


Figure 7.10 Association of iNKT cells with CD27IgD- B cells in healthy controls

In the group of 10 healthy controls, the percentage of $CD27^{+}IgD^{-}$ did not differ significantly between the 2 groups. This suggests that the difference in the CVID setting is disease specific and that iNKT number is a surrogate for class switched B cell memory status in such individuals.

7.3.3 Summary

•The number of iNKT cells is significantly reduced in CVID patients compared to healthy controls. Almost 50% of the patients have no detectable iNKT cells present in their peripheral blood.

•The majority of CVID patients (78%) failed to proliferate following of α-GC activation. This suggests the iNKT cells are truly absent in these patients.

•Those CVID patients with >80 iNKT cells retain multi-cytokine capacity but with a diminished IL-17 responsiveness and to increased IFN-γ production to CD1d ligands.

•An association between iNKT cell number and class switched B cell memory number is seen, the latter of which is known to be a surrogate for more severe autoimmune and lymphoproliferative disease.

7.4 Discussion and conclusion

It has been more than 50 years since the first case of CVID was reported (365). Although different cellular defect and genetic defects has been defined, the mechanism of the disease is far from understood (320). In this study, we have identified another cell deficiency that is present in the majority of CVID patients but not in the related disorder XLA (366).

Around 40% of the patients were shown to have a complete defect in the number of iNKT cells and a further 40% were shown to have a defect in NKT cells function. Therefore iNKT deficiency is present in over three quarters of all CVID patients tested in this large cohort.

Previous studies have identified that CVID patients can be grouped according to the number of class-switching memory cells they have. Low class-switch memory cells being seen in group I CVID patients, whilst group II CVID patients were normal. In our study, we found that those with normal iNKT cells were most similar to group II CVID patients, whilst group I CVID patients had a deficiency of iNKT cells and class switched B cells.

It is tempting to speculate that these observations are related given the B cell help that iNKT cells are thought to provide. As discussed above iNKT cells, can act in a number of ways to help B cell proliferation and sustain antibody production. Figure 7.11 illustrates how iNKT cells may promote antibody responses through cognate and non-cognate interactions.

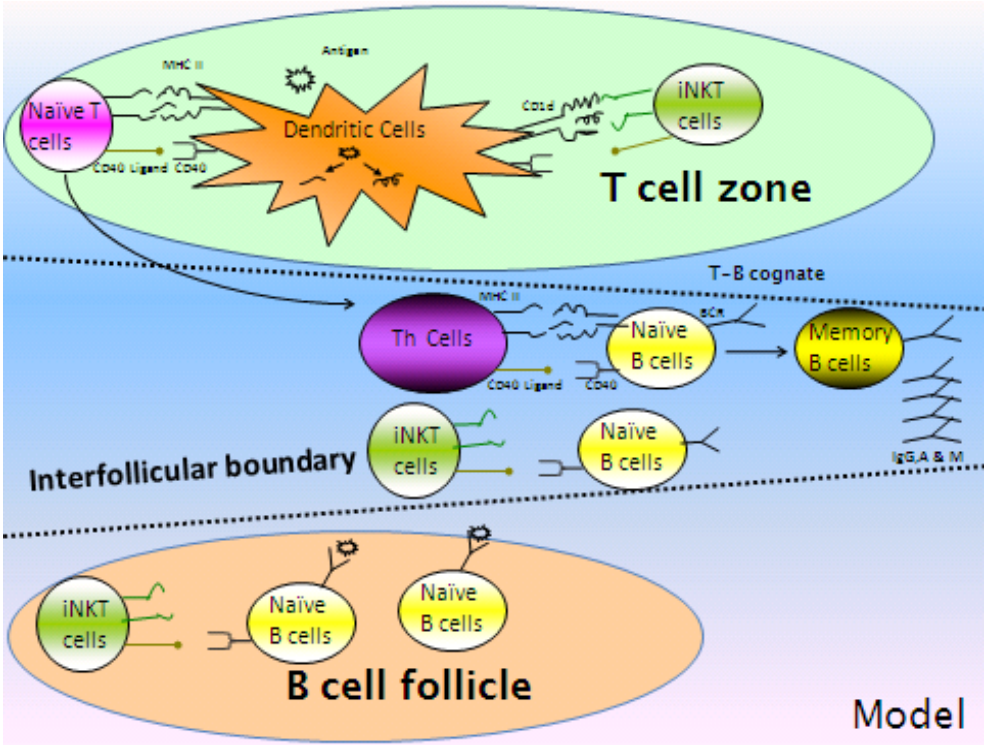


Figure 7.11 iNKT cells may provide "help" to B cells in a number of ways. iNKT cells can provide help to B cells in both cognate and non-cognate pathway. iNKT provide cognate help to B cells via Dendritic cells and the activation of naïve T cells, which efficiently up-regulate co-stimulatory molecules in dendritic cells and further lead to memory B cell development and antibody production. In non-cognate system, iNKT cells can act as helper T cells when and lead to the activation of naïve B cells.

In CVID patients the lack of iNKT cells, may impact upon the activation of antigen presenting cells to support a cognate engagement of conventional T helper cells. This may also occur within the B cell follicle through direct cognate interaction. Alternatively an iNKT cell absence may impair the non-cognate activation of memory B cells that are important for sustaining antibody production and survival. These possible consequences of iNKT cell absence will require further targeted experiments in future work. Finally further studies are required to assess whether the iNKT defects described are primary or secondary

to the CVID disease course. Longitudinal studies of such cohorts and an evaluation of paediatric CVID patients may target these questions in the future.

Chapter 8 Conclusions and future directions

During the tenure of this project several new areas of iNKT biology have been addressed and novel observations made regarding human iNKT cell behavior in health and disease. The first observation to consider in more detail concerns the demonstration that the previous dogma regarding the association between the CD4 co-receptor usage of individual iNKT cell lines and their cytokine production, is part of a larger interrelationship between iNKT cells, conventional T cells and their respective enumeration and CD4 co receptor dominance. We have confirmed the cytokine skewing of CD4 positive iNKT cells towards a Th2 response, compared to DN iNKT cells, on ex vivo cells but have now linked this co receptor phenotype to the absolute number of iNKT cells present in an individual. Indeed at a population level, high and low iNKT donors are seen to exist and this co associates with a fixed phenotype and functional imprinting (Figure 8.1). Furthermore this skewed co receptor usage is shared with conventional T cells such that those individuals with a CD4:CD8 ratio of >2.5, which is normal in the population, reproducibly have a low number of iNKT cells which are CD4 biased. Conversely those with a high number of iNKT cells have a reduced CD4:CD8 ratio in their conventional T cells and a paucity of CD4 positive iNKT cells in their repertoire.

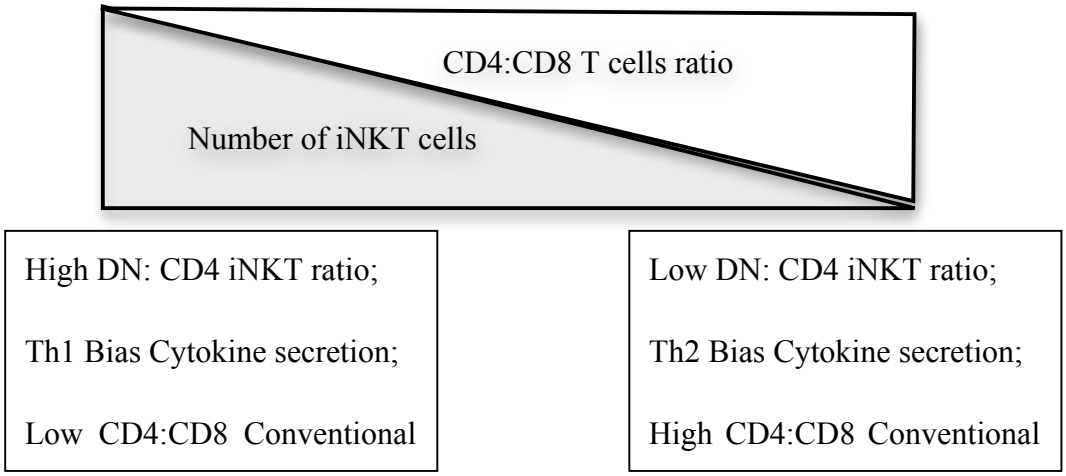


Figure 8.1. Relationship between iNKT cell number, iNKT co receptor phenotype and conventional T cell CD4:CD8 ratio.

In addition to predicting the multi cytokine profile potential of an individual from the number of iNKT cells present, our study has also shown that on a one to one cell basis identical phenotypic iNKT subsets differ in the amount of cytokines they express across high and low donors. This suggests that parameters such as iNKT TCR affinity or functional affinity differs between donors and that this relates to the central selection or peripheral maintenance mechanisms that influence the homeostatic number of iNKT cells that are present.

Several hypotheses may be evaluated from these observations and we would initially propose a central thymic selection mechanism which links these iNKT and conventional T cells observations (Figure 8.2). It is known that in chimeric mice, artificial expression of MHC class II on thymocytes reduces iNKT cell numbers compared to wild type mice (238). It has also become recently recognized that the transcription factor ThPOK is essential for CD4 iNKT and CD4 conventional T cell selection (367-369). Therefore it is tempting to speculate that the expression of MHC class II and signaling through CD4 may modify both iNKT selection and CD4 skewing across a population. The typical DN phenotype of iNKT cells has been previously attributed to the redundancy of co receptor usage for T cells that posses a relatively high affinity for self ligand-CD1d complexes, thus avoiding negative selection imposed by integrated TCR and co receptor signaling. In the case of most healthy individuals a CD4:CD8 ratio of greater than 2 is often seen and we have co associated this with the presence of a low number of iNKT cells that are CD4 biased. We propose that the expression of MHC class II on DP thymocytes influences iNKT selection through a non polymorphic interaction with CD4 and downstream ThPOK signaling leading to a notable negative selection of iNKT cells which have a high affinity for co expressed CD1d on double positive thymocytes. The iNKT cells that do emerge have an attenuated affinity for the CD1d complex through their TCR which is also supported by a dominant CD4 MHC class II interaction. It is this CD4 MHC class II interaction that shapes the CD4 repertoire of conventional T cells in association with their recognition of distinct peptides presented in the context of the polymorphic binding groove of MHC class II. In the case of those individuals with a high number of iNKT cells this CD4-MHC

class II ‘gain of function’ interaction is not apparent and therefore this is little negative selection. The negative selection process of these iNKT cells can tolerate a higher affinity TCR CD1d lipid interaction leading to distinct functional features in the periphery. The absent of a dominating CD4 MHC class II effect is also seen at the conventional T cell level with a less marked CD4:CD8 ratio.

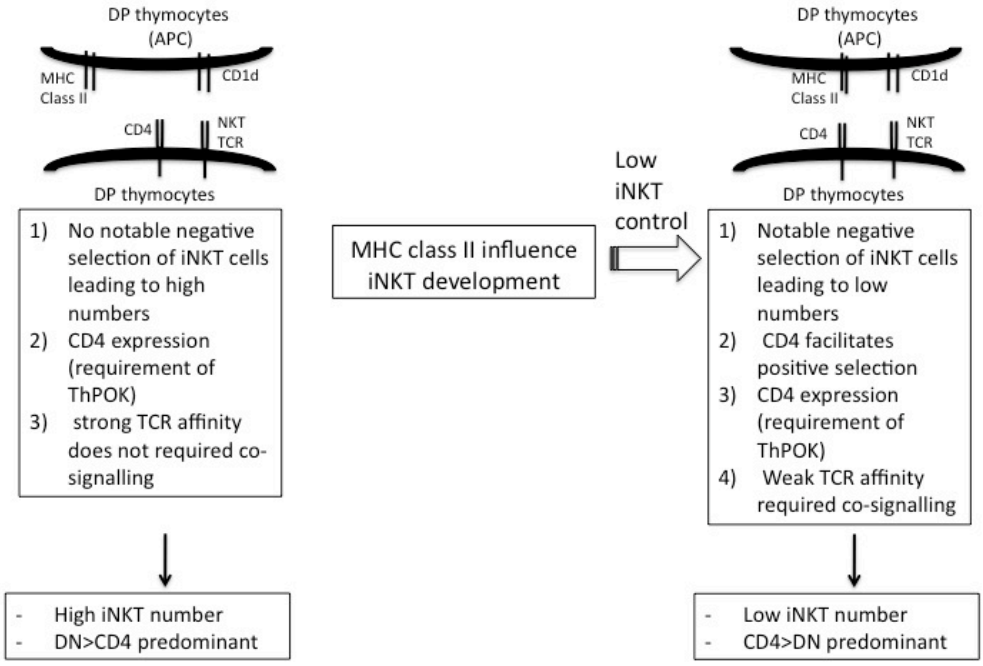


Figure 8.2. Hypothetical model of the factors that may influence the selection of iNKT cells and the CD4:CD8 ratio of conventional T cells.

This distinction between those with high numbers of iNKT cells and those with low numbers was further developed in Chapter 4 through a review of the CD1d APC dependence of the 2 groups of individuals. It was a surprise to find that most individuals, i.e. low donors, failed to proliferate (or produce cytokines) in the presence of CD1d positive B cells whilst those with higher numbers of iNKT cells were unaffected (370). This monocyte dependence for iNKT cells of low

donors is again difficult to reconcile with our understanding of antigen presentation to iNKT cells. We do not believe it was simply an effect of too few iNKT cells for a putatively reduced antigen expression of B cells. Indeed B cells express more surface CD1d than monocytes and when we corrected the iNKT numbers in vitro, there was no rescue of iNKT proliferation. It is unlikely that the B cells of high donors are different to those of low donors, but this could be tested with criss-cross experiments using B cells of high and low donors, however, alloreactivity might be present (370) . It is notable that the processing of lipids by B cells is different to that of monocytes as B cells are poor at internalizing exogenous lipid but may bind at the cell surface. Previous experiments have established that lipids with shorter acyl and sphingosine chains are more easily loaded at the cell surface of B cells but this observation was not relevant to our studies that used α-GC, as an optimal ligand (271). The influence of other factors to effective iNKT responses in low and high donors may also relate to the differences between these two APC populations. We show that the activity of IL-15 upon iNKT cells is monocyte dependent in both high and low donors. This appears to relate to the expression of the IL15Rα in monocytes and it is possible that differences such as this influence the iNKT populations in high and low donors. Future experiments would look at transfecting the IL15Rα gene into B cells to see if this would permit iNKT proliferation of low donors. Lastly, it is worth considering the iNKT themselves as showing selectivity for the manner in which lipids are presented to them. It is possible that the low donors have been selected to recognize only endogenously processed lipid, typically by phagocytic APC i.e. monocytes, whilst high donors may respond to that loaded at the cell surface (B cells) or following uptake and intracellular loading. Future studies could explore this through a number of experimental approaches including artificially loaded CD1d dimmers, B cell lipid transfection or inhibition of phagocytosis in monocytes.

Having established that the iNKT responses of healthy controls are multilayered, we sought to reduce this variability to evaluate novel analogues for differences in

functional readouts. The overarching principle that emerges from our work is that no single assay can be used to identify a 'good ligand'. Our exploration of proliferative capacity, multicytokine productivity, cytotoxicity and anergy revealed a complex effector response portfolio to the ligands tested. The acyl and sphingosine compounds were perhaps the most interesting as these uncoupled proliferation from cytokine production whilst increasing the previously under explored IL-17 cytokine. Our assessment of the 6' derivatised galactose analogues revealed a surprising effectiveness of all the ligands with an intriguing relationship between the induction of PD-1 and proliferation potential for some of the ligands. It is also worth noting that the comparison of our results with those previously published on the 6' derivatised galactose analogues in mice argues for a screening platform for both mice and humans as the responses to the ligands are not identical across the species (293). Our future work will be directed at utilising these different ligands to influence the cytokine milieu at the time of priming to a protein antigen to establish whether they may be truly adjunctive in skewing conventional T cell responses to a Th1, Th2 or Th17 direction.

Another interesting aspect of iNKT biology we have identified with our ligands is that iNKT cells can be ligand specific, despite minor differences in the antigenic structure of individual glycolipids. This specificity showed a partial recognition across the 2 most similar analogues but limited reactivity to α-GC. How such invariant cells develop antigen specificity is intriguing and follow up studies should identify if this specificity is through TCR differences in the Vα or Vβ CDRs or reflects important CD1d ligand conformations that can be explored through crystal structures or in silico modeling. The derivation of CD1d-analogue specific tetramers would also be a useful approach to evaluate if they may bind to the respective iNKT cell lines. A reciprocal experiment may also be undertaken where the TCR's of the different lines are cloned and used to probe analogue loaded complexes for cross reactivity.

Lastly we chose to look at iNKT cells in a human disease characterized by antibody deficiency and immune dysregulation, where iNKT deficiency had been

previously noted in related disorder. Our examination of these cells in a pure B cell disorder (XLA) revealed a numerical and functional similarity to healthy controls. In our CVID patients a profound deficiency of iNKT cells was identified in the majority of patients. This correlated with the absence of memory B cells, an association normally linked to a more severe clinical outcome. Whether this abnormality is a primary or secondary event to the underlying cause of CVID is unclear but future studies may now assess whether this cellular deficiency is a risk factor for more severe clinical disease or additional complications i.e. malignancy. An examination of known monogenic forms of CVID will also illuminate whether this iNKT deficiency is common to the disease progression or its onset.

In summary our evaluation of iNKT biology has provided novel insights into iNKT development, function, and manipulation in a newly configured ex vivo testing platform. Our ligand evaluation programme has identified the complexities of monitoring iNKT responses and the limited potential of single assay readouts of function. Lastly our formal assessment of these cells in one disease setting provides a template to further explore this intriguing T cell subset in the other diverse immunological diseases of host defence, autoimmunity, allergy and tumour immunity.

Chapter 9 References

1. Janeway CA, Jr., Medzhitov R. 2002. Innate immune recognition. *Annu Rev Immunol* 20: 197-216
2. Boyton RJ, Openshaw PJ. 2002. Pulmonary defences to acute respiratory infection. *Br Med Bull* 61: 1-12
3. Agerberth B, Gudmundsson GH. 2006. Host antimicrobial defence peptides in human disease. *Curr Top Microbiol Immunol* 306: 67-90
4. Doss M, White MR, Tecle T, Hartshorn KL. 2010. Human defensins and LL-37 in mucosal immunity. *J Leukoc Biol* 87: 79-92
5. Bustamante J, Boisson-Dupuis S, Jouanguy E, Picard C, Puel A, Abel L, Casanova JL. 2008. Novel primary immunodeficiencies revealed by the investigation of paediatric infectious diseases. *Curr Opin Immunol* 20: 39-48
6. Biron CA, Byron KS, Sullivan JL. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* 320: 1731-5
7. Crozat K, Vivier E, Dalod M. 2009. Crosstalk between components of the innate immune system: promoting anti-microbial defenses and avoiding immunopathologies. *Immunol Rev* 227: 129-49
8. Gold MC, Ehlinger HD, Cook MS, Smyk-Pearson SK, Wille PT, Ungerleider RM, Lewinsohn DA, Lewinsohn DM. 2008. Human innate Mycobacterium tuberculosis-reactive alphabetaTCR+ thymocytes. *PLoS Pathog* 4: e39
9. Zelante T, De Luca A, D'Angelo C, Moretti S, Romani L. 2009. IL-17/Th17 in anti-fungal immunity: what's new? *Eur J Immunol* 39: 645-8
10. Silva MT. 2010. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J Leukoc Biol* 87: 93-106
11. Hampton MB, Kettle AJ, Winterbourn CC. 1998. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* 92: 3007-17

12. De Filippo K, Henderson RB, Laschinger M, Hogg N. 2008. Neutrophil chemokines KC and macrophage-inflammatory protein-2 are newly synthesized by tissue macrophages using distinct TLR signaling pathways. *J Immunol* 180: 4308-15
13. Knudsen E, Iversen PO, Van Rooijen N, Benestad HB. 2002. Macrophage-dependent regulation of neutrophil mobilization and chemotaxis during development of sterile peritonitis in the rat. *Eur J Haematol* 69: 284-96
14. Kiessling R, Klein E, Pross H, Wigzell H. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* 5: 117-21
15. Strowig T, Brilot F, Munz C. 2008. Noncytotoxic functions of NK cells: direct pathogen restriction and assistance to adaptive immunity. *J Immunol* 180: 7785-91
16. Bryceson YT, Long EO. 2008. Line of attack: NK cell specificity and integration of signals. *Curr Opin Immunol* 20: 344-52
17. Cooper MA, Colonna M, Yokoyama WM. 2009. Hidden talents of natural killers: NK cells in innate and adaptive immunity. *EMBO Rep* 10: 1103-10
18. Moretta L, Ferlazzo G, Bottino C, Vitale M, Pende D, Mingari MC, Moretta A. 2006. Effector and regulatory events during natural killer-dendritic cell interactions. *Immunol Rev* 214: 219-28
19. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. 2008. Functions of natural killer cells. *Nat Immunol* 9: 503-10
20. Ferlazzo G, Munz C. 2004. NK cell compartments and their activation by dendritic cells. *J Immunol* 172: 1333-9
21. Cooper MA, Yokoyama WM. 2010. Memory-like responses of natural killer cells. *Immunol Rev* 235: 297-305
22. Cooper MA, Elliott JM, Keyel PA, Yang L, Carrero JA, Yokoyama WM. 2009. Cytokine-induced memory-like natural killer cells. *Proc Natl Acad Sci U S A* 106: 1915-9

23. Koka R, Burkett PR, Chien M, Chai S, Chan F, Lodolce JP, Boone DL, Ma A. 2003. Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice. *J Exp Med* 197: 977-84

24. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, Diefenbach A. 2009. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol* 10: 83-91

25. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, Mention JJ, Thiam K, Cerf-Bensussan N, Mandelboim O, Eberl G, Di Santo JP. 2008. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* 29: 958-70

26. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, Doherty JM, Mills JC, Colonna M. 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457: 722-5

27. Hedrick SM. 2008. Thymus lineage commitment: a single switch. *Immunity* 28: 297-9

28. Kashiwagi Y, Kawashima H, Kato N, Takekuma K, Hoshika A, Kumaki S. 2009. A mutation in the IL-2 receptor gamma chain gene associated with X-linked severe combined immunodeficiency accompanying opisthotonus. *Tohoku J Exp Med* 218: 1-3

29. Laky K, Fleischacker C, Fowlkes BJ. 2006. TCR and Notch signaling in CD4 and CD8 T-cell development. *Immunol Rev* 209: 274-83

30. Laky K, Fowlkes BJ. 2008. Notch signaling in CD4 and CD8 T cell development. *Curr Opin Immunol* 20: 197-202

31. Jenkinson EJ, Jenkinson WE, Rossi SW, Anderson G. 2006. The thymus and T-cell commitment: the right niche for Notch? *Nat Rev Immunol* 6: 551-5

32. Oltz EM, Osipovich O. 2007. Targeting V(D)J recombinase: putting a PHD to work. *Immunity* 27: 539-41

33. Bonilla FA, Oettgen HC. 2010. Adaptive immunity. *J Allergy Clin Immunol* 125: S33-40
34. Dustin ML. 2009. The cellular context of T cell signaling. *Immunity* 30: 482-92
35. Feske S. 2007. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol* 7: 690-702
36. Jenkins MK, Schwartz RH, Pardoll DM. 1988. Effects of cyclosporine A on T cell development and clonal deletion. *Science* 241: 1655-8
37. Gao EK, Lo D, Cheney R, Kanagawa O, Sprent J. 1988. Abnormal differentiation of thymocytes in mice treated with cyclosporin A. *Nature* 336: 176-9
38. Bueno OF, Brandt EB, Rothenberg ME, Molkentin JD. 2002. Defective T cell development and function in calcineurin A beta -deficient mice. *Proc Natl Acad Sci U S A* 99: 9398-403
39. Neilson JR, Winslow MM, Hur EM, Crabtree GR. 2004. Calcineurin B1 is essential for positive but not negative selection during thymocyte development. *Immunity* 20: 255-66
40. Vig M, Kinet JP. 2009. Calcium signaling in immune cells. *Nat Immunol* 10: 21-7
41. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136: 2348-57
42. Kaminuma O, Kitamura F, Miyatake S, Yamaoka K, Miyoshi H, Inokuma S, Tatsumi H, Nemoto S, Kitamura N, Mori A, Hiroi T. 2009. T-box 21 transcription factor is responsible for distorted T(H)2 differentiation in human peripheral CD4+ T cells. *J Allergy Clin Immunol* 123: 813-23 e3
43. Steinman L. 2007. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* 13: 139-45

44. Elyaman W, Bradshaw EM, Uyttenhove C, Dardalhon V, Awasthi A, Imitola J, Bettelli E, Oukka M, van Snick J, Renauld JC, Kuchroo VK, Khoury SJ. 2009. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci U S A* 106: 12885-90

45. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, Mitsdoerffer M, Strom TB, Elyaman W, Ho IC, Khoury S, Oukka M, Kuchroo VK. 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol* 9: 1347-55

46. Dong C. 2006. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. *Nat Rev Immunol* 6: 329-33

47. Bacchetta R, Gambineri E, Roncarolo MG. 2007. Role of regulatory T cells and FOXP3 in human diseases. *J Allergy Clin Immunol* 120: 227-35; quiz 36-7

48. Chatila TA. 2005. Role of regulatory T cells in human diseases. *J Allergy Clin Immunol* 116: 949-59; quiz 60

49. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, Martin B, Wilhelm C, Stockinger B. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9: 1341-6

50. Schmitt E, Germann T, Goedert S, Hoehn P, Huels C, Koelsch S, Kuhn R, Muller W, Palm N, Rude E. 1994. IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *J Immunol* 153: 3989-96

51. Ma CS, Tangye SG, Deenick EK. 2010. Human Th9 cells: inflammatory cytokines modulate IL-9 production through the induction of IL-21. *Immunol Cell Biol*

52. Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, Gerlitzki B, Hoffmann M, Ulges A, Taube C, Dehzad N, Becker M, Stassen M,

Steinborn A, Lohoff M, Schild H, Schmitt E, Bopp T. 2010. Interferon-Regulatory Factor 4 Is Essential for the Developmental Program of T Helper 9 Cells. *Immunity*

53. Oreshkova T, Wang H, Seier AM, Sindrilaru A, Varga G, Grabbe S, Scharffetter-Kochanek K, Peters T. 2009. Beta(2) integrin deficiency yields unconventional double-negative T cells distinct from mature classical natural killer T cells in mice. *Immunology* 128: 271-86
54. Fuxa M, Skok JA. 2007. Transcriptional regulation in early B cell development. *Curr Opin Immunol* 19: 129-36
55. Wang H, Lee CH, Qi C, Tailor P, Feng J, Abbasi S, Atsumi T, Morse HC, 3rd. 2008. IRF8 regulates B-cell lineage specification, commitment, and differentiation. *Blood* 112: 4028-38
56. Kerr WG, Hendershot LM, Burrows PD. 1991. Regulation of IgM and IgD expression in human B-lineage cells. *J Immunol* 146: 3314-21
57. LeBien TW, Tedder TF. 2008. B lymphocytes: how they develop and function. *Blood* 112: 1570-80
58. Dorshkind K, Montecino-Rodriguez E. 2007. Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential. *Nat Rev Immunol* 7: 213-9
59. Steinman RM, Cohn ZA. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137: 1142-62
60. Katsnelson A. 2006. Kicking off adaptive immunity: the discovery of dendritic cells. *J Exp Med* 203: 1622
61. Fazekas de St Groth B. 1998. The evolution of self-tolerance: a new cell arises to meet the challenge of self-reactivity. *Immunol Today* 19: 448-54
62. Spits H, Lanier LL. 2007. Natural killer or dendritic: what's in a name? *Immunity* 26: 11-6
63. Lo J, Clare-Salzler MJ. 2006. Dendritic cell subsets and type I diabetes: focus upon DC-based therapy. *Autoimmun Rev* 5: 419-23

64. King PD, Ibrahim MA, Katz DR. 1993. Adhesion molecules: co-stimulators and co-mitogens in dendritic cell-T cell interaction. *Adv Exp Med Biol* 329: 53-8

65. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18: 767-811

66. Heath WR, Belz GT, Behrens GM, Smith CM, Forehan SP, Parish IA, Davey GM, Wilson NS, Carbone FR, Villadangos JA. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev* 199: 9-26

67. Coquerelle C, Moser M. 2010. DC subsets in positive and negative regulation of immunity. *Immunol Rev* 234: 317-34

68. De Becker G, Moulin V, Tielemans F, De Mattia F, Urbain J, Leo O, Moser M. 1998. Regulation of T helper cell differentiation in vivo by soluble and membrane proteins provided by antigen-presenting cells. *Eur J Immunol* 28: 3161-71

69. Neparidze N, Dhodapkar MV. 2009. Harnessing CD1d-restricted T cells toward antitumor immunity in humans. *Ann N Y Acad Sci* 1174: 61-7

70. Makino Y, Kanno R, Ito T, Higashino K, Taniguchi M. 1995. Predominant expression of invariant V alpha 14+ TCR alpha chain in NK1.1+ T cell populations. *Int Immunol* 7: 1157-61

71. Seaman WE. 2000. Natural killer cells and natural killer T cells. *Arthritis Rheum* 43: 1204-17

72. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. 2004. NKT cells: what's in a name? *Nat Rev Immunol* 4: 231-7

73. McMichael AJ, Pilch JR, Galfre G, Mason DY, Fabre JW, Milstein C. 1979. A human thymocyte antigen defined by a hybrid myeloma monoclonal antibody. *Eur J Immunol* 9: 205-10

74. Brigl M, Brenner MB. 2004. CD1: antigen presentation and T cell function. *Annu Rev Immunol* 22: 817-90

75. Martin LH, Calabi F, Lefebvre FA, Bilsland CA, Milstein C. 1987. Structure and expression of the human thymocyte antigens CD1a, CD1b, and CD1c. *Proc Natl Acad Sci U S A* 84: 9189-93

76. Longley J, Kraus J, Alonso M, Edelson R. 1989. Molecular cloning of CD1a (T6), a human epidermal dendritic cell marker related to class I MHC molecules. *J Invest Dermatol* 92: 628-31

77. van der Wel NN, Sugita M, Fluitsma DM, Cao X, Schreibelt G, Brenner MB, Peters PJ. 2003. CD1 and major histocompatibility complex II molecules follow a different course during dendritic cell maturation. *Mol Biol Cell* 14: 3378-88

78. Han M, Hannick LI, DiBrino M, Robinson MA. 1999. Polymorphism of human CD1 genes. *Tissue Antigens* 54: 122-7

79. Oteo M, Parra JF, Mirones I, Gimenez LI, Setien F, Martinez-Naves E. 1999. Single strand conformational polymorphism analysis of human CD1 genes in different ethnic groups. *Tissue Antigens* 53: 545-50

80. Jones DC, Gelder CM, Ahmad T, Campbell IA, Barnardo MC, Welsh KI, Marshall SE, Bunce M. 2001. CD1 genotyping of patients with Mycobacterium malmoense pulmonary disease. *Tissue Antigens* 58: 19-23

81. Cohen NR, Garg S, Brenner MB. 2009. Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity. *Adv Immunol* 102: 1-94

82. Kasinrerk W, Baumruker T, Majdic O, Knapp W, Stockinger H. 1993. CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. *J Immunol* 150: 579-84

83. Porcelli S, Morita CT, Brenner MB. 1992. CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen. *Nature* 360: 593-7

84. Caux C, Vanbervliet B, Massacrier C, Dezutter-Dambuyant C, de Saint-Vis B, Jacquet C, Yoneda K, Imamura S, Schmitt D, Banchereau J. 1996. CD34+ hematopoietic progenitors from human cord blood

differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. *J Exp Med* 184: 695-706

85. Gatti E, Velleca MA, Biedermann BC, Ma W, Unternaehrer J, Ebersold MW, Medzhitov R, Pober JS, Mellman I. 2000. Large-scale culture and selective maturation of human Langerhans cells from granulocyte colony-stimulating factor-mobilized CD34+ progenitors. *J Immunol* 164: 3600-7
86. Ochoa MT, Loncaric A, Krutzik SR, Becker TC, Modlin RL. 2008. "Dermal dendritic cells" comprise two distinct populations: CD1+ dendritic cells and CD209+ macrophages. *J Invest Dermatol* 128: 2225-31
87. Cernadas M, Cavallari M, Watts G, Mori L, De Libero G, Brenner MB. 2010. Early recycling compartment trafficking of CD1a is essential for its intersection and presentation of lipid antigens. *J Immunol* 184: 1235-41
88. Cernadas M, Lu J, Watts G, Brenner MB. 2009. CD1a expression defines an interleukin-12 producing population of human dendritic cells. *Clin Exp Immunol* 155: 523-33
89. Chang CC, Wright A, Punnonen J. 2000. Monocyte-derived CD1a+ and CD1a- dendritic cell subsets differ in their cytokine production profiles, susceptibilities to transfection, and capacities to direct Th cell differentiation. *J Immunol* 165: 3584-91
90. Liu YJ. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23: 275-306
91. Plebani A, Proserpio AR, Guarneri D, Buscaglia M, Cattoretti G. 1993. B and T lymphocyte subsets in fetal and cord blood: age-related modulation of CD1c expression. *Biol Neonate* 63: 1-7
92. Smith ME, Thomas JA, Bodmer WF. 1988. CD1c antigens are present in normal and neoplastic B-cells. *J Pathol* 156: 169-77

93. Porcelli S, Brenner MB, Greenstein JL, Balk SP, Terhorst C, Bleicher PA. 1989. Recognition of cluster of differentiation 1 antigens by human CD4-CD8-cytolytic T lymphocytes. *Nature* 341: 447-50

94. Faure F, Miossec C, Jitsukawa S, Triebel F, Hercend T. 1990. The gamma/delta T cell receptor expressed on human peripheral blood lymphocytes. *Eur Cytokine Netw* 1: 65-9

95. Faure F, Jitsukawa S, Miossec C, Hercend T. 1990. CD1c as a target recognition structure for human T lymphocytes: analysis with peripheral blood gamma/delta cells. *Eur J Immunol* 20: 703-6

96. Spada FM, Grant EP, Peters PJ, Sugita M, Melian A, Leslie DS, Lee HK, van Donselaar E, Hanson DA, Krensky AM, Majdic O, Porcelli SA, Morita CT, Brenner MB. 2000. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *J Exp Med* 191: 937-48

97. Grant EP, Beckman EM, Behar SM, Degano M, Frederique D, Besra GS, Wilson IA, Porcelli SA, Furlong ST, Brenner MB. 2002. Fine specificity of TCR complementarity-determining region residues and lipid antigen hydrophilic moieties in the recognition of a CD1-lipid complex. *J Immunol* 168: 3933-40

98. Melian A, Watts GF, Shamshiev A, De Libero G, Clatworthy A, Vincent M, Brenner MB, Behar S, Niazi K, Modlin RL, Almo S, Ostrov D, Nathenson SG, Porcelli SA. 2000. Molecular recognition of human CD1b antigen complexes: evidence for a common pattern of interaction with alpha beta TCRs. *J Immunol* 165: 4494-504

99. Russano AM, Bassotti G, Agea E, Bistoni O, Mazzocchi A, Morelli A, Porcelli SA, Spinozzi F. 2007. CD1-restricted recognition of exogenous and self-lipid antigens by duodenal gammadelta+ T lymphocytes. *J Immunol* 178: 3620-6

100. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. 1994. Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. *Nature* 372: 691-4

101. Moody DB, Young DC, Cheng TY, Rosat JP, Roura-Mir C, O'Connor PB, Zajonc DM, Walz A, Miller MJ, Levery SB, Wilson IA, Costello CE, Brenner MB. 2004. T cell activation by lipopeptide antigens. *Science* 303: 527-31

102. Stenger S, Mazzaccaro RJ, Uyemura K, Cho S, Barnes PF, Rosat JP, Sette A, Brenner MB, Porcelli SA, Bloom BR, Modlin RL. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science* 276: 1684-7

103. Rosat JP, Grant EP, Beckman EM, Dascher CC, Sieling PA, Frederique D, Modlin RL, Porcelli SA, Furlong ST, Brenner MB. 1999. CD1-restricted microbial lipid antigen-specific recognition found in the CD8+ alpha beta T cell pool. *J Immunol* 162: 366-71

104. Gilleron M, Stenger S, Mazorra Z, Wittke F, Mariotti S, Bohmer G, Prandi J, Mori L, Puzo G, De Libero G. 2004. Diacylated sulfoglycolipids are novel mycobacterial antigens stimulating CD1-restricted T cells during infection with Mycobacterium tuberculosis. *J Exp Med* 199: 649-59

105. Beckman EM, Melian A, Behar SM, Sieling PA, Chatterjee D, Furlong ST, Matsumoto R, Rosat JP, Modlin RL, Porcelli SA. 1996. CD1c restricts responses of mycobacteria-specific T cells. Evidence for antigen presentation by a second member of the human CD1 family. *J Immunol* 157: 2795-803

106. Blumberg RS, Terhorst C, Bleicher P, McDermott FV, Allan CH, Landau SB, Trier JS, Balk SP. 1991. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. *J Immunol* 147: 2518-24

107. Canchis PW, Bhan AK, Landau SB, Yang L, Balk SP, Blumberg RS. 1993. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. *Immunology* 80: 561-5

108. Zeng Z, Castano AR, Segelke BW, Stura EA, Peterson PA, Wilson IA. 1997. Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. *Science* 277: 339-45

109. Borg NA, Wun KS, Kjer-Nielsen L, Wilce MC, Pellicci DG, Koh R, Besra GS, Bharadwaj M, Godfrey DI, McCluskey J, Rossjohn J. 2007. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature* 448: 44-9

110. Koch M, Stronge VS, Shepherd D, Gadola SD, Mathew B, Ritter G, Fersht AR, Besra GS, Schmidt RR, Jones EY, Cerundolo V. 2005. The crystal structure of human CD1d with and without alpha-galactosylceramide. *Nat Immunol* 6: 819-26

111. Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* 7: 529-34

112. De Libero G, Collmann A, Mori L. 2009. The cellular and biochemical rules of lipid antigen presentation. *Eur J Immunol* 39: 2648-56

113. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, Tsuji M, Kawahara K, Wong CH, Kronenberg M. 2005. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 434: 520-5

114. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, 3rd, Zhou D, Saint-Mezard P, Wang V, Gao Y, Yin N, Hoebe K, Schneewind O, Walker D, Beutler B, Teyton L, Savage PB, Bendelac A. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434: 525-9

115. Sriram V, Du W, Gervay-Hague J, Brutkiewicz RR. 2005. Cell wall glycosphingolipids of Sphingomonas paucimobilis are CD1d-specific ligands for NKT cells. *Eur J Immunol* 35: 1692-701

116. Blomqvist M, Rhost S, Teneberg S, Lofbom L, Osterbye T, Brigl M, Mansson JE, Cardell SL. 2009. Multiple tissue-specific isoforms of sulfatide activate CD1d-restricted type II NKT cells. *Eur J Immunol* 39: 1726-35

117. Yuan W, Kang SJ, Evans JE, Cresswell P. 2009. Natural lipid ligands associated with human CD1d targeted to different subcellular compartments. *J Immunol* 182: 4784-91

118. Zhou D, Mattner J, Cantu C, 3rd, Schrantz N, Yin N, Gao Y, Sagiv Y, Hudspeth K, Wu YP, Yamashita T, Teneberg S, Wang D, Proia RL, Levery SB, Savage PB, Teyton L, Bendelac A. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 306: 1786-9

119. Van Kaer L, Joyce S. 2010. The hunt for iNKT cell antigens: alpha-galactosidase-deficient mice to the rescue? *Immunity* 33: 143-5

120. Gapin L. 2010. iNKT cell autoreactivity: what is 'self' and how is it recognized? *Nat Rev Immunol* 10: 272-7

121. van den Elzen P, Garg S, Leon L, Brigl M, Leadbetter EA, Gumperz JE, Dascher CC, Cheng TY, Sacks FM, Illarionov PA, Besra GS, Kent SC, Moody DB, Brenner MB. 2005. Apolipoprotein-mediated pathways of lipid antigen presentation. *Nature* 437: 906-10

122. Bendelac A, Savage PB, Teyton L. 2007. The biology of NKT cells. *Annu Rev Immunol* 25: 297-336

123. Salio M, Silk JD, Cerundolo V. 2010. Recent advances in processing and presentation of CD1 bound lipid antigens. *Curr Opin Immunol* 22: 81-8

124. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, Zajonc DM, Ben-Menachem G, Ainge GD, Painter GF, Khurana A, Hoebe K, Behar SM, Beutler B, Wilson IA, Tsuji M, Sellati TJ, Wong CH, Kronenberg M. 2006. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* 7: 978-86

125. Yuan W, Dasgupta A, Cresswell P. 2006. Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. *Nat Immunol* 7: 835-42

126. Raftery MJ, Winau F, Giese T, Kaufmann SH, Schaible UE, Schonrich G. 2008. Viral danger signals control CD1d de novo synthesis and NKT cell activation. *Eur J Immunol* 38: 668-79

127. De Libero G, Moran AP, Gober HJ, Rossy E, Shamshiev A, Chelnokova O, Mazorra Z, Vendetti S, Sacchi A, Prendergast MM, Sansano S, Tonevitsky A, Landmann R, Mori L. 2005. Bacterial infections promote T cell recognition of self-glycolipids. *Immunity* 22: 763-72

128. Lantz O, Bendelac A. 1994. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med* 180: 1097-106

129. Sumida T, Takei I, Taniguchi M. 1984. Activation of acceptor-suppressor hybridoma with antigen-specific suppressor T cell factor of two-chain type: requirement of the antigen- and the I-J-restricting specificity. *J Immunol* 133: 1131-6

130. Sumida T, Taniguchi M. 1985. Novel mechanisms of specific suppression of anti-hapten antibody response mediated by monoclonal anti-carrier antibody. *J Immunol* 134: 3675-81

131. Imai K, Kanno M, Kimoto H, Shigemoto K, Yamamoto S, Taniguchi M. 1986. Sequence and expression of transcripts of the T-cell antigen receptor alpha-chain gene in a functional, antigen-specific suppressor-T-cell hybridoma. *Proc Natl Acad Sci U S A* 83: 8708-12

132. Budd RC, Miescher GC, Howe RC, Lees RK, Bron C, MacDonald HR. 1987. Developmentally regulated expression of T cell receptor beta chain variable domains in immature thymocytes. *J Exp Med* 166: 577-82

133. Fowlkes BJ, Kruisbeek AM, Ton-That H, Weston MA, Coligan JE, Schwartz RH, Pardoll DM. 1987. A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family. *Nature* 329: 251-4

134. Dellabona P, Padovan E, Casorati G, Brockhaus M, Lanzavecchia A. 1994. An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med* 180: 1171-6

135. Godfrey DI, Stankovic S, Baxter AG. 2010. Raising the NKT cell family. *Nat Immunol* 11: 197-206

136. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M. 1997. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278: 1623-6

137. Matangkasombut P, Pichavant M, Saez DE, Giliani S, Mazzolari E, Finocchi A, Villa A, Sobacchi C, Cortes P, Umetsu DT, Notarangelo LD. 2008. Lack of iNKT cells in patients with combined immune deficiency due to hypomorphic RAG mutations. *Blood* 111: 271-4

138. Marrella V, Poliani PL, Casati A, Rucci F, Frascoli L, Gougeon ML, Lemercier B, Bosticardo M, Ravanini M, Battaglia M, Roncarolo MG, Cavazzana-Calvo M, Facchetti F, Notarangelo LD, Vezzoni P, Grassi F, Villa A. 2007. A hypomorphic R229Q Rag2 mouse mutant recapitulates human Omenn syndrome. *J Clin Invest* 117: 1260-9

139. Guo J, Hawwari A, Li H, Sun Z, Mahanta SK, Littman DR, Krangel MS, He YW. 2002. Regulation of the TCRalpha repertoire by the survival window of CD4(+)CD8(+) thymocytes. *Nat Immunol* 3: 469-76

140. Bezbradica JS, Hill T, Stanic AK, Van Kaer L, Joyce S. 2005. Commitment toward the natural T (iNKT) cell lineage occurs at the CD4+8+ stage of thymic ontogeny. *Proc Natl Acad Sci U S A* 102: 5114-9

141. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, Bendelac A, Littman DR. 2005. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* 22: 705-16

142. Wei DG, Lee H, Park SH, Beaudoin L, Teyton L, Lehuen A, Bendelac A. 2005. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *J Exp Med* 202: 239-48

143. Chun T, Page MJ, Gapin L, Matsuda JL, Xu H, Nguyen H, Kang HS, Stanic AK, Joyce S, Koltun WA, Chorney MJ, Kronenberg M, Wang CR.

2003. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J Exp Med* 197: 907-18

144. Pellicci DG, Uldrich AP, Kyparissoudis K, Crowe NY, Brooks AG, Hammond KJ, Sidobre S, Kronenberg M, Smyth MJ, Godfrey DI. 2003. Intrathymic NKT cell development is blocked by the presence of alpha-galactosylceramide. *Eur J Immunol* 33: 1816-23
145. Veillette A. 2006. Immune regulation by SLAM family receptors and SAP-related adaptors. *Nat Rev Immunol* 6: 56-66
146. Latchman Y, McKay PF, Reiser H. 1998. Identification of the 2B4 molecule as a counter-receptor for CD48. *J Immunol* 161: 5809-12
147. Griewank K, Borowski C, Rietdijk S, Wang N, Julien A, Wei DG, Mamchak AA, Terhorst C, Bendelac A. 2007. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* 27: 751-62
148. Sayos J, Wu C, Morra M, Wang N, Zhang X, Allen D, van Schaik S, Notarangelo L, Geha R, Roncarolo MG, Oettgen H, De Vries JE, Aversa G, Terhorst C. 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* 395: 462-9
149. Latour S, Gish G, Helgason CD, Humphries RK, Pawson T, Veillette A. 2001. Regulation of SLAM-mediated signal transduction by SAP, the X-linked lymphoproliferative gene product. *Nat Immunol* 2: 681-90
150. Pasquier B, Yin L, Fondaneche MC, Relouzat F, Bloch-Queyrat C, Lambert N, Fischer A, de Saint-Basile G, Latour S. 2005. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J Exp Med* 201: 695-701
151. Nichols KE, Hom J, Gong SY, Ganguly A, Ma CS, Cannons JL, Tangye SG, Schwartzberg PL, Koretzky GA, Stein PL. 2005. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med* 11: 340-5

152. Chung B, Aoukaty A, Dutz J, Terhorst C, Tan R. 2005. Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. *J Immunol* 174: 3153-7

153. Dose M, Sleckman BP, Han J, Bredemeyer AL, Bendelac A, Gounari F. 2009. Intrathymic proliferation wave essential for Valpha14+ natural killer T cell development depends on c-Myc. *Proc Natl Acad Sci U S A* 106: 8641-6

154. Mycko MP, Ferrero I, Wilson A, Jiang W, Bianchi T, Trumpp A, MacDonald HR. 2009. Selective requirement for c-Myc at an early stage of V(alpha)14i NKT cell development. *J Immunol* 182: 4641-8

155. Dose M, Gounari F. 2009. The My(c)stery of iNKT cell ontogeny. *Cell Cycle* 8: 3082-5

156. Satoh Y, Matsumura I, Tanaka H, Ezoe S, Sugahara H, Mizuki M, Shibayama H, Ishiko E, Ishiko J, Nakajima K, Kanakura Y. 2004. Roles for c-Myc in self-renewal of hematopoietic stem cells. *J Biol Chem* 279: 24986-93

157. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, Lantz O, Bendelac A. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29: 391-403

158. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, Chua K, Eidson M, Kim HJ, Im JS, Pandolfi PP, Sant'Angelo DB. 2008. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol* 9: 1055-64

159. Kreslavsky T, Savage AK, Hobbs R, Gounari F, Bronson R, Pereira P, Pandolfi PP, Bendelac A, von Boehmer H. 2009. TCR-inducible PLZF transcription factor required for innate phenotype of a subset of gammadelta T cells with restricted TCR diversity. *Proc Natl Acad Sci U S A* 106: 12453-8

160. Bendelac A, Savage PB, Teyton L. 2006. The Biology of NKT Cells. *Annu Rev Immunol*

161. Prussin C, Foster B. 1997. TCR V alpha 24 and V beta 11 coexpression defines a human NK1 T cell analog containing a unique Th0 subpopulation. *J Immunol* 159: 5862-70

162. Hammond KJ, Kronenberg M. 2003. Natural killer T cells: natural or unnatural regulators of autoimmunity? *Curr Opin Immunol* 15: 683-9

163. Gumperz JE, Miyake S, Yamamura T, Brenner MB. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* 195: 625-36

164. Karadimitris A, Patterson S, Spanoudakis E. 2006. Natural killer T cells and haemopoiesis. *Br J Haematol* 134: 263-72

165. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, von Blomberg BM, Scheper RJ, van der Vliet HJ, van den Eertwegh AJ, Roelvink M, Beijnen J, Zwierzina H, Pinedo HM. 2002. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 8: 3702-9

166. Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, Iizasa T, Nakayama T, Taniguchi M, Fujisawa T. 2005. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* 11: 1910-7

167. Newman DJ, Cragg GM. 2004. Marine natural products and related compounds in clinical and advanced preclinical trials. *J Nat Prod* 67: 1216-38

168. Araki M, Kondo T, Gumperz JE, Brenner MB, Miyake S, Yamamura T. 2003. Th2 bias of CD4+ NKT cells derived from multiple sclerosis in remission. *Int Immunol* 15: 279-88

169. Kojo S, Tsutsumi A, Goto D, Sumida T. 2003. Low expression levels of soluble CD1d gene in patients with rheumatoid arthritis. *J Rheumatol* 30: 2524-8

170. Jahng AW, Maricic I, Pedersen B, Burdin N, Naidenko O, Kronenberg M, Koezuka Y, Kumar V. 2001. Activation of natural killer T cells

potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* 194: 1789-99

171. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. 2000. NKT cells: facts, functions and fallacies. *Immunol Today* 21: 573-83
172. Hansen DS, Siomos MA, Buckingham L, Scalzo AA, Schofield L. 2003. Regulation of murine cerebral malaria pathogenesis by CD1d-restricted NKT cells and the natural killer complex. *Immunity* 18: 391-402
173. Johnson TR, Hong S, Van Kaer L, Koezuka Y, Graham BS. 2002. NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. *J Virol* 76: 4294-303
174. Levy O, Orange JS, Hibberd P, Steinberg S, LaRussa P, Weinberg A, Wilson SB, Shaulov A, Fleisher G, Geha RS, Bonilla FA, Exley M. 2003. Disseminated varicella infection due to the vaccine strain of varicella-zoster virus, in a patient with a novel deficiency in natural killer T cells. *J Infect Dis* 188: 948-53
175. Xia C, Schumann J, Emmanuel R, Zhang Y, Chen W, Zhang W, Libero GD, Wang PG. 2007. Modification of the ceramide moiety of isoglobotrihexosylceramide on its agonist activity in stimulation of invariant natural killer T cells. *J Med Chem* 50: 3489-96
176. Yamamura T, Miyamoto K, Illes Z, Pal E, Araki M, Miyake S. 2004. NKT cell-stimulating synthetic glycolipids as potential therapeutics for autoimmune disease. *Curr Top Med Chem* 4: 561-7
177. Zajonc DM, Cantu C, 3rd, Mattner J, Zhou D, Savage PB, Bendelac A, Wilson IA, Teyton L. 2005. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat Immunol* 6: 810-8
178. Ndonye RM, Izmirian DP, Dunn MF, Yu KO, Porcelli SA, Khurana A, Kronenberg M, Richardson SK, Howell AR. 2005. Synthesis and evaluation of sphinganine analogues of KRN7000 and OCH. *J Org Chem* 70: 10260-70

179. Tsuji M. 2006. Glycolipids and phospholipids as natural CD1d-binding NKT cell ligands. *Cell Mol Life Sci* 63: 1889-98

180. Stanic AK, Shashidharamurthy R, Bezbradica JS, Matsuki N, Yoshimura Y, Miyake S, Choi EY, Schell TD, Van Kaer L, Tevethia SS, Roopenian DC, Yamamura T, Joyce S. 2003. Another view of T cell antigen recognition: cooperative engagement of glycolipid antigens by Va14Ja18 natural T(iNKT) cell receptor [corrected]. *J Immunol* 171: 4539-51

181. Sidobre S, Hammond KJ, Benazet-Sidobre L, Maltsev SD, Richardson SK, Ndonye RM, Howell AR, Sakai T, Besra GS, Porcelli SA, Kronenberg M. 2004. The T cell antigen receptor expressed by Valpha14i NKT cells has a unique mode of glycosphingolipid antigen recognition. *Proc Natl Acad Sci U S A* 101: 12254-9

182. Balato A, Unutmaz D, Gaspari AA. 2009. Natural killer T cells: an unconventional T-cell subset with diverse effector and regulatory functions. *J Invest Dermatol* 129: 1628-42

183. Skold M, Behar SM. 2003. Role of CD1d-restricted NKT cells in microbial immunity. *Infect Immun* 71: 5447-55

184. Tupin E, Kinjo Y, Kronenberg M. 2007. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol* 5: 405-17

185. Tessmer MS, Fatima A, Paget C, Trottein F, Brossay L. 2009. NKT cell immune responses to viral infection. *Expert Opin Ther Targets* 13: 153-62

186. Rigaud S, Fondaneche MC, Lambert N, Pasquier B, Mateo V, Soulas P, Galicier L, Le Deist F, Rieux-Laucat F, Revy P, Fischer A, de Saint Basile G, Latour S. 2006. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature* 444: 110-4

187. Locci M, Draghici E, Marangoni F, Bosticardo M, Catucci M, Aiuti A, Cancrini C, Marodi L, Espanol T, Bredius RG, Thrasher AJ, Schulz A, Litzman J, Roncarolo MG, Casorati G, Dellabona P, Villa A. 2009. The

Wiskott-Aldrich syndrome protein is required for iNKT cell maturation and function. *J Exp Med* 206: 735-42

188. Motsinger A, Haas DW, Stanic AK, Van Kaer L, Joyce S, Unutmaz D. 2002. CD1d-restricted human natural killer T cells are highly susceptible to human immunodeficiency virus 1 infection. *J Exp Med* 195: 869-79
189. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278: 1626-9
190. Motoki K, Morita M, Kobayashi E, Uchida T, Akimoto K, Fukushima H, Koezuka Y. 1995. Immunostimulatory and antitumor activities of monoglycosylceramides having various sugar moieties. *Biol Pharm Bull* 18: 1487-91
191. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, Yagita H, Godfrey DI. 2002. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* 99: 1259-66
192. Hayakawa Y, Godfrey DI, Smyth MJ. 2004. Alpha-galactosylceramide: potential immunomodulatory activity and future application. *Curr Med Chem* 11: 241-52
193. Crowe NY, Smyth MJ, Godfrey DI. 2002. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med* 196: 119-27
194. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. 1999. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* 163: 2387-91
195. Berzofsky JA, Terabe M. 2009. The contrasting roles of NKT cells in tumor immunity. *Curr Mol Med* 9: 667-72
196. Ishihara S, Nieda M, Kitayama J, Osada T, Yabe T, Kikuchi A, Koezuka Y, Porcelli SA, Tadokoro K, Nagawa H, Juji T. 2000. Alpha-

glycosylceramides enhance the antitumor cytotoxicity of hepatic lymphocytes obtained from cancer patients by activating CD3-CD56+ NK cells in vitro. *J Immunol* 165: 1659-64

197. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, Hutchinson A, Geller M, Liu N, Annable R, Shay J, Kirchhoff K, Nishi N, Ando Y, Hayashi K, Hassoun H, Steinman RM, Dhodapkar MV. 2005. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* 201: 1503-17
198. Spanoudakis E, Hu M, Naresh K, Terpos E, Melo V, Reid A, Kotsianidis I, Abdalla S, Rahemtulla A, Karadimitris A. 2009. Regulation of multiple myeloma survival and progression by CD1d. *Blood* 113: 2498-507
199. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, Balk SP, Exley MA. 2001. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol* 167: 4046-50
200. Crough T, Purdie DM, Okai M, Maksoud A, Nieda M, Nicol AJ. 2004. Modulation of human Valpha24(+)Vbeta11(+) NKT cells by age, malignancy and conventional anticancer therapies. *Br J Cancer* 91: 1880-6
201. Fujii S, Shimizu K, Klimek V, Geller MD, Nimer SD, Dhodapkar MV. 2003. Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br J Haematol* 122: 617-22
202. Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. 2002. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. *J Immunol* 168: 6494-9
203. van der Vliet HJ, Wang R, Yue SC, Koon HB, Balk SP, Exley MA. 2008. Circulating myeloid dendritic cells of advanced cancer patients result in reduced activation and a biased cytokine profile in invariant NKT cells. *J Immunol* 180: 7287-93

204. Dhodapkar KM, Cirignano B, Chamian F, Zagzag D, Miller DC, Finlay JL, Steinman RM. 2004. Invariant natural killer T cells are preserved in patients with glioma and exhibit antitumor lytic activity following dendritic cell-mediated expansion. *Int J Cancer* 109: 893-9

205. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, Imamura M. 2005. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res* 11: 7322-7

206. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, Taniguchi M, Grusby MJ, DeKruyff RH, Umetsu DT. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* 9: 582-8

207. Kaneko Y, Harada M, Kawano T, Yamashita M, Shibata Y, Gejyo F, Nakayama T, Taniguchi M. 2000. Augmentation of Valpha14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of concanavalin A-induced hepatitis. *J Exp Med* 191: 105-14

208. Lee PT, Putnam A, Benlagha K, Teyton L, Gottlieb PA, Bendelac A. 2002. Testing the NKT cell hypothesis of human IDDM pathogenesis. *J Clin Invest* 110: 793-800

209. Wilson SB, Kent SC, Patton KT, Orban T, Jackson RA, Exley M, Porcelli S, Schatz DA, Atkinson MA, Balk SP, Strominger JL, Hafler DA. 1998. Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. *Nature* 391: 177-81

210. Arrenberg P, Halder R, Kumar V. 2009. Cross-regulation between distinct natural killer T cell subsets influences immune response to self and foreign antigens. *J Cell Physiol* 218: 246-50

211. Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B, Kronenberg M, Seeger RC. 2001. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol* 167: 3114-22

212. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, Sato M, Takeda K, Okumura K, Van Kaer L, Kawano T, Taniguchi M, Nishimura T. 1999. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* 189: 1121-8

213. Fujii S, Shimizu K, Kronenberg M, Steinman RM. 2002. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 3: 867-74

214. Liu K, Idoyaga J, Charalambous A, Fujii S, Bonito A, Mordoh J, Wainstok R, Bai XF, Liu Y, Steinman RM. 2005. Innate NKT lymphocytes confer superior adaptive immunity via tumor-capturing dendritic cells. *J Exp Med* 202: 1507-16

215. Shimizu K, Goto A, Fukui M, Taniguchi M, Fujii S. 2007. Tumor cells loaded with alpha-galactosylceramide induce innate NKT and NK cell-dependent resistance to tumor implantation in mice. *J Immunol* 178: 2853-61

216. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S. 2007. Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. *J Exp Med* 204: 2641-53

217. Molling JW, Moreno M, de Groot J, van der Vliet HJ, von Blomberg BM, van den Eertwegh AJ, Scheper RJ, Bontkes HJ. 2008. Chronically stimulated mouse invariant NKT cell lines have a preserved capacity to enhance protection against experimental tumor metastases. *Immunol Lett* 118: 36-43

218. Giaccone G. 2002. Clinical impact of novel treatment strategies. *Oncogene* 21: 6970-81

219. Okai M, Nieda M, Tazbirkova A, Horley D, Kikuchi A, Durrant S, Takahashi T, Boyd A, Abraham R, Yagita H, Juji T, Nicol A. 2002. Human peripheral blood Valpha24+ Vbeta11+ NKT cells expand

following administration of alpha-galactosylceramide-pulsed dendritic cells. *Vox Sang* 83: 250-3

220. Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, Abraham R, Juji T, Macfarlane DJ, Nicol AJ. 2004. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* 103: 383-9
221. Watarai H, Nakagawa R, Omori-Miyake M, Dashtsoodol N, Taniguchi M. 2008. Methods for detection, isolation and culture of mouse and human invariant NKT cells. *Nat Protoc* 3: 70-8
222. Metelitsa LS. 2004. Flow cytometry for natural killer T cells: multi-parameter methods for multifunctional cells. *Clin Immunol* 110: 267-76
223. Chan AC, Serwecinska L, Cochrane A, Harrison LC, Godfrey DI, Berzins SP. 2009. Immune characterization of an individual with an exceptionally high natural killer T cell frequency and her immediate family. *Clin Exp Immunol* 156: 238-45
224. Lee PT, Benlagha K, Teyton L, Bendelac A. 2002. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med* 195: 637-41
225. Sidobre S, Kronenberg M. 2002. CD1 tetramers: a powerful tool for the analysis of glycolipid-reactive T cells. *J Immunol Methods* 268: 107-21
226. Kita H, Naidenko OV, Kronenberg M, Ansari AA, Rogers P, He XS, Koning F, Mikayama T, Van De Water J, Coppel RL, Kaplan M, Gershwin ME. 2002. Quantitation and phenotypic analysis of natural killer T cells in primary biliary cirrhosis using a human CD1d tetramer. *Gastroenterology* 123: 1031-43
227. Montoya CJ, Pollard D, Martinson J, Kumari K, Wasserfall C, Mulder CB, Rugeles MT, Atkinson MA, Landay AL, Wilson SB. 2007. Characterization of human invariant natural killer T subsets in health

and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. *Immunology* 122: 1-14

228. Schuster V, Kreth HW. 2000. X-linked lymphoproliferative disease is caused by deficiency of a novel SH2 domain-containing signal transduction adaptor protein. *Immunol Rev* 178: 21-8
229. Schmidt-Supprian M, Tian J, Grant EP, Pasparakis M, Maehr R, Ovaa H, Ploegh HL, Coyle AJ, Rajewsky K. 2004. Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. *Proc Natl Acad Sci U S A* 101: 4566-71
230. Sivakumar V, Hammond KJ, Howells N, Pfeffer K, Weih F. 2003. Differential requirement for Rel/nuclear factor kappa B family members in natural killer T cell development. *J Exp Med* 197: 1613-21
231. Molling JW, Kolgen W, van der Vliet HJ, Boomsma MF, Kruizenga H, Smorenburg CH, Molenkamp BG, Langendijk JA, Leemans CR, von Blomberg BM, Scheper RJ, van den Eertwegh AJ. 2005. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *Int J Cancer* 116: 87-93
232. Sandberg JK, Fast NM, Palacios EH, Fennelly G, Dobroszycki J, Palumbo P, Wiznia A, Grant RM, Bhardwaj N, Rosenberg MG, Nixon DF. 2002. Selective loss of innate CD4(+) V alpha 24 natural killer T cells in human immunodeficiency virus infection. *J Virol* 76: 7528-34
233. van der Vliet HJ, von Blomberg BM, Hazenberg MD, Nishi N, Otto SA, van Benthem BH, Prins M, Claessen FA, van den Eertwegh AJ, Giaccone G, Miedema F, Scheper RJ, Pinedo HM. 2002. Selective decrease in circulating V alpha 24+V beta 11+ NKT cells during HIV type 1 infection. *J Immunol* 168: 1490-5
234. van der Vliet HJ, van Vonderen MG, Molling JW, Bontkes HJ, Reijm M, Reiss P, van Agtmael MA, Danner SA, van den Eertwegh AJ, von Blomberg BM, Scheper RJ. 2006. Cutting edge: Rapid recovery of NKT

cells upon institution of highly active antiretroviral therapy for HIV-1 infection. *J Immunol* 177: 5775-8

235. de Lalla C, Galli G, Aldrighetti L, Romeo R, Mariani M, Monno A, Nuti S, Colombo M, Callea F, Porcelli SA, Panina-Bordignon P, Abrignani S, Casorati G, Dellabona P. 2004. Production of profibrotic cytokines by invariant NKT cells characterizes cirrhosis progression in chronic viral hepatitis. *J Immunol* 173: 1417-25
236. van der Vliet HJ, Molling JW, von Blomberg BM, Kolgen W, Stam AG, de Gruijl TD, Mulder CJ, Janssen HL, Nishi N, van den Eertwegh AJ, Scheper RJ, van Nieuwkerk CJ. 2005. Circulating Valpha24+Vbeta11+ NKT cell numbers and dendritic cell CD1d expression in hepatitis C virus infected patients. *Clin Immunol* 114: 183-9
237. Brutkiewicz RR. 2006. CD1d ligands: the good, the bad, and the ugly. *J Immunol* 177: 769-75
238. Li W, Sofi MH, Wei DG, Du W, Gervay-Hague J, Renukaradhya GJ, Brutkiewicz RR, Chang CH. 2009. MHC class II-expressing thymocytes suppress invariant NKT cell development. *Immunol Cell Biol* 87: 186-9
239. Lee YJ, Jung KC, Park SH. 2009. MHC class II-dependent T-T interactions create a diverse, functional and immunoregulatory reaction circle. *Immunol Cell Biol* 87: 65-71
240. Kronenberg M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol* 23: 877-900
241. Taniguchi M, Tashiro T, Dashtsoodol N, Hongo N, Watarai H. 2010. The specialized iNKT cell system recognizes glycolipid antigens and bridges the innate and acquired immune systems with potential applications for cancer therapy. *Int Immunol* 22: 1-6
242. Onoe K, Yanagawa Y, Minami K, Iijima N, Iwabuchi K. 2007. Th1 or Th2 balance regulated by interaction between dendritic cells and NKT cells. *Immunol Res* 38: 319-32
243. McCarthy C, Shepherd D, Fleire S, Stronge VS, Koch M, Illarionov PA, Bossi G, Salio M, Denkberg G, Reddington F, Tarlton A, Reddy BG,

Schmidt RR, Reiter Y, Griffiths GM, van der Merwe PA, Besra GS, Jones EY, Batista FD, Cerundolo V. 2007. The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. *J Exp Med* 204: 1131-44

244. Venkataswamy MM, Porcelli SA. 2010. Lipid and glycolipid antigens of CD1d-restricted natural killer T cells. *Semin Immunol* 22: 68-78

245. Yu KO, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, Fujiwara N, Arias I, Miyake S, Yamamura T, Chang YT, Besra GS, Porcelli SA. 2005. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc Natl Acad Sci U S A* 102: 3383-8

246. Fujii S, Shimizu K, Steinman RM, Dhodapkar MV. 2003. Detection and activation of human Valpha24+ natural killer T cells using alpha-galactosyl ceramide-pulsed dendritic cells. *J Immunol Methods* 272: 147-59

247. van der Vliet HJ, Molling JW, Nishi N, Masterson AJ, Kolgen W, Porcelli SA, van den Eertwegh AJ, von Blomberg BM, Pinedo HM, Giaccone G, Scheper RJ. 2003. Polarization of Valpha24+ Vbeta11+ natural killer T cells of healthy volunteers and cancer patients using alpha-galactosylceramide-loaded and environmentally instructed dendritic cells. *Cancer Res* 63: 4101-6

248. Roark JH, Park SH, Jayawardena J, Kavita U, Shannon M, Bendelac A. 1998. CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J Immunol* 160: 3121-7

249. Barral P, Eckl-Dorna J, Harwood NE, De Santo C, Salio M, Illarionov P, Besra GS, Cerundolo V, Batista FD. 2008. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. *Proc Natl Acad Sci U S A* 105: 8345-50

250. Matsuda JL, Gapin L. 2005. Developmental program of mouse Valpha14i NKT cells. *Curr Opin Immunol* 17: 122-30

251. Ohteki T, Ho S, Suzuki H, Mak TW, Ohashi PS. 1997. Role for IL-15/IL-15 receptor beta-chain in natural killer 1.1+ T cell receptor-alpha beta+ cell development. *J Immunol* 159: 5931-5

252. Matsuda JL, Gapin L, Sidobre S, Kieper WC, Tan JT, Ceredig R, Surh CD, Kronenberg M. 2002. Homeostasis of V alpha 14i NKT cells. *Nat Immunol* 3: 966-74

253. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, Matsuki N, Charrier K, Sedger L, Willis CR, Brasel K, Morrissey PJ, Stocking K, Schuh JC, Joyce S, Peschon JJ. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med* 191: 771-80

254. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, Ma A. 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* 9: 669-76

255. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, Gapin L, Glimcher LH. 2004. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 20: 477-94

256. Matsuda JL, Gapin L, Baron JL, Sidobre S, Stetson DB, Mohrs M, Locksley RM, Kronenberg M. 2003. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc Natl Acad Sci U S A* 100: 8395-400

257. Martin SM, Mehta IK, Yokoyama WM, Thomas ML, Lorenz RG. 2001. Development of intestinal intraepithelial lymphocytes, NK cells, and NK 1.1+ T cells in CD45-deficient mice. *J Immunol* 166: 6066-73

258. Vinay DS, Choi BK, Bae JS, Kim WY, Gebhardt BM, Kwon BS. 2004. CD137-deficient mice have reduced NK/NKT cell numbers and function, are resistant to lipopolysaccharide-induced shock syndromes, and have lower IL-4 responses. *J Immunol* 173: 4218-29

259. Sato H, Nakayama T, Tanaka Y, Yamashita M, Shibata Y, Kondo E, Saito Y, Taniguchi M. 1999. Induction of differentiation of pre-NKT

cells to mature Valpha14 NKT cells by granulocyte/macrophage colony-stimulating factor. *Proc Natl Acad Sci U S A* 96: 7439-44

260. Bessoles S, Fouret F, Dudal S, Besra GS, Sanchez F, Lafont V. 2008. IL-2 triggers specific signaling pathways in human NKT cells leading to the production of pro- and anti-inflammatory cytokines. *J Leukoc Biol* 84: 224-33
261. Lin H, Nieda M, Nicol AJ. 2004. Differential proliferative response of NKT cell subpopulations to in vitro stimulation in presence of different cytokines. *Eur J Immunol* 34: 2664-71
262. Van Der Vliet HJ, Nishi N, Koezuka Y, Peyrat MA, Von Blomberg BM, Van Den Eertwegh AJ, Pinedo HM, Giaccone G, Scheper RJ. 1999. Effects of alpha-galactosylceramide (KRN7000), interleukin-12 and interleukin-7 on phenotype and cytokine profile of human Valpha24+ Vbeta11+ T cells. *Immunology* 98: 557-63
263. Yang OO, Racke FK, Nguyen PT, Gausling R, Severino ME, Horton HF, Byrne MC, Strominger JL, Wilson SB. 2000. CD1d on myeloid dendritic cells stimulates cytokine secretion from and cytolytic activity of V alpha 24J alpha Q T cells: a feedback mechanism for immune regulation. *J Immunol* 165: 3756-62
264. Santegoets SJ, Masterson AJ, van der Sluis PC, Lougheed SM, Fluitsma DM, van den Eertwegh AJ, Pinedo HM, Scheper RJ, de Gruijl TD. 2006. A CD34(+) human cell line model of myeloid dendritic cell differentiation: evidence for a CD14(+)CD11b(+) Langerhans cell precursor. *J Leukoc Biol* 80: 1337-44
265. Montoya CJ, Jie HB, Al-Harthi L, Mulder C, Patino PJ, Rugeles MT, Krieg AM, Landay AL, Wilson SB. 2006. Activation of plasmacytoid dendritic cells with TLR9 agonists initiates invariant NKT cell-mediated cross-talk with myeloid dendritic cells. *J Immunol* 177: 1028-39
266. Stonier SW, Schluns KS. 2010. Trans-presentation: a novel mechanism regulating IL-15 delivery and responses. *Immunol Lett* 127: 85-92

267. Schmieg J, Yang G, Franck RW, Tsuji M. 2003. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med* 198: 1631-41

268. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, Carbone DP, Paul WE, Berzofsky JA. 2000. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* 1: 515-20

269. Novak J, Beaudoin L, Park S, Griseri T, Teyton L, Bendelac A, Lehuen A. 2007. Prevention of type 1 diabetes by invariant NKT cells is independent of peripheral CD1d expression. *J Immunol* 178: 1332-40

270. Miyamoto K, Miyake S, Yamamura T. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 413: 531-4

271. Cerundolo V, Barral P, Batista FD. 2010. Synthetic iNKT cell-agonists as vaccine adjuvants--finding the balance. *Curr Opin Immunol* 22: 417-24

272. Scott-Browne JP, Matsuda JL, Mallevaey T, White J, Borg NA, McCluskey J, Rossjohn J, Kappler J, Marrack P, Gapin L. 2007. Germline-encoded recognition of diverse glycolipids by natural killer T cells. *Nat Immunol* 8: 1105-13

273. Oki S, Chiba A, Yamamura T, Miyake S. 2004. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J Clin Invest* 113: 1631-40

274. Im JS, Arora P, Bricard G, Molano A, Venkataswamy MM, Baine I, Jerud ES, Goldberg MF, Baena A, Yu KO, Ndonye RM, Howell AR, Yuan W, Cresswell P, Chang YT, Illarionov PA, Besra GS, Porcelli SA. 2009. Kinetics and cellular site of glycolipid loading control the outcome of natural killer T cell activation. *Immunity* 30: 888-98

275. Fujio M, Wu D, Garcia-Navarro R, Ho DD, Tsuji M, Wong CH. 2006. Structure-based discovery of glycolipids for CD1d-mediated NKT cell

activation: tuning the adjuvant versus immunosuppression activity. *J Am Chem Soc* 128: 9022-3

276. Lee T, Cho M, Ko SY, Youn HJ, Baek DJ, Cho WJ, Kang CY, Kim S. 2007. Synthesis and evaluation of 1,2,3-triazole containing analogues of the immunostimulant alpha-GalCer. *J Med Chem* 50: 585-9
277. Silk JD, Salio M, Reddy BG, Shepherd D, Gileadi U, Brown J, Masri SH, Polzella P, Ritter G, Besra GS, Jones EY, Schmidt RR, Cerundolo V. 2008. Cutting edge: nonglycosidic CD1d lipid ligands activate human and murine invariant NKT cells. *J Immunol* 180: 6452-6
278. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, Kronenberg M. 1998. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med* 188: 1521-8
279. Kjer-Nielsen L, Borg NA, Pellicci DG, Beddoe T, Kostenko L, Clements CS, Williamson NA, Smyth MJ, Besra GS, Reid HH, Bharadwaj M, Godfrey DI, Rossjohn J, McCluskey J. 2006. A structural basis for selection and cross-species reactivity of the semi-invariant NKT cell receptor in CD1d/glycolipid recognition. *J Exp Med* 203: 661-73
280. Goff RD, Gao Y, Mattner J, Zhou D, Yin N, Cantu C, 3rd, Teyton L, Bendelac A, Savage PB. 2004. Effects of lipid chain lengths in alpha-galactosylceramides on cytokine release by natural killer T cells. *J Am Chem Soc* 126: 13602-3
281. Godfrey DI, McCluskey J, Rossjohn J. 2005. CD1d antigen presentation: treats for NKT cells. *Nat Immunol* 6: 754-6
282. Gadola SD, Koch M, Marles-Wright J, Lissin NM, Shepherd D, Matulis G, Harlos K, Villiger PM, Stuart DI, Jakobsen BK, Cerundolo V, Jones EY. 2006. Structure and binding kinetics of three different human CD1d-alpha-galactosylceramide-specific T cell receptors. *J Exp Med* 203: 699-710
283. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, Joyce S, Van Kaer L. 2005. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* 115: 2572-83

284. Uldrich AP, Crowe NY, Kyparissoudis K, Pellicci DG, Zhan Y, Lew AM, Bouillet P, Strasser A, Smyth MJ, Godfrey DI. 2005. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J Immunol* 175: 3092-101

285. Chang WS, Kim JY, Kim YJ, Kim YS, Lee JM, Azuma M, Yagita H, Kang CY. 2008. Cutting edge: Programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. *J Immunol* 181: 6707-10

286. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, Kumar V, Wu L, Kaer LV. 2009. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* 182: 2816-26

287. Wang J, Cheng L, Wondimu Z, Swain M, Santamaria P, Yang Y. 2009. Cutting edge: CD28 engagement releases antigen-activated invariant NKT cells from the inhibitory effects of PD-1. *J Immunol* 182: 6644-7

288. Moll M, Kuylenstierna C, Gonzalez VD, Andersson SK, Bosnjak L, Sonnerborg A, Quigley MF, Sandberg JK. 2009. Severe functional impairment and elevated PD-1 expression in CD1d-restricted NKT cells retained during chronic HIV-1 infection. *Eur J Immunol* 39: 902-11

289. O'Hagan DaR, HS. 1997. Some influences of Fluorine in Bioorganic chemistry. *Chem. Commun.*: 645-52

290. Leung L, Tomassi C, Van Beneden K, Decruy T, Trappeniers M, Elewaut D, Gao Y, Elliott T, Al-Shamkhani A, Ottensmeier C, Werner JM, Williams A, Van Calenbergh S, Linclau B. 2009. The synthesis and in vivo evaluation of 2',2'-difluoro KRN7000. *ChemMedChem* 4: 329-34

291. Trappeniers M, Goormans S, Van Beneden K, Decruy T, Linclau B, Al-Shamkhani A, Elliott T, Ottensmeier C, Werner JM, Elewaut D, Van Calenbergh S. 2008. Synthesis and in vitro evaluation of alpha-GalCer epimers. *ChemMedChem* 3: 1061-70

292. Leung L, Tomassi C, Van Beneden K, Decruy T, Elewaut D, Elliott T, Al-Shamkhani A, Ottensmeier C, Van Calenbergh S, Werner J, Williams T, Linclau B. 2008. Synthesis and in vivo evaluation of 4-deoxy-4,4-difluoro-KRN7000. *Org Lett* 10: 4433-6

293. Trappeniers M, Beneden KV, Decruy T, Hillaert U, Linclau B, Elewaut D, Calenbergh SV. 2008. 6'-Derivatised alpha-GalCer Analogues Capable of Inducing Strong CD1d-Mediated Th1-Biased NKT Cell Responses in Mice. *J Am Chem Soc*

294. Joyee AG, Uzonna J, Yang X. 2010. Invariant NKT cells preferentially modulate the function of CD8 alpha+ dendritic cell subset in inducing type 1 immunity against infection. *J Immunol* 184: 2095-106

295. Reyes NJ, Mayhew E, Chen PW, Niederkorn JY. 2010. NKT cells are necessary for maximal expression of allergic conjunctivitis1. *Int Immunol*

296. Wermeling F, Lind SM, Jordo ED, Cardell SL, Karlsson MC. 2010. Invariant NKT cells limit activation of autoreactive CD1d-positive B cells. *J Exp Med* 207: 943-52

297. Bousso P, Albert ML. 2010. Signal 0 for guided priming of CTLs: NKT cells do it too. *Nat Immunol* 11: 284-6

298. Yue SC, Shaulov A, Wang R, Balk SP, Exley MA. 2005. CD1d ligation on human monocytes directly signals rapid NF-kappaB activation and production of bioactive IL-12. *Proc Natl Acad Sci U S A* 102: 11811-6

299. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, Kondo E, Harada M, Koseki H, Nakayama T, Tanaka Y, Taniguchi M. 1998. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci U S A* 95: 5690-3

300. Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, Kronenberg M, Koezuka Y, Delovitch TL, Gombert JM, Leite-De-Moraes M, Gouarin C, Zhu R, Hameg A, Nakayama T, Taniguchi M, Lepault F, Lehuen A, Bach JF, Herbelin A. 2001. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the

onset and recurrence of autoimmune Type 1 diabetes. *Nat Med* 7: 1057-62

301. Zhang W, Zheng X, Xia C, Perali RS, Yao Q, Liu Y, Zheng P, Wang PG. 2008. Alpha-lactosylceramide as a novel "sugar-capped" CD1d ligand for natural killer T cells: biased cytokine profile and therapeutic activities. *Chembiochem* 9: 1423-30
302. Chang DH, Deng H, Matthews P, Krasovsky J, Ragupathi G, Spisek R, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV. 2008. Inflammation-associated lysophospholipids as ligands for CD1d-restricted T cells in human cancer. *Blood* 112: 1308-16
303. Zhou D. 2006. The immunological function of iGb3. *Curr Protein Pept Sci* 7: 325-33
304. Christiansen D, Milland J, Mouhtouris E, Vaughan H, Pellicci DG, McConville MJ, Godfrey DI, Sandrin MS. 2008. Humans lack iGb3 due to the absence of functional iGb3-synthase: implications for NKT cell development and transplantation. *PLoS Biol* 6: e172
305. Shiratsuchi T, Schneck J, Kawamura A, Tsuji M. 2009. Human CD1 dimeric proteins as indispensable tools for research on CD1-binding lipids and CD1-restricted T cells. *J Immunol Methods* 345: 49-59
306. Im JS, Yu KO, Illarionov PA, LeClair KP, Storey JR, Kennedy MW, Besra GS, Porcelli SA. 2004. Direct measurement of antigen binding properties of CD1 proteins using fluorescent lipid probes. *J Biol Chem* 279: 299-310
307. Cantu C, 3rd, Benlagha K, Savage PB, Bendelac A, Teyton L. 2003. The paradox of immune molecular recognition of alpha-galactosylceramide: low affinity, low specificity for CD1d, high affinity for alpha beta TCRs. *J Immunol* 170: 4673-82
308. Naidenko OV, Maher JK, Ernst WA, Sakai T, Modlin RL, Kronenberg M. 1999. Binding and antigen presentation of ceramide-containing glycolipids by soluble mouse and human CD1d molecules. *J Exp Med* 190: 1069-80

309. biosciences B. 2008. DimerX I: Recombinant Soluble Dimeric Mouse CD1d:Ig Fusion Protein. In *BD Technical Data Sheet*

310. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, Koezuka Y, Kronenberg M. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* 192: 741-54

311. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. 2000. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J Exp Med* 191: 1895-903

312. Karadimitris A, Gadola S, Altamirano M, Brown D, Woolfson A, Klenerman P, Chen JL, Koezuka Y, Roberts IA, Price DA, Dusheiko G, Milstein C, Fersht A, Luzzatto L, Cerundolo V. 2001. Human CD1d-glycolipid tetramers generated by in vitro oxidative refolding chromatography. *Proc Natl Acad Sci U S A* 98: 3294-8

313. Gadola SD, Karadimitris A, Zaccai NR, Salio M, Dulphy N, Shepherd D, Jones EY, Cerundolo V. 2003. Generation of CD1 tetramers as a tool to monitor glycolipid-specific T cells. *Philos Trans R Soc Lond B Biol Sci* 358: 875-7

314. Li D, Chen N, McMichael AJ, Screaton GR, Xu XN. 2008. Generation and characterisation of CD1d tetramer produced by a lentiviral expression system. *J Immunol Methods* 330: 57-63

315. Castano AR, Tangri S, Miller JE, Holcombe HR, Jackson MR, Huse WD, Kronenberg M, Peterson PA. 1995. Peptide binding and presentation by mouse CD1. *Science* 269: 223-6

316. Dal Porto J, Johansen TE, Catipovic B, Parfiit DJ, Tuveson D, Gether U, Kozlowski S, Fearon DT, Schneck JP. 1993. A soluble divalent class I major histocompatibility complex molecule inhibits alloreactive T cells at nanomolar concentrations. *Proc Natl Acad Sci U S A* 90: 6671-5

317. Rauch J, Gumperz J, Robinson C, Skold M, Roy C, Young DC, Lafleur M, Moody DB, Brenner MB, Costello CE, Behar SM. 2003. Structural features of the acyl chain determine self-phospholipid antigen

recognition by a CD1d-restricted invariant NKT (iNKT) cell. *J Biol Chem* 278: 47508-15

318. Kang SJ, Cresswell P. 2004. Saposins facilitate CD1d-restricted presentation of an exogenous lipid antigen to T cells. *Nat Immunol* 5: 175-81
319. Prigozy TI, Naidenko O, Qasba P, Elewaut D, Brossay L, Khurana A, Natori T, Koezuka Y, Kulkarni A, Kronenberg M. 2001. Glycolipid antigen processing for presentation by CD1d molecules. *Science* 291: 664-7
320. Park MA, Li JT, Hagan JB, Maddox DE, Abraham RS. 2008. Common variable immunodeficiency: a new look at an old disease. *Lancet* 372: 489-502
321. Di Renzo M, Pasqui AL, Auteri A. 2004. Common variable immunodeficiency: a review. *Clin Exp Med* 3: 211-7
322. Bonilla FA, Bernstein IL, Khan DA, Ballas ZK, Chinen J, Frank MM, Kobrynski LJ, Levinson AI, Mazer B, Nelson RP, Jr., Orange JS, Routes JM, Shearer WT, Sorensen RU. 2005. Practice parameter for the diagnosis and management of primary immunodeficiency. *Ann Allergy Asthma Immunol* 94: S1-63
323. Cunningham-Rundles C, Bodian C. 1999. Common variable immunodeficiency: clinical and immunological features of 248 patients. *Clin Immunol* 92: 34-48
324. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, Eibel H, Fischer B, Schaffer AA, Mages HW, Kroczek RA, Peter HH. 2003. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol* 4: 261-8
325. Salzer U, Maul-Pavicic A, Cunningham-Rundles C, Urschel S, Belohradsky BH, Litzman J, Holm A, Franco JL, Plebani A, Hammarstrom L, Skrabl A, Schwinger W, Grimbacher B. 2004. ICOS deficiency in patients with common variable immunodeficiency. *Clin Immunol* 113: 234-40

326. Castigli E, Wilson S, Garibyan L, Rachid R, Bonilla F, Schneider L, Morra M, Curran J, Geha R. 2007. Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. *Nat Genet* 39: 430-1

327. Castigli E, Wilson SA, Elkhal A, Ozcan E, Garibyan L, Geha RS. 2007. Transmembrane activator and calcium modulator and cyclophilin ligand interactor enhances CD40-driven plasma cell differentiation. *J Allergy Clin Immunol* 120: 885-91

328. Castigli E, Wilson SA, Garibyan L, Rachid R, Bonilla F, Schneider L, Geha RS. 2005. TACI is mutant in common variable immunodeficiency and IgA deficiency. *Nat Genet* 37: 829-34

329. Losi CG, Silini A, Fiorini C, Soresina A, Meini A, Ferrari S, Notarangelo LD, Lougaris V, Plebani A. 2005. Mutational analysis of human BAFF receptor TNFRSF13C (BAFF-R) in patients with common variable immunodeficiency. *J Clin Immunol* 25: 496-502

330. van Zelm MC, Reisli I, van der Burg M, Castano D, van Noesel CJ, van Tol MJ, Woellner C, Grimbacher B, Patino PJ, van Dongen JJ, Franco JL. 2006. An antibody-deficiency syndrome due to mutations in the CD19 gene. *N Engl J Med* 354: 1901-12

331. Greenwald RJ, Freeman GJ, Sharpe AH. 2005. The B7 family revisited. *Annu Rev Immunol* 23: 515-48

332. Ling V, Wu PW, Finnerty HF, Agostino MJ, Graham JR, Chen S, Jussiff JM, Fisk GJ, Miller CP, Collins M. 2001. Assembly and annotation of human chromosome 2q33 sequence containing the CD28, CTLA4, and ICOS gene cluster: analysis by computational, comparative, and microarray approaches. *Genomics* 78: 155-68

333. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397: 263-6

334. Yagi J, Arimura Y, Dianzani U, Uede T, Okamoto T, Uchiyama T. 2003. Regulatory roles of IL-2 and IL-4 in H4/inducible costimulator

expression on activated CD4+ T cells during Th cell development. *J Immunol* 171: 783-94

335. Wassink L, Vieira PL, Smits HH, Kingsbury GA, Coyle AJ, Kapsenberg ML, Wierenga EA. 2004. ICOS expression by activated human Th cells is enhanced by IL-12 and IL-23: increased ICOS expression enhances the effector function of both Th1 and Th2 cells. *J Immunol* 173: 1779-86
336. Okamoto N, Tezuka K, Kato M, Abe R, Tsuji T. 2003. PI3-kinase and MAP-kinase signaling cascades in AILIM/ICOS- and CD28-costimulated T-cells have distinct functions between cell proliferation and IL-10 production. *Biochem Biophys Res Commun* 310: 691-702
337. Beier KC, Hutloff A, Dittrich AM, Heuck C, Rauch A, Buchner K, Ludewig B, Ochs HD, Mages HW, Kroczek RA. 2000. Induction, binding specificity and function of human ICOS. *Eur J Immunol* 30: 3707-17
338. Salzer U, Grimbacher B. 2005. TACItly changing tunes: farewell to a yin and yang of BAFF receptor and TACI in humoral immunity? New genetic defects in common variable immunodeficiency. *Curr Opin Allergy Clin Immunol* 5: 496-503
339. Kanegane H, Agematsu K, Futatani T, Sira MM, Suga K, Sekiguchi T, van Zelm MC, Miyawaki T. 2007. Novel mutations in a Japanese patient with CD19 deficiency. *Genes Immun* 8: 663-70
340. Tedder TF, Poe JC, Fujimoto M, Haas KM, Sato S. 2005. The CD19-CD21 signal transduction complex of B lymphocytes regulates the balance between health and autoimmune disease: systemic sclerosis as a model system. *Curr Dir Autoimmun* 8: 55-90
341. Salzer U, Jennings S, Grimbacher B. 2007. To switch or not to switch--the opposing roles of TACI in terminal B cell differentiation. *Eur J Immunol* 37: 17-20

342. Mackay F, Ambrose C. 2003. The TNF family members BAFF and APRIL: the growing complexity. *Cytokine Growth Factor Rev* 14: 311-24

343. Mackay F, Schneider P, Rennert P, Browning J. 2003. BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* 21: 231-64

344. Ng LG, Sutherland AP, Newton R, Qian F, Cachero TG, Scott ML, Thompson JS, Wheway J, Chtanova T, Groom J, Sutton IJ, Xin C, Tangye SG, Kalled SL, Mackay F, Mackay CR. 2004. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. *J Immunol* 173: 807-17

345. Avery DT, Kalled SL, Ellyard JI, Ambrose C, Bixler SA, Thien M, Brink R, Mackay F, Hodgkin PD, Tangye SG. 2003. BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. *J Clin Invest* 112: 286-97

346. Novak AJ, Darce JR, Arendt BK, Harder B, Henderson K, Kindsvogel W, Gross JA, Greipp PR, Jelinek DF. 2004. Expression of BCMA, TACI, and BAFF-R in multiple myeloma: a mechanism for growth and survival. *Blood* 103: 689-94

347. von Bulow GU, Bram RJ. 1997. NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. *Science* 278: 138-41

348. Seshasayee D, Valdez P, Yan M, Dixit VM, Tumas D, Grewal IS. 2003. Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLyS receptor. *Immunity* 18: 279-88

349. Castigli E, Scott S, Dedeoglu F, Bryce P, Jabara H, Bhan AK, Mizoguchi E, Geha RS. 2004. Impaired IgA class switching in APRIL-deficient mice. *Proc Natl Acad Sci U S A* 101: 3903-8

350. Conley ME, Dobbs AK, Farmer DM, Kilic S, Paris K, Grigoriadou S, Coustan-Smith E, Howard V, Campana D. 2009. Primary B cell

immunodeficiencies: comparisons and contrasts. *Annu Rev Immunol* 27: 199-227

351. Zhang L, Radigan L, Salzer U, Behrens TW, Grimbacher B, Diaz G, Bussel J, Cunningham-Rundles C. 2007. Transmembrane activator and calcium-modulating cyclophilin ligand interactor mutations in common variable immunodeficiency: clinical and immunologic outcomes in heterozygotes. *J Allergy Clin Immunol* 120: 1178-85
352. Sasaki Y, Casola S, Kutok JL, Rajewsky K, Schmidt-Supprian M. 2004. TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. *J Immunol* 173: 2245-52
353. Schneider P, Takatsuka H, Wilson A, Mackay F, Tardivel A, Lens S, Cachero TG, Finke D, Beermann F, Tschopp J. 2001. Maturation of marginal zone and follicular B cells requires B cell activating factor of the tumor necrosis factor family and is independent of B cell maturation antigen. *J Exp Med* 194: 1691-7
354. Bayry J, Hermine O, Webster DA, Levy Y, Kaveri SV. 2005. Common variable immunodeficiency: the immune system in chaos. *Trends Mol Med* 11: 370-6
355. Livaditi O, Giamarellos-Bourboulis EJ, Kakkas I, Kapsimali V, Lymberi P, Papastariades C, Douzinas EE. 2007. Grouping of patients with common variable immunodeficiency based on immunoglobulin biosynthesis: comparison with a classification system on CD4-naive cells. *Immunol Lett* 114: 103-9
356. Giovannetti A, Pierdominici M, Mazzetta F, Marziali M, Renzi C, Mileo AM, De Felice M, Mora B, Esposito A, Carello R, Pizzuti A, Paggi MG, Paganelli R, Malorni W, Aiuti F. 2007. Unravelling the complexity of T cell abnormalities in common variable immunodeficiency. *J Immunol* 178: 3932-43
357. Moratto D, Gulino AV, Fontana S, Mori L, Pirovano S, Soresina A, Meini A, Imberti L, Notarangelo LD, Plebani A, Badolato R. 2006. Combined decrease of defined B and T cell subsets in a group of

common variable immunodeficiency patients. *Clin Immunol* 121: 203-14

358. Yu GP, Chiang D, Song SJ, Hoyte EG, Huang J, Vanishsarn C, Nadeau KC. 2009. Regulatory T cell dysfunction in subjects with common variable immunodeficiency complicated by autoimmune disease. *Clin Immunol* 131: 240-53
359. Horn J, Manguiat A, Berglund LJ, Knerr V, Tahami F, Grimbacher B, Fulcher DA. 2009. Decrease in phenotypic regulatory T cells in subsets of patients with common variable immunodeficiency. *Clin Exp Immunol* 156: 446-54
360. Vitetta ES, Fernandez-Botran R, Myers CD, Sanders VM. 1989. Cellular interactions in the humoral immune response. *Adv Immunol* 45: 1-105
361. Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, Besra GS, Brenner MB. 2008. NK T cells provide lipid antigen-specific cognate help for B cells. *Proc Natl Acad Sci U S A* 105: 8339-44
362. Galli G, Pittoni P, Tonti E, Malzone C, Uematsu Y, Tortoli M, Maione D, Volpini G, Finco O, Nuti S, Tavarini S, Dellabona P, Rappuoli R, Casorati G, Abrignani S. 2007. Invariant NKT cells sustain specific B cell responses and memory. *Proc Natl Acad Sci U S A* 104: 3984-9
363. Tonti E, Galli G, Malzone C, Abrignani S, Casorati G, Dellabona P. 2009. NKT-cell help to B lymphocytes can occur independently of cognate interaction. *Blood* 113: 370-6
364. Marsh RA, Villanueva J, Kim MO, Zhang K, Marmer D, Risma KA, Jordan MB, Bleesing JJ, Filipovich AH. 2009. Patients with X-linked lymphoproliferative disease due to BIRC4 mutation have normal invariant natural killer T-cell populations. *Clin Immunol* 132: 116-23
365. Janeway CA, Apt L, Gitlin D. 1953. Agammaglobulinemia. *Trans Assoc Am Physicians* 66: 200-2
366. Spickett GP, Farrant J, North ME, Zhang JG, Morgan L, Webster AD. 1997. Common variable immunodeficiency: how many diseases? *Immunol Today* 18: 325-8

367. Chakravarti S, Godfrey DI. 2010. Directing traffic on the NKT-cell highway: A key role for ThPOK. *Eur J Immunol* 40: 2372-5

368. Wang L, Carr T, Xiong Y, Wildt KF, Zhu J, Feigenbaum L, Bendelac A, Bosselut R. 2010. The sequential activity of Gata3 and Thpok is required for the differentiation of CD1d-restricted CD4(+) NKT cells. *Eur J Immunol* 40: 2385-90

369. Wang L, Wildt KF, Zhu J, Zhang X, Feigenbaum L, Tessarollo L, Paul WE, Fowlkes BJ, Bosselut R. 2008. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. *Nat Immunol* 9: 1122-30

370. Patterson S, Chaidos A, Neville DC, Poggi A, Butters TD, Roberts IA, Karadimitris A. 2008. Human invariant NKT cells display alloreactivity instructed by invariant TCR-CD1d interaction and killer Ig receptors. *J Immunol* 181: 3268-76

Appendix

Number of iNKT cells	CD4:CD8 ratio in conventional Vβ11 T cells	CD4:CD8 ratio in conventional Vα 24 T cells	%CD4 Conventional Vβ 11T cells	%CD4 Conventional Vα24 T cells
1	3.6	6.8	63.4	66.9
1	1.9	4.2	54.5	41.2
3	2.6	3.9	38.7	57
4	1.1	2.3	50.6	54.9
4	8.4	21.9	87.4	94
7	3.2	10.2	76.2	89
19	3.7	4.4	55.3	56.4
19	8.5	17.7	89.3	93.6
22	7.4	19.7	85.2	86.7
23	3.0	2.1	71.4	65.9
25	1.6	10.1	42.9	18.2
32	5.7	14.0	80	80
35	3.2	5.3	73.4	80.6
37	1.3	1.1	49.3	40.9
39	4.0	5.0	75	79.5
43	4.1	2.8	78	70.4
44	1.9	2.6	63.9	70.2

45	1.4	0.9	44	21.5
53	2.6	3.1	68.6	63.4
57	4.1	5.6	76.9	78.7
87	0.6	4.0	36.7	56.7
89	3.6	3.4	71.4	69.8
99	2.0	3.3	62.3	68.9
106	4.5	3.1	79	76.4
123	2.8	3.5	62.2	56.6
128	1.5	1.6	33.4	23.6
161	2.3	4.4	61.9	67.6
163	2.8	11.9	56.6	33.3
192	2.2	3.3	62.8	55.6
198	8.1	7.3	81.1	58.9
213	3.9	5.3	75.7	72.5
221	1.9	1.3	47.1	27.2
235	1.9	2.8	51.4	54.8
245	1.2	7.1	53.7	83.3
275	0.4	1.1	25.2	36.6
283	2.1	2.7	45.4	39
345	3.9	5.6	61.8	59.6
379	2.0	2.1	35.5	22

406	3.6	4.3	31.6	17.9
476	3.0	0.9	41.9	13.8
646	0.7	3.2	27.5	29.7
646	2.1	2.1	36.3	32.9
659	1.2	1.7	25.2	23.8
669	4.1	10.7	62.9	63.9
692	1.5	1.9	39.3	29.2
1110	2.1	2.2	36.1	33.6
1329	1.8	2.2	43.1	42.5

Table 1 CD4/CD8 analysis on Vα24 and Vβ11