Characterisation of the immunomodulatory properties of human natural killer T cells

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Invariant natural killer (iNKT) cells are a small subset of innate cells which express an invariant T cell receptor and NK surface molecules and recognize glycolipid antigen presented by CD1. iNKT cells have shown potent anti-tumour properties when stimulated with the iNKT cell agonist α-galactosylceramide (αGalCer) in murine models. However iNKT cell based immunotherapies have proved disappointing in phase I and II clinical trials to date. Humans have a wider repertoire of CD1-restricted T cells compared to mice. These CD1 restricted T cells are poorly defined in healthy human blood. In this study we investigated the reactivity of CD1 in human blood, we identified in healthy donors the majority of CD1 reactive T cells were identified as iNKT cells. We optimized a method to readily isolate and expand iNKT from human peripheral blood.

iNKT stimulated with synthetic analogues of αGalCer have shown the potential as tailored adjuvants for dendritic cell (DC) based immunotherapy. We established a system to test the immunostimulatory ability of iNKT cell analogues on DC maturation and function. A novel αGalCer analogue α-S-GalCer stimulated iNKT cell line to produce a Th1 biased cytokine response and lyse tumour cells. α-S-GalCer stimulated iNKT cells also induced maturation of monocyte derived DC, and stimulated DC to produce Th1 biased cytokines. Furthermore DC matured by α-S-GalCer stimulated iNKT cells proliferated allogeneic T cell and induced Th1 cytokine production by the same.

This data shows that the novel iNKT agonist α-S-GalCer stimulates iNKT cell lines in a Th1 biased fashion and induces DC maturation. Results suggests that α-S-GalCer may possibly be an attractive adjuvant for iNKT/DC based cell immunotherapy for malignancies and viruses.

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ABBREVIATIONS

Abbreviation	Description
ºC	Degrees Centigrade
αGalCer	Alpha Galactosylceramide
^{51}Cr	^{51}Chromium
aa	Amino Acid
Abs	Absorbance
Ag	Antigen
AO	Acridine Orange
APC	Antigen Presenting Cell
BCR	B Cell Receptor
BM	Bone Marrow
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CFSE	5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester
Cpm	Counts Per Minute
CSF	Colony Stimulating Factor
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DN	Double negative
DNA	Deoxyribonucleic Acid
E/T	Effector/Target Ratio
EB	Ethidium Bromide
EDTA	Ethylenediaminetetra-Acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum

Abbreviations

FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HS	Human Serum
iDC	Immature Dendritic Cell
iGb3	Isoglobotrihexosylceramide
IL	Interleukin
iNKT	Invariant Natural Killer T cell
KO	Knock Out
mDC	Mature Dendritic cell
MHC	Major Histocompatibility Complex
NK	Natural Killer Cell
NKT	Natural Killer T cell
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PHA	Phytohaemaglutinin
PMA	Phorbol Myristate Acetate
Rh	Recombinant
RPMI	Roswell Park Memorial Institute
TCM	T cell Medium
TCR	T cell Receptor
TGF	Transforming Growth Factor
Th1	T Helper 1
Th2	T Helper 2
Th17	T Helper 17
TNF	Tumour Necrosis Factor

Abbreviations

Treg   Regulatory T cell

1.1 Overview of innate and adaptive immunity

The human body has evolved a highly complex group of defence mechanisms that are collectively termed the immune system. The function of the immune system is to protect the host from a vast array of micro-organisms such as viruses, bacteria, fungi, large parasitic eukaryotes known generally as parasites, and cancer. Classically the immune system has been divided into two separate but interlinked sections; the innate and adaptive immune systems. The innate immune system recognizes conserved components of pathogens using germ-line encoded receptors and responds rapidly. The adaptive immune system discriminates individual antigens using rearranged antigen receptors and elicits a specific response resulting in memory. In no way is this separation of the two systems literal. The innate and adaptive arms coexist and in many ways are complementary to each other, each relying on many shared response mediators (Rouse & Sehrawat, 2010, Einsenbarth & Flavell, 2009).

The immune system provides defence on a number of levels and is composed of a number of important organs and several different cell types. All cellular components of the blood including the cells of the immune system develop from pluripotent haematopoietic stem cells in the bone marrow. White blood cell or leukocyte production proceeds along two main pathways of differentiation. The myeloid lineage produces polymorphonuclear leucocytes and monocytes as well as other elements of the blood such as erythrocytes and platelets. The lymphoid lineage develops from a common lymphoid progenitor stem cell and gives rise to the lymphocytes; T lymphocytes, B lymphocytes, and natural killer cells (NK cells).

1.2 Pathogen recognition by the innate immune system

The outstanding feature of innate immunity is its direct recognition of molecular structures that signify danger. These include conserved motifs on microbes known as pattern associated molecular patterns (PAMPs) and host molecules which are induced or released in response to infection, damage or tumour transformation. Cells of the innate immune system use a restricted set of germ-line-encoded pattern recognition receptors (PRRs) such as Toll-like receptors (TLR), scavenger receptors, collectins & ficolins, RIG I like receptors (RLRs) and proteins containing nucleotide oligomerization domains (NOD) (Einsenbarth & Flavell, 2009). These receptors recognize PAMPs but recognition is not pathogen specific. Unlike the adaptive immune response, activation of innate cells is immediate and of relatively short duration. There is no immunological memory associated with the innate immune response (Pichlmair & Reise de Sousa, 2007, Iwasaki & Medzhitov, 2004).

Collectins and ficolins are C-type lectin containing molecules which recognise a variety of PAMPs and act as opsonins, ultimately leading to complement assisted phagocytosis (Holmskov *et al,* 2003). Scavenger receptors such as mannose receptor allow phagocytes to bind directly to microbes; they consist of a similar C-type lectin domain to that found in collectins & ficolins. Mannose receptors recognise mannan in bacterial cell walls and are expressed by mononuclear phagocytes. NOD like receptors (NLRs) are intracellular receptors for PAMPs such as microbial wall degradation products. Ligation of these receptors induces the production of pro-inflammatory cytokines from cells such as the macrophage.

The TLR family of receptors are a group of PRRs which upon binding by a PAMP can activate the innate immune response. Transmembrane protein Toll was first identified in studies examining development in the fruit fly, *Drosophila melanogaster*. It was noted that mutants lacking Toll were very susceptible to infection by gram positive bacteria and fungi (Lemaitre *et al*, 1996). Subsequent studies identified the presence of Toll like receptors in mammals. There are at least 11 identified TLRs to date in humans (Garantziotis *et al* 2008). Cellular expression of TLR repertoires varies and TLRs can be surface expressed (TLR 1, 2, 4, 5, 6 and 11) or intracellular (TLR 3, 7, 8 and 9). TLR expression levels can be modulated by inflammatory stimuli (interferons). The ligands recognised by TLRs range from lipopeptides (TLR 1, 2 and 6), double stranded RNA (TLR 3), lipopolysaccaride (TLR 4), flagellin (TLR 5), single stranded RNA (TLR 7 & 8), CpG (TLR 9), prokaryotic DNA (TLR 10) to profiling like protein (TLR 11) (Table 1). Upon ligation, signaling through adaptor molecules and expression of transcription factors such as NFκB leads to cellular activation (O'Neill & Bowie, 2007) (Figure 1.1).

Table 1: Human TLRs and their respective PAMPs		
TLR	**PAMP**	**Microorganism**
TLR1	TLR2 co-factor	*Neisseria, Borrelia, Mycobacterium*
TLR2	lipoprotein, peptidoglycan, HSP60	Gram-positive, *Mycobacterium,* spirochetes, mycoplasm
TLR3	dsRNA	viruses
TLR4	endotoxin (lipopolysaccharide)	Gram-negative and respiratory syncytial virus
TLR5	flagellin	bacteria
TLR6	peptidoglycan and zymosan	fungi
TLR7	imidazoquinoline in vitro	unknown in vivo
TLR8	imidazoquinoline in vitro	unknown in vivo
TLR9	bacterial DNA (unmethylated CpG motif)	bacteria
TLR10	prokaryotic DNA	bacteria
TLR11	profilin-like protein	*Toxoplasma gondii*, uropathogenic *E. coli*

Table 1: Adapted from Garantziotis *et al* 2008.

Human Toll like receptors are transmembrane proteins characterised by multiple copies of a leucine rich motif in their extracellular domains and a shared TIR (Toll IL-1 receptor) motif in their cytoplasmic domain (O'Neill & Bowie, 2007). Cell activation via the transcription factor NFκB is initiated by the TIR domain. Upon TLR4 ligation, a conformational change occurs in the TIR domain allowing it to bind one of a family of five adaptor proteins which also contain TIR domains. This results in the activation of kinases and transcription factors, for example NFκB, is liberated from its inhibitor IκB and NFκB is rendered free to translocate to the nucleus (O'Neill & Bowie 2007). TLR4 signalling is detailed as an example of TLR signalling due to the presence of this pathway in dendritic cell (DCs) activation, detailed in later chapters (Figure 1.2). TLR4 signalling utilizes the MyD88, TRIF, TRAM or Mal dependant pathway, which recruits IRAK-4 and TRAF-6, leading to the activation of TAK1. Activated transforming

growth factor-β kinase 1 (TAK1) activates the IKK complex which catalyses IκBs. IκBs are degraded allowing translocation of NFκB in the nucleus and the transcription of innate immune response genes such as those encoding pro-inflammatory cytokines. This is one of several TLR-4 pathways (Kawai and Akira, 2006).
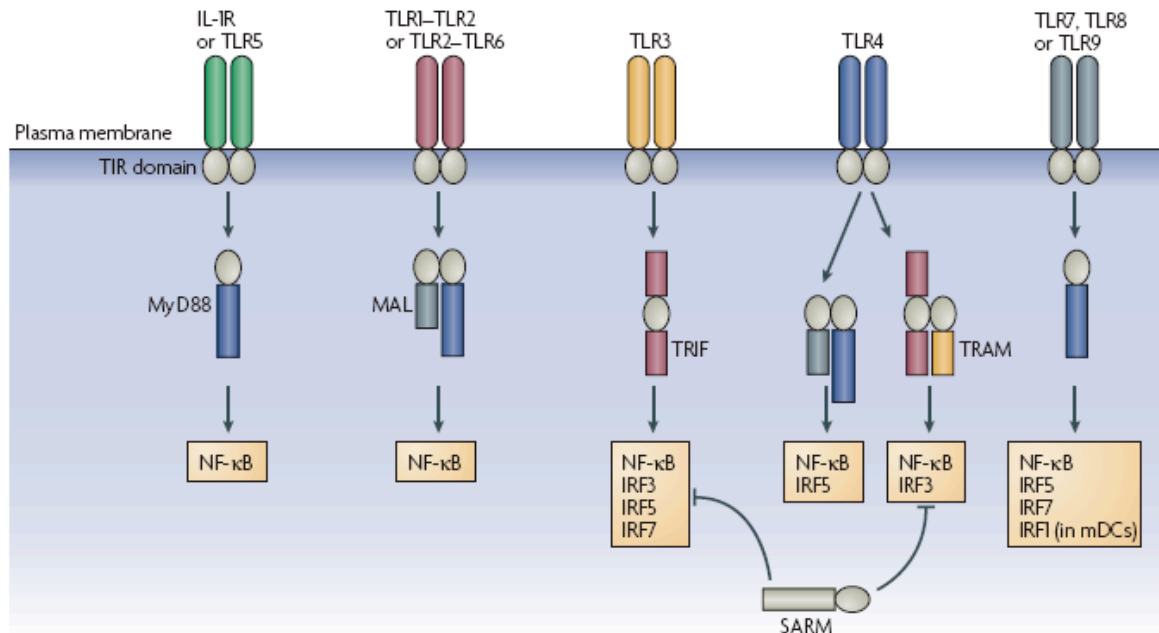
Figure 1.1: The TLR signalling pathways. Schematic outlining nine TLR signalling pathways. Diagrams shows pathways utilized by surface expressed TLRs 1, 2, 4, 5 and 6 and intracellular TLRs 3, 7, 8 and 9. Reproduced from Bowie & O'Neill, Nat Imm 2007.
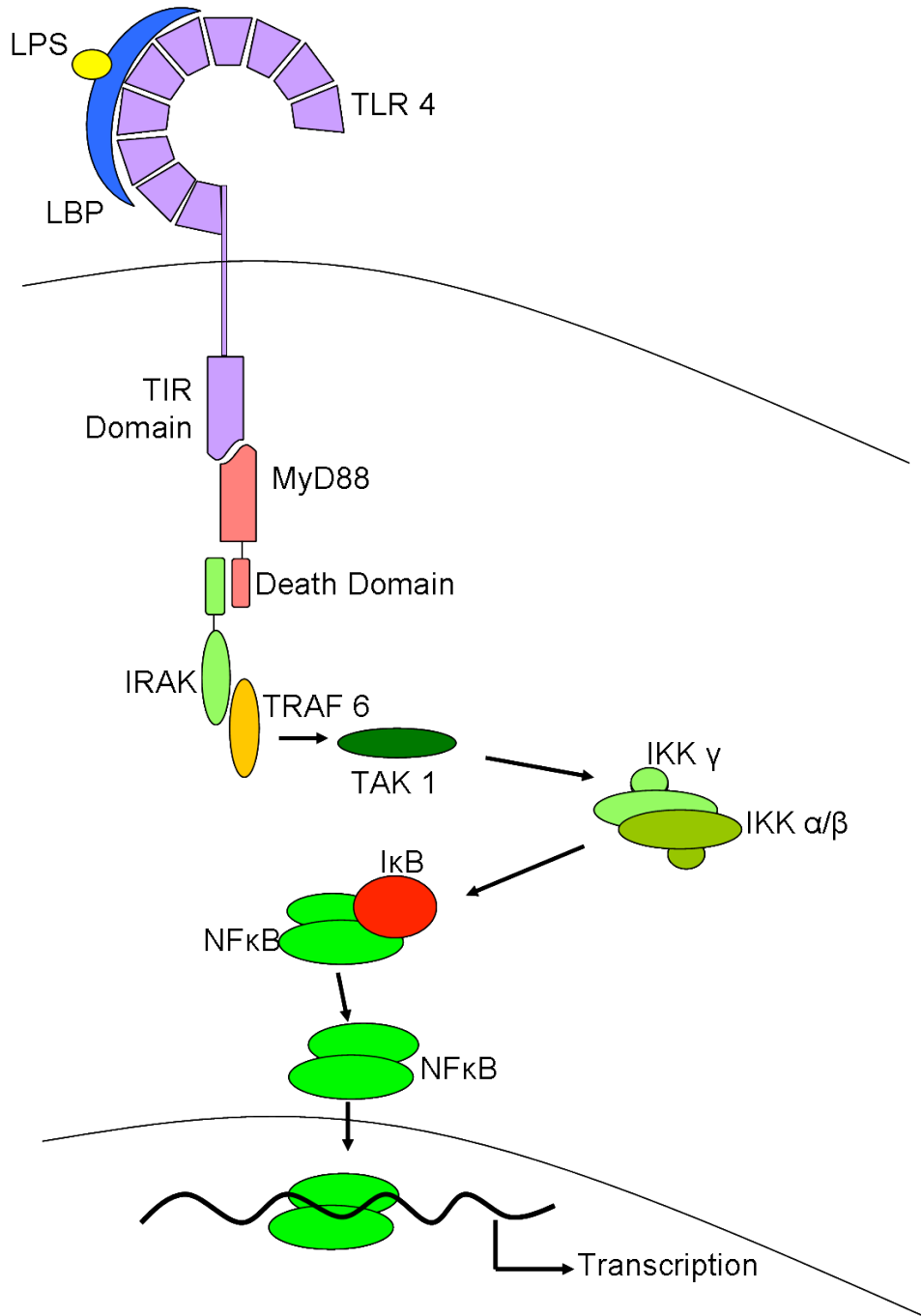
Figure 1.2: The TLR 4 signalling pathway. TLR4 utilizes the MyD88 dependant pathway, which recruits IRAK-4 and TRAF-6, leading to the activation of TAK1. Activated transforming growth factor-β kinase 1 (TAK1) activates IKK complex which catalyses IκBs. IκBs are degraded allowing translocation of NFκB in the nucleus. Adapted from Kawai and Akira, 2006.

1.3 Physical barriers and defensins

The first line of defence is provided by a physical barrier to the outside world and invading micro-organisms provided by the epithelia. Epithelial cells are held together in tight junctions which effectively form a tight seal against the external environment. Epithelia make up the skin, and the linings of the gastrointestinal, respiratory and urogenital tracts. The internal epithelia are known as the mucosal epithelia, due to their secretion of a thick viscous fluid called mucus. This mucus contains many glycoproteins called mucins. Mucus lines the epithelia and prevents micro-organisms binding to the surfaces. Mucus also aids in the expulsion of invading micro-organisms via the mucus flow driven by the beating of the epithelial cilia. Epithelial cells also produce several microbicidal chemicals such as histatins, cryptdins and defensins. Defensins are secreted in the small intestine in the form of α-defensins, and in the respiratory, urogenital tracts and skin in the form of β-defensins. Defensins' mode of function is cationic which disrupts the cell walls of invading micro-organisms (Metz-Boutigue *et al,* 2010).

1.4 Effector cells of the innate immune system

The principle cells of the innate immune system include cytotoxic lymphocytes (NK cells and some T cells), toxin releasing cells of myeloid origin such as mast cells, basophils and eosinophils, and the phagocytic cells of the monocyte/macrophage lineage, neutrophils and myeloid dendritic cells (DCs).

1.4.1 Phagocytes - Neutrophils and Macrophages

Macrophages are long lived tissue resident phagocytes that mature from circulating monocytes before migrating to tissues. In contrast, neutrophils are released from bone marrow in response to infection and abundantly found in the blood.

1.4.2 Eosinophils, basophils and mast cells

Eosinophils and basophils have noted importance in defence against parasitic infection and are recruited to sites of allergic inflammation. Tissue resident mast cells at these sites release many chemical mediators, triggering local inflammation and blood vessel dilation in response to allergens.

1.4.3 Natural killer cells

NK cells are bone marrow derived lymphocytes which share a common progenitor with T cells. They play key roles in the recognition and destruction of virus infected and tumour cells, and in early cytokine secretion (Lanier 1998). NK cells are potent cytotoxic effector cells, and have been shown to lyse target cells *in vitro* and *in vivo*. NK cells also rapidly produce cytokines (e.g IFN-γ) which promote Th1 biased immune responses. Various stimuli are required to activate NK cell functions and this process is tightly regulated by inhibitory receptors and cytokines that control and potentially terminate the NK response. NK function is therefore regulated by a balance of signals which are stimulatory or inhibitory. Virus infected and tumour cells are identified by changes in MHC class I expression and NK cells can recognise and kill cells with altered MHC class I expression (Karre *et al* 1986), (Ljunggren & Karre, 1990).

NK cells have several families of receptors which provide the activatory/inhibitory signals that control NK function. NK1.1 is a member of C-type lectin superfamily found on the surface of murine NK cells. Ligation of NK1.1 activates cytokine production and cytotoxicity by some NK cells. Murine NK cells also express the Ly49 family of receptors which recognise MHC class I (human homologue called HLA) and activate or inhibit NK cell cytotoxicity. Another stimulatory/inhibitory receptor found on murine and also human NK cells is the CD94/NKG2 receptor complex which recognise the non-classical MHC class I molecule HLA-E. Mice and humans have four NKG2 receptors which can recognise HLA class I molecules and some associate with CD94 which acts as a chaperone for the surface expression of NKG2A and NKG2C. NKG2D and NKG2C have been reported to have stimulatory function, whereas the CD94/NKG2A complex is inhibitory (Lanier, 1998).

Killer cell immunoglobulin like cell receptor (KIRs) genes encode glycoproteins of the Ig superfamily which bind HLA class I ligands in humans. There are 2 subsets of KIRs based on the number of domains in the extracellular region. KIR3D and KIR2D have 3 and 2 domains respectively. The intracellular cytoplasmic tail can be long or short (KIR3DL or KIR3DS) and signals through these resulting in inhibition or stimulation, respectively, of the NK cell (Lanier, 1998).

1.4.5 Dendritic cells

Dendritic cells (DCs) are professional antigen presentation cells (APC) and unique in their ability to induce a primary immune response resulting in immunological memory (Figure 1.4) (Banchereau & Steinman, 1998). DCs are considered the professional antigen presenting cells, because they possess the ability to carry a large antigen load. This ability to capture and process large quantities of antigen is paired with low rates of antigen degradation. DCs can induce qualitatively different adaptive immune responses (Palucka *et al*, 2007).
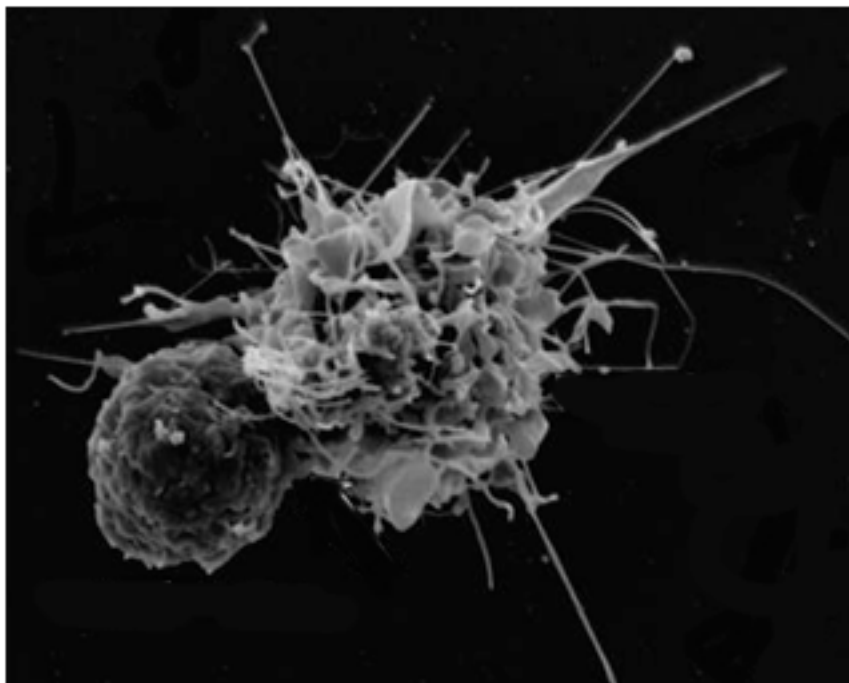
Figure 1.4: Dendritic Cell: Electron microscope picture showing a mature dendritic cell interacting with a lymphocyte. Reproduced from University of California, San Diego.

Several subsets of DC exist in humans; plasmacytoid, thymic, LN resident, skin resident Langerhans cells and myeloid DCs (the focus of this work). Plasmacytoid DC arise from a lymphoid origin and are CD14 negative CD11c negative cells. They express intracellular TLRs 7 and 9 and are potent producers of type I interferons. DC progenitors are found in bone marrow and give rise to blood circulating precursors which home to the tissues where they reside as immature DCs (iDC) with high phagocytic capacities. Chemokines at sites of inflammation draw tissue resident iDC to the site of antigen (Ag) deposition. The dendritic cell proceeds to capture antigen by one of several methods; phagocytosis, receptor mediated endocytosis or macropinocytosis. PRR ligation and antigen capture by the iDC induces maturation of the cell; this involves the phenotypic and functional change from tissue resident Ag capture cell to APC (Banchereau & Steinman, 1998).

1.5 Effector cells of the adaptive immune system

The adaptive immune response is mediated by T and B lymphocytes that express antigen specific receptors and display immunological memory. T lymphocytes and B lymphocytes derive from the bone marrow and undergo genetic rearrangement during development in order to select cells with diversity of receptors that are specific for antigen. T lymphocytes are distinguished from B lymphocytes firstly by their place of maturation during development. Both types originate in the bone marrow but T lymphocytes mature in the thymus where they are differentiate into $CD4^+$ and $CD8^+$ cells and are selected on the basis of their ability to recognize self HLA and to remain unreactive with self antigen. B cells on the other hand mature in the bone marrow and are stimulated by T cells, specifically $CD4^+$ T helper cells to proliferate and secrete antibodies. Self-reactive B and T cells, die by apoptosis and thereby the body prevents the development of autoimmune disorders.

In the event that a pathogen or tumour successfully evades the innate immune defences, inflammation is not resolved and an adaptive immune response is initiated. Professional antigen presenting cells (APCs) such as dendritic cells are the important link between the innate and adaptive immune systems. Upon presentation of foreign antigen to complementary antigen receptors on T and B lymphocytes, they initiate clonal expansion and differentiation into effector T and B cells, the beginning of an adaptive immune response.

Activation or priming of the adaptive immune response is slow to develop but once activated infection is usually resolved and a state of immunological memory is established. This memory may last a lifetime and is the result of the pre-existence of populations of clonally expanded antigen specific lymphocytes known as immune memory effector cells. Immunological memory along with antigen specificity is a defining feature of the adaptive immune response.

1.5.5 B cells

B cells express an antigen specific receptor on their surface, the B cell receptor (BCR). The BCR is a immunoglobulin, composed of 2 identical light chains and 2 identical heavy chains, the genes specifying them are found in the 'V' (variable) region and the 'C' (constant) region. In the heavy-chain 'V' region there are three segments; V, D and J, which recombine randomly, giving a massive repertoire of unique BCRs (Tonegawa *et al,* 1983).

Upon binding of BCR to specific antigen, the B cell becomes activated and with signals from the T helper cell (Th cell) differentiates into an antibody secreting plasma cells (Reth & Wienands, 1997). The secreted antibodies are truncated BCRs with the same antigen specificity and function by neutralizing, opsonisation of targets for removal by phagocytes and activation of the complement system. B cells also function as antigen processing and presenting cells, although not as efficiently as the "professional" APC; the dendritic cell.

1.5.6   T cells

T cells function by killing virus infected or tumour cells, and activating or regulating other cells of the immune system via the secretion of cytokines. There are several forms of T cells; cytotoxic, helper and regulatory, all express a cell surface T cell receptor which is specific for antigen. Classical T cells can be divided into 2 separate groups depending on the accessory molecules expressed; CD4 T helper cells and CD8 cytotoxic T cells. CD4 T helper cells recognize MHC II (HLA in humans) molecules and have been divided into several groups including Th1, Th2, Tregs, Th17 cells. Th1 cells are mainly involved in immunity against intracellular pathogens and tumors, and secrete IFN-γ and IL-2 amongst others. Th1 cells can also induce the production of antibody involved in macrophage activation. Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 and are mainly involved in B cell, neutralizing antibody, mast cell and eosinophil opsonisation immune responses. Regulatory T cells were the third defined subset of T cells described; the main populations are CD4+ cells which also express CD25 and the transcriptional regulator FOX-P3. As the name suggests they have a regulatory role and are associated with IL-10 and TGF-β secretion which inhibits the classical Th1 and Th2 profiles (Bluestone, 2009).

From 2003 to 2005 several groups reported a new lineage of T cells defined by the production of IL-17 (Aggarwal *et al*, 2003, Langrish *et al*, 2005, Park *et al*, 2005). This Th17 T cell subset was characterized in many studies as being CD4+ T cells which produce IL-17A (a member of the IL-17 cytokine family). The factors required

for Th17 polarization were identified as IL-6 and TGF-β with IL-21, with IL-23 needed for expanding and sustaining Th17 T cells.

1.5.7   Cytokines

Interferons are well known for their anti-viral, anti-proliferative and immunomodulatory functions. IFN-γ is the classical Th1 cytokine and has antiviral, immunoregulatory, and anti-tumour properties. Amongst the effects are: suppression of Th2 cell differentiation, the upregulation of MHC class II expression, increased antigen presentation and lysosome activity of macrophages, and the activation of NK cells. Interleukins 4 and 13 are described as Th2 cytokines and promote proliferation and development of B cells and IgE class switching during parasitic infection. It also leads to the upregulation of MHC class II. IL-10 is classed as a regulatory cytokine which has anti inflammatory functions. IL-10 mainly secreted by monocytes, DCs and regulatory T cells. IL-17 produced by Th17 cells induces the release of chemotactic factors for neutrophils and inflammatory mediators. IL-17 is highly pathogenic during the inflammation process, with opposite effects to those of the developmentally linked regulatory T cells (Awasthi & Kuchroo, 2009).

1.5.4 Antigen recognition by T cells

The TCR is a hetrodimer composed of 2 transmembrane glycoprotein chains. Both TCR chains have 'V' (variable) region which interacts with antigen, a 'C' (constant) region and a short hinge region, each chain has transmembrane domain. The vast TCR repertoire is made using VDJ recombination; as a result each TCR is specific for a

unique Ag. The concentration of TCR diversity occurs in both the Vα and Vβ CDR3 loops. Structural data confirms that the CDR3 loops sit largely over the centre of the antigenic peptides (Davis *et al,* 1998*)*. The transmembrane domain, has positive charges and these play important roles in the association with the invariant signaling molecule CD3 (Clevers *et al,* 1988). CD3 is composed of 3 chains; CD3γ, CD3δ, and CD3ε which form the CD3 complex and associate with ζ chain which is disulfide-linked homodimer (Janeway *et al*, 2008). The ζ chain contains 3 immunoreceptor tyrosine-based activation motif (ITAM) regions and function in signal transduction and activation of signaling kinases (Samelson & Klausner,1992).

TCRs respond to major histocompatibility complex (MHC) proteins loaded with peptide fragments of protein Ag. The loading of peptide antigen onto MHC molecules has been shown to be a highly regulated process, MHC class I (York & Rock,1996) and MHC class II (Cresswell, 1997) molecules use different pathways for the processing and loading of antigen. TAP and tapasin facilitate the loading of cytosolic peptide onto MHC class I in the endoplasmic reticulum (ER). Invariant chain (Ii) and HLA-DM catalyze the loading of endogenously processed peptide derivatives from extracellular antigen onto MHC class II molecules in lysosome like multivesicular compartments (MIIC). To stabilize the interaction between the TCR and antigen loaded MHC molecules, T cells have accessory molecules, CD4 and CD8 and depending which molecule is expressed determines cell effector functions. Both molecules bind sites on the MHC molecule and are required for effective response. CD4 is a single chain molecule composed of four immunoglobulin like domains. The intracellular portion of

CD4 is linked to signaling kinase (discussed later). CD4 recognizes MHC class II molecules and is expressed by T helper set of T cells. CD8 is a disulfide linked dimer, composed of an α and a β immunoglobulin like domain expressed by cytotoxic T cells (Figure 1.3). CD8 recognizes MHC class I molecules. The intracellular portion of CD8 is linked to the same signaling kinase as CD4.
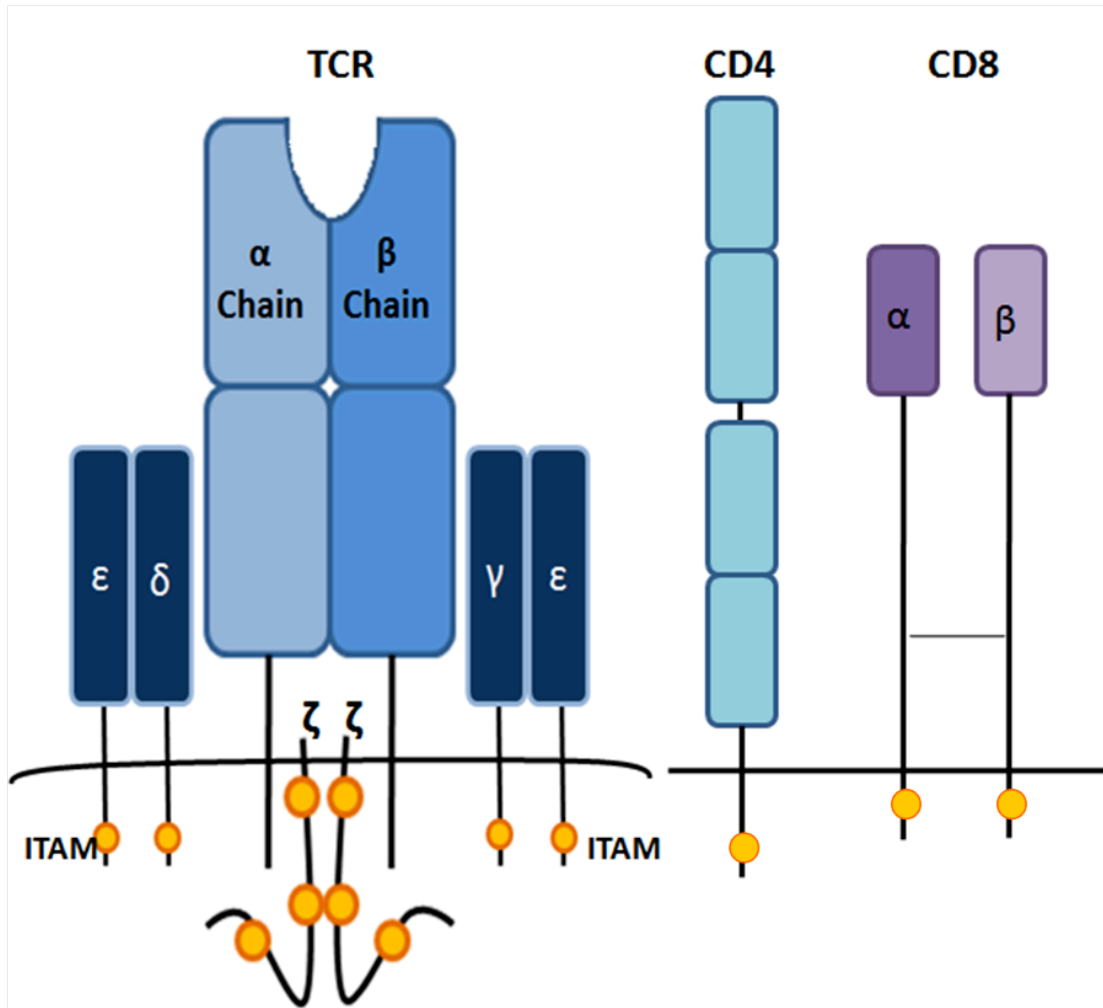
Figure 1.3: The T cell receptor complex: Diagrammatic representation of the TCR and accessory molecules CD3, CD4 & CD8. CD3 has extracellular domains (rectangles) and 2 transmembrane ζ domains. Immunoreceptor tyrosine based activating motifs (ITAMs) are denoted in yellow. CD4 has 4 extracellular domains, whilst CD8 has 2 extracellular domains.

1.6 Initiation of adaptive immune responses

1.6.1 Dendritic cell maturation

Maturation of DCs is associated with several co-ordinated events including; the loss of endocytic/phagocytic receptors and the upregulation of MHC and T & B cell co-stimulatory molecules such as CD40, CD80 and CD86 (Banchereau & Steinman, 1998). Maturation results in the changes in the Ag processing compartments such as MHC compartments (Cella *et al*, 1997). Maturation also results in the induction and upregulation of receptors for chemokines such as CCL-2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) which regulate DC trafficking to the lymphoid tissues and recruitment of DC progenitors. Maturation also results in chemokine secretion which recruits effector cells to the site of Ag capture (CCR7, CCL19, and CCL21) (Sozzani *et* al, 2005). Finally they undergo a change in morphology to a mature cell expressing the classical dendrite projections which earn the DC its name.

The maturing DC migrates via the lymphatics to the lymphoid organs where it presents and cross-presents antigen to specific CD8+ and CD4+ T cells via MHC class I & II molecules repectively. During migration, internalized capture antigen is directed to specific MHC compartments (Sallusto & Lanzavecchia, 1994) for processing and loading onto MHC molecules. These loaded MHC molecules are quickly trafficked to the cell surface and remain stable and ready for T cell interaction for days.

Mature dendritic cells (mDCs) express a unique repertoire of functional surface expressed molecules. Some are constitutively expressed on the immature DCs and are

up-regulated upon activation, others are absent on the iDC and expressed only on the mature form. DC maturation signals the change from immature antigen capturing cell to the mature antigen presenting form, and begins with the upregulation of MHC class I and II expression. The MHC molecules present antigen on the dendritic cell surface to T helper cells. This interaction provides "signal 1" (TCR-MHC-Ag) to the effector T cell. The induction of co stimulatory molecule expression is the next step of the DC maturation process and co-stimulatory molecules are essential to fully activate a naïve T cell response. If the dendritic cell provides signal 1 but no signal 2 to the responding T cell, the T cell will go into a state of unresponsiveness known as anergy (Janeway *et al*, 2008). CD80 and CD86 are co stimulatory molecules found on DCs, which are also known as the B7 molecules. They bind to the same pair of receptors, CD28 and cytotoxic T lymphocyte antigen-4 (CTLA-4). CD28 is constitutively expressed on the surface of T cells whereas CTLA-4 is up-regulated following T cell activation. CTLA-4 has a 10-100 fold higher affinity for the B7 molecules. Signalling through CD28 results in T cell activation, in contrast signalling through CTLA-4 results in the inhibition of the T cell response (Greenwald *et al*, 2005, Alegre *et al*, 2001).

CD40 is a member of the TNF-receptor superfamily found on monocytes, dendritic cells and B cells. The binding of CD40L on T helper cells to CD40 activates antigen presenting cells and ultimately T helper responses. CD40 is essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation (Van Kooten & Banchereau, 2000). CD40 ligation results in the production

and secretion of IL-1, IL-6, IL-10 and IL-12 by DCs, and the upregulation of; CD54, CD80 and CD86 (Banchereau & Steinman, 1998). Interupting CD40-CD40L interaction during T cell and DC co-cultures *in vitro* results in reduction in T cell proliferation (Van Kooten & Banchereau, 2000).

Once a DC encounters an effector cell, adhesion molecules bind and result in cell to cell contact. CD54, also known as intracellular adhesion molecule 1 (ICAM-1), is an adhesion molecule found on most leukocytes including monocytes and DCs. During interaction between DCs and T cells, CD54 binds weakly to LFA-1 on the T cell, upon TCR signal the affinity increases and the contact is prolonged between the 2 cells. (Springer, 1990, Janeway *et al*, 2008).

CD83 is a member of the immunoglobulin (Ig) super-family with one V type Ig domain. It is expressed by human circulating and tissue resident DCs (Zhou & Tedder, 1995, Zhou *et al*, 1992). The function of CD83 is unclear and a number studies are aiming to elucidate a ligand (Prazma & Tedder, 2008). High density CD83 expression is however recognised as a marker of DC maturation and correlates with the upregulation of HLA class II expression (Zhou *et al*, 1992). Monocyte derived DC (generated using GM-CSF and IL-4) express low levels of CD83 on the cell surface but upon activation upregulate the protein (Zhou & Tedder, 1996).

1.6.2   DC based immunotherapy

The DC system is designed to shape the recognition repertoire of T cells, consisting of billions of different lymphocytes, each with distinct but randomly arranged antigen receptors. This repertoire in turn represents a virtually infinite 'drug library' for specific therapies that increase or decrease T cell function (Steinman & Banchereau, 2007). Due to this powerful ability of initiating specific immune responses, DCs have been targeted for immunotherapy (Figure 1.5).

As well as priming naïve T cells for maturation into effector cells, DCs also have regulatory role for antigen activated T cells. Tolergenic DCs induce regulatory CD4+ T cells, and are described as partial or semi matured DCs, whereas fully matured DC are immunogenic. The decisive signal is hypotheised to be the release of proinflammatory cytokines from DCs (Lutz & Schuler, 2002). These tolerogenic DCs have the ability to block or delete T cells by signalling through inhibitory receptors such as PD-1 and CTLA-4 interactions. If deletion is avoided the selected T cell clone undergoes differentiation and elicits a specific response (Probst *et al*, 2005).

The importance of DC in resistance to infection has been demonstrated in studies, where microbial antigens were injected in association with DC into mice, and the animals acquire adaptive immune responses specific to the antigen (Steinman & Banchereau, 2007). Vaccination of healthy volunteers with *ex-vivo* generated DCs that were pulsed with either the keyhole limpet hemocyanin protein, tetanus toxin or

influenza matrix protein, then subsequently activated, can lead to Ag specific immune responses (Dhodapkar *et al*, 1999, Dhodapkar *et al*, 2000).

One of the main targets of DC-based immunotherapy has been on tumour therapy (Figure 1.5). Tumours contain many potential antigens which can be loaded onto DCs with the aim to generate an adaptive immune response (Figdor *et al*, 2004, Banchereau & Palucka, 2005). Several approaches have been investigated; the *in-vivo* targeting of DC with antigen and adjuvant, anti-DC antibodies conjugated with antigen, and the *ex-vivo* generation of DC loaded with antigen and injected into patients (Palucka *et al*, 2007). Several studies have focused on the approach consisting of adoptive transfer of tumour antigen pulsed DCs, in a range of human cancers including colorectal cancer (Rains *et al*, 2001), melonoma (Palucka *et al*, 2006, Butterfield *et al*, 2008) and hepatocellular carcinoma (Palmer *et al*, 2009).

To date DC vaccinations for tumour therapy has been somewhat disappointing and resulted in a failure to generate anti-tumour immune responses due to T cell anergy or induction of specific response but without the regression of the tumour (6 trials are outlined in a review by Palucka *et al*, 2007). Three possible reasons offered for this include (1) the generation of a weak T cell responses, where tolerance or a non protective T cell response is initiated, (2) altered trafficking of T cells or (3) inhibition of T cell responses by elements in the tumour micro-enviroment such as vascular endothelial growth factor (VEG-F) and transforming growth factor beta (TGF-β), which inhibit DC maturation and function (Palucka *et al*, 2007, Steinman &

Banchereau, 2007). However the use of dendritic cells in conjunction with or as an adjuvant remains an attractive target for therapy especially in human cancers. This will be discussed in detail in section 1.11.
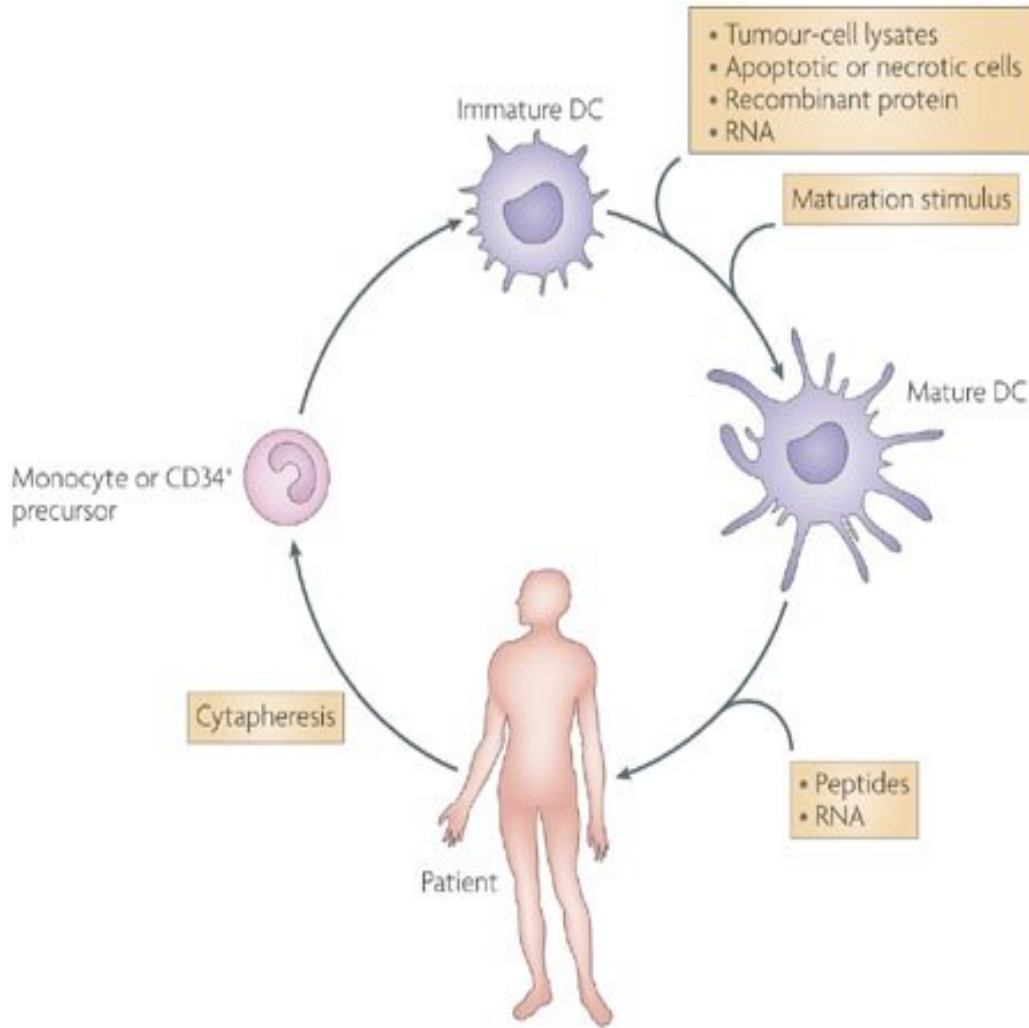
Figure 1.5: DC adoptive transfer strategy: *Schematic detailing DC adoptive transfer therapy. Monocytes are taken from patient and induced to differentiate into immature DC. The tumour lysate and maturation stimuli such as LPS, are cultured with the iDC, which is then infused back into the patient. Reproduced from Steinman & Banchereau Nat Imm 2008*

1.7   Innate T cells

Innate T cells are a small proportion of CD3+ T cells which are MHC (HLA) unrestricted and recognise conserved Ag. Innate like rapid activation is a defining feature of these subsets. The various subsets of innate T cells will be discussed in the forthcoming sections.

1.7.1   Mucosal associated invariant T cells

Mucosal associated invariant T cells (MAIT cells) are gut resident innate T cells. They express a semi invariant TCR (Vα19 Jα33 in mice and Vα7.2 Jα33 in humans) which recognises the monomorphic MHC class Ib molecule MR1. MAIT cells express NK cell surface markers such as CD161 and can produce multiple cytokines such as IFN-γ, IL-4, IL-5 and IL-10 (Godfrey, 2010, Lantz, 2010).

1.7.2 $\gamma\delta$ T cells

$\gamma\delta$ T cells are a subset of T cells which express a TCR composed of γ and δ chains as opposed to the conventional $\alpha\beta$ arrangement. They account for 1-5% of peripheral T cells and 25-60% of gut T cells (Hayday, 2000). $\gamma\delta$ T cells are innate like effector T cells which express a limited repertoire of TCRs but which include a subset of memory cells, a function not normally associated with innate immunity. Antigen recognition by the $\gamma\delta$ TCR seems to be direct or through a non MHC molecule, and it occurs in the tissue, as $\gamma\delta$ T cells are generally absent from the lymph nodes (Hayday, 2000). $\gamma\delta$ T cells are predominantly CD8+ in the tissues and double negative for CD4 and CD8 in

the periphery although a minority are CD4+. The CD8 molecule on γδ T cells is composed of a CD8α homodimer opposed to heterodimers found in most αβ T cells (Hayday, 2000).

Human γδ T cells can be broadly divided into 3 groups based on the Vδ chain selection. Vδ1 cells are mostly tissue resident and express a naïve phenotype. Studies into their function suggest a role in tumour surveillance (Eberl *et al* 2006). Vδ2 cells are unique to higher primates and account for the majority of peripheral γδ T cells. They are activated in an MHC-independent manner by non-peptide antigens such as phosphoantigens. This subset expands in response to bacteria and tumors and release cytokine (IFN-γ, TNF-α) and can kill bacteria and tumour cells. Vδ2 cells have also been observed to have characteristics of professional APC (Brandes *et al* 2005). Vδ3 cells are found mainly in the liver (Kenna *et al* 2004*)*. The exact function of Vδ3+ T cells is unknown but they are thought to recognize cytomegalovirus (Dechanet *et al* 1999).

1.7.3   Natural Killer T cells

Natural Killer T (NKT) cells are innate T cell subsets which express a TCR and NK cell receptors. NKT cells recognise glycolipid antigen presented by the MHC like molecules CD1.

1.7.4   CD1

CD1 molecules are a family of glycoproteins resembling major histocompatibility complex class 1 (MHC I) in overall structure, consisting of a transmembrane heavy chain composed of 3 domains; α1, α2 and α3 which associate with β_2-microglobulin (Gumperz Traffic 2006). The binding sites of CD1 molecules are deep hydrophobic pockets which bind lipids and glycolipids and present them to T cells (Zeng *et al* 1997) (Figure 1.6 A-C).

CD1 isotypes have been found in all mammals examined to date. Humans express 5 isoforms of CD1, while mice and rats have lost their CD1a, CD1b, CD1c and CD1e isoforms but have duplicated CD1d genes, CD1d1 and CD1d2 (Dascher & Brenner, 2003) (Figure 1.7). Studies in chickens led to the indentification of a CD1 orthologue which suggests CD1 is evolutionary ancient, predating the divergence of birds and mammals (Miller *et al* 2005, Salomonsen *et al*, 2005).
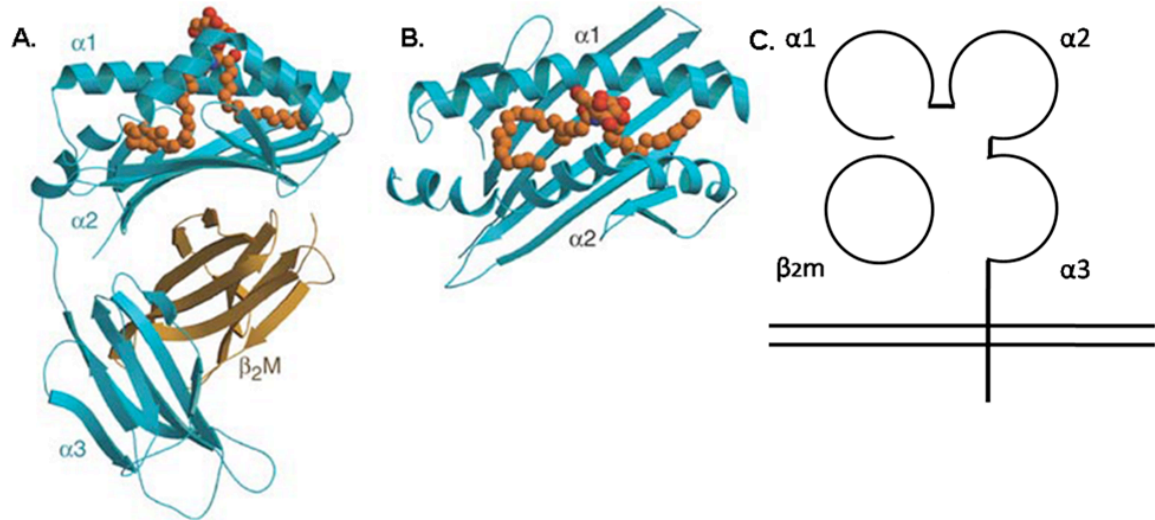
Figure 1.6: Structure of CD1: (A) *Crystal structure of the CD1d molecule with bound alpha galactoslyceramide (αGalCer) showing the 3 α domains (blue) paired with a β₂Microglobulin domain (Yellow).* **(B)** *Molecule and Lipid shown from the point of view of T cell (Koch et al, 2005).* **(C)** *Schematic showing the structure of a CD1 molecule, showing the 3 α domains paired with a β₂-microglobulin domain, and a transmembrane cytoplasmic tail. Redproduced from Gumperz 2006.*
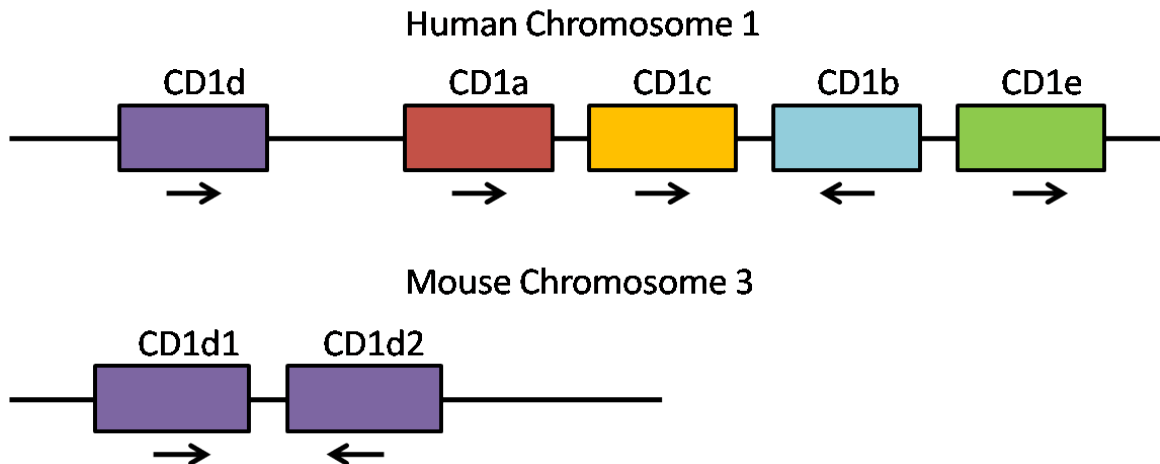
Figure 1.7: CD1 gene organisation in humans and mice: Schematic representation of the location and orientation of the 5 human CD1 genes; CD1a-e, on human chromosome 1 and the duplicated murine CD1d genes on mouse chromosome 3. The direction of transcription is indicated by arrows.

Human CD1a-d function in the presentation of lipid derived antigen to CD1 restricted T cells. CD1e differs in its location and function; being an intracellular chaperone molecule (Angenieux *et al* 2000). Human CD1 molecules are split, based on sequence homology, into 2 groups termed group 1 and 2. Group 1 CD1 molecules include CD1a, b and c whilst group 2 contains CD1d. Group 1 molecules are widely distributed, and are commonly used as markers for human DCs. Langerhans cells express CD1a and low levels of CD1c, but not CD1c or CD1d. Dermal DCs express CD1b and CD1d detected (Brigl & Brenner, 2004, Pena-Cruz *et al*, 2003, Gerlini *et al*, 2001). CD1c and CD1d are expressed by circulating B cells (Brigl & Brenner, 2004, Exley *et al*, 2000). CD1d is expressed on monocytes, iDC and mDCs at low but functional levels. The in-vivo generation of monocyte derived DC results in a reduction of CD1d expression but remain functional (Spada *et al*, 2000, Brigl & Brenner, 2004). CD1d is also found on epithelial, hepatocytes and some parenchymal cells (Bleicher *et al*, 1990).

1.7.5 Antigen processing and presentation by CD1

The intracellular pathway taken by a CD1 isoform will ultimately determine the lipid processing and loading. Studies into this processing and loading have shown the CD1 heavy chains are folded and assembled with β_2-microglobulin in the endoplasmic reticulum (Sugita *et al*, 1997), by a mechanism which is dependant on calnexin and calreticulin but independent of transporter associated with antigen processing (TAP) (Kang *et al,* 2002). From the ER the molecules are transported via the Golgi to the cell surface using the secretory pathway. The surface expressed molecules are then re-

internalised through clathrin-coated pits (Sugita *et al*, 1999) and the different isoforms undergo different intracellular trafficking and processing pathways (Dascher *et al*, 2002) as shown in figure 1.8.
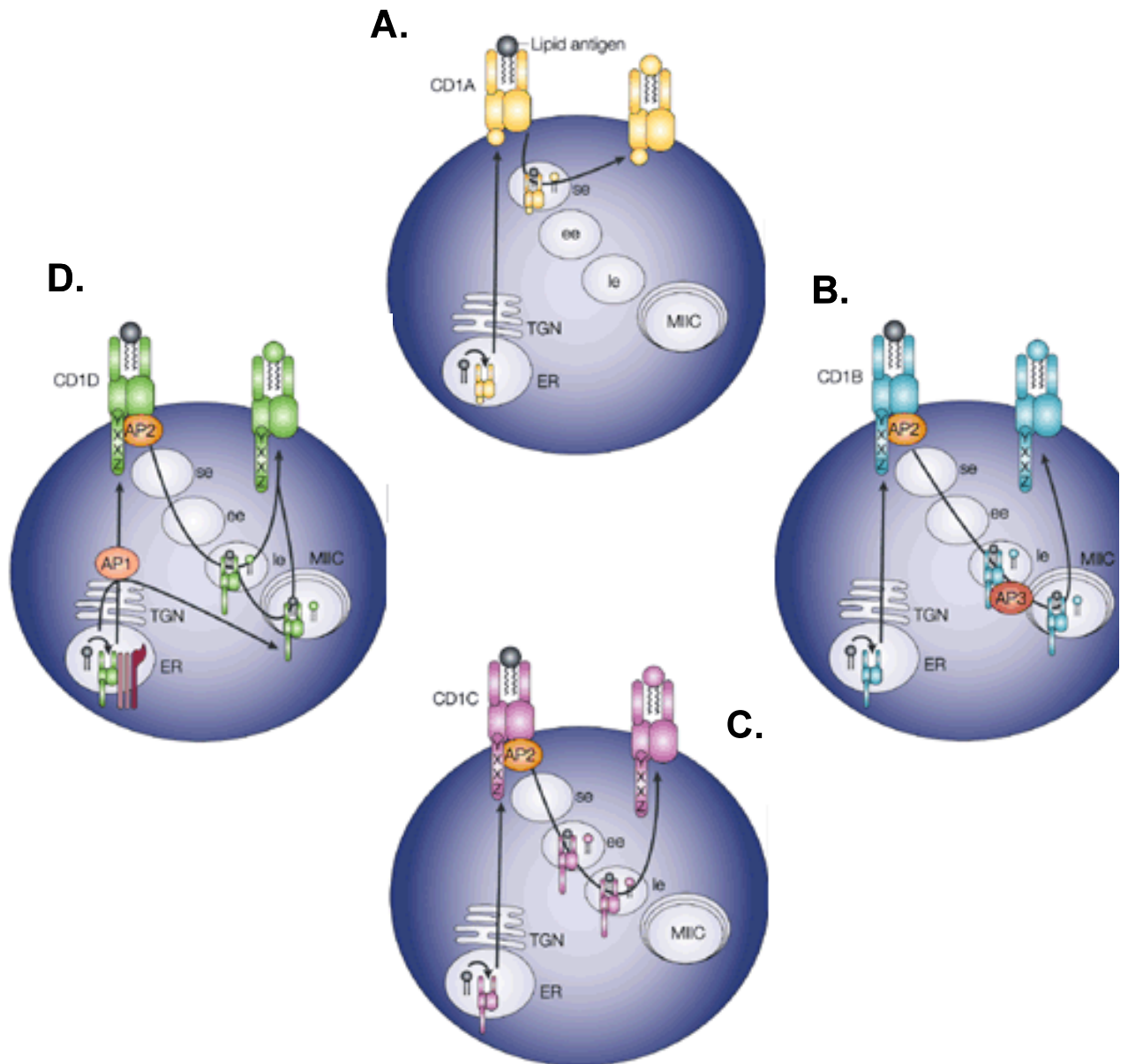
Figure 1.8: CD1 trafficking pathways: Schematic showing the different CD1 trafficking pathways. (A) CD1a is trafficked to the surface via the endoplasmic reticulum (ER) and recycled through the early endosome (se and ee). (B) CD1b is trafficked to the surface via the ER and recycles through the late endosome (le) and the MIIC compartments with the aid of the adaptor proteins (AP) 2 and 3. (C) CD1c traffics to the surface via the ER and recycles with the aid of AP2 through the ee and the le. (D) CD1d traffics with the aid of AP1 to the surface and subsequently traffics with the aid of AP2 through the le and MIIC compartments. (Reproduced from Brigl and Brenner, 2003)

The intracellular pathway selected appears to depend on the cytoplasmic amino acid sequence of CD1 (Sugita *et al*, 1996). The cytoplasmic tails of most isoforms contain tyrosine-based motifs of the pattern YXXZ, where Y is tyrosine, X is any amino acid and Z is a bulky hydrophobic amino acid (Sugita *et al*, 2004, Gumprez, 2006). CD1a is truncated and lacks this motif. CD1a finds its way to the early endosome, and subsequently traffics through the recycling endosome. The YXXZ motif of the other molecules bind adaptor proteins (AP). AP-2 directs the CD1b, CD1c and CD1d molecules to the early and late endosomes. CD1b associates with AP-3 and results in the molecule trafficking to the lysosomal compartment (Sugita *et al*, 2004). CD1b is the only human CD1 isoform which associates with AP-3 but a point to note is that murine CD1d also associates with AP-3 and can traffic to the lysosome. The endocytic compartments differ in pH resulting in differing antigen processing (Brenner, 2003). Studies into the trafficking of CD1 during DC maturation have shown CD1a, CD1b and CD1c localization doesn't change between the immature and mature form (Cao *et al*, 2002, van der Wel *et al*, 2003). Human CD1d unlike its murine homologue does not bind AP-3 so can not access the lysosomal compartments. However studies by Kang *et al* (2002) reported that human CD1d can sometime associate with MHC II molecules and end up in the lysosomal compartments. After DC maturation CD1d molecules may lose access to these compartments, resulting in altered presentation (Gumperz, 2006).

Understanding the processing of intracellular lipids onto CD1 molecules raises the question; how are extracellular glycolipids internalized and processed? Studies have shown that antigen presenting cells (APCs) such as DCs can internalize lipid antigen

using their mannose receptors, and these antigens are successfully delivered to the endosomal compartments for loading onto CD1b (Prigozy *et al*, 1997). Van der Elzen *et al* (2005) reported that apolipoprotein E (ApoE) is the serum factor that binds lipids and mediates their uptake by DCs through the lipoprotein receptor for presentation by CD1 molecules CD1b, CD1c and CD1d. This is a possible route for foreign lipid or extracellular self lipid uptake.

1.7.6   Lipid antigens that bind to CD1 molecules

The repertoire of lipids which can be loaded successfully onto the different CD1 molecules is an expanding area of research. The lipid tails which are loaded deep into the groove of the CD1 molecule can be composed of mycolic acids (Beckman *et al*, 1994), diacylglycerols (Sieling *et al*, 1995), ceramides (Kawano *et al*, 1997), polyisoprenols (Moody *et al*, 2004), polyketides (Matsunaga *et al*, 2004), phthioceranate (Gilleron *et al*, 2004) or fatty acid chains (Moody *et al*, 2004). The length of these chains varies greatly and sometimes exceeds the capacity of the CD1 molecule antigen binding groove. The greatest mismatch is CD1b which can present diacylglycerols, sphingolipids and polyacylated carbohydrates ranging in length from C_{12} to C_{80} (Moody *et al*, 1997, Shamshiev *et al*, 1999, Gilleron *et al*, 2004). The identification of the crystal structure of CD1b loaded with a glucose monomycolate (GMM) antigen by Batuwangala *et al* (2004) showed the C_{56} long chain occupied almost the entire groove which could optimally accommodate a lipid length of C_{60}-C_{64} (Cheng *et al*, 2006). Studies by Ulrichs *et al* have shown CD1b loaded with antigen with lipid length of C_{80} which exceeds the predicted capacity of CD1b (Ulrichs *et al*,

2003). Following up on these reports Cheng *et al* demonstrated that the lipid chains aren't trimmed but the excess carbons protrude through a portal acting as a "trapdoor" in the binding groove of the CD1b molecule. The studies also identified low pH as an important factor in the formation of CD1b antigen complexes (Cheng *et al*, 2006). The successfully loaded CD1 molecules are trafficked to the surface for recognition by CD1 restricted T cells.

1.7.7   Lipid antigens presented to CD1 restricted T cells

Glycolipid antigens contain a hydrophilic head and hydrophobic tails. The molecular structure of the CD1 molecule reveals an Ag-binding superdomain that contains 2 pockets (A' and F') lined by hydrophobic residues which bind the lipid tails (Zeng *et al,* 1997). The T cells restricted by CD1a, CD1b and CD1c express various TCR arrangements (Grant *et al,* 1999, Shamshiev *et al*, 1999, Shamshiev *et al*, 2002). CD1d restricted T cells can express an invariant or semi-invariant TCR. It has been proposed that the CDR3 loop of the TCR is the point of contact between the lipid glycosyl group and the TCR. CDR3 residue mutagenesis has confirmed that CD1 restricted T cell TCRs are responsible for recognition of lipid antigens presented by the CD1 family of molecules. Minor changes in the CDR3 loop of the TCR abrogated the ability of the TCR to respond to CD1 bound lipid antigen (Grant *et al*, 1999, Grant *et al,* 2002).

Several classes of lipid antigens have been shown to be presented by CD1 molecules to specific T cells; mycolates, glycosphinolipids, phospholipids, sulfoglycolipids and lipopeptides (Figure 1.8) (Lawton & Kronenberg, 2004). The first lipids shown to be recognized by CD1-restricted T cells were mycobacterial lipids. CD1b presents

mycolic acids found in *Mycobacterium tuberculosis* and *Mycobacterium leprae* cell walls. These mycolic acids include glucose monomycolates and lipoarabinomanan (Figure 1.9) (Beckman *et al*, 1994, Sieling *et al*, 1995, Moody *et al*, 1997). CD1c was also reported to present lipids found in mycobacterial cell walls, such as mannosyl-phosphopolyprenols (Figure 1.9) (Moody *et al*, 2000). Gilleron *et al* reported a CD1 dependant T cell lipid Ag derived from mycobacteria called diacylated sulfoglycolipid (Ac$_2$SGL). Ac$_2$SGL was found to be presented by both CD1b and CD1c molecules. In CD1 blocking experiments, the blocking of CD1b using anti-CD1b antibodies resulted in 70% reduction in secreted IFN-γ and cytotoxicity whilst blocking of CD1c using mAb resulted in 35% reduction. CD1a blocking did not affect IFN-γ secretion and cytotoxicity against mycobacterium infected cells (Gilleron *et al*, 2004). CD1a can present lipids from mycobacterial cell walls such as lipopeptides. The lipopeptide didhydroxymycobactin (DMM) is thought to be an intermediate in mycobactin synthesis and structural studies have shown the T cell recognizes both the tail and head of the lipid (Moody *et al*, 2004) (Figure 1.9).

Many CD1a, CD1b and CD1c restricted T cells do not require exogenous antigens for their activation. CD1 restricted T cells can also recognize "self antigen" presented by CD1 molecules such as the gangioside GM1 (Figure 1.9) (Shamshiev *et al*, 1999).

Microbial lipid antigen presented by CD1d is not as well documented; several microbial lipids such as lipoarabinomannan (LAM) and glycosyl-phosphatidylinositols (GP1) bind CD1d but do not elicit T cell responses (Burdin *et al,* 1998, (Joyce *et al*,

1998, Molano *et al,* 2000 and Procopio *et al,* 2002). CD1d can also present the autologus disialogangalioside (GD3) to a subgroup of human CD1d restricted T cells however GD3 is not expressed on normal human tissues but only on tumours of neuroectodermal origin. The responding cells have invariant TCR α chains (Vα14 Jα18 in mice and Vα24 Jα18 in humans) also respond to α-galactosylceramide and iGb3, and are denoted invariant NKT cells (iNKT). iNKT cells will be discussed in a separate section.

1.8   Invariant natural killer T cells

1.8.1   iNKT cell history

The term NK T cell was first published in 1995 by Taniguchi *et al* and was used to define a subset of mouse T cells that shared some characteristics with NK cells, such as surface expression of NK1.1. The term NKT cell is now applied generally to CD1 restricted T cells which express a TCR and NK cell associated receptors.

The identification of murine Vα14+ NKT cells was first reported in 1986 when Imai *et al* isolated cDNA encoding the Vα14 TCR chain from a suppressor T cell hybridoma (Imai *et al*, 1986). Several groups published studies describing distinct subsets of αβ TCR+ T cells in mice which lacked the expression of CD4 and CD8 (Budd *et al,* 1987, Fowlkes *et al*, 1987, Newman *et al*, 1987). Other studies confirmed that this population of αβ T cells expressed NK1.1 (Sykes *et al*, 1990). NK1.1 had been previously thought to be expressed exclusively by NK cells. Vα14 Vβ8 T cell hybridomas which expressed CD4 or were double negative for CD4/CD8 and NK1.1 were made by Lantz and Bendelac (J Exp Med 1994).
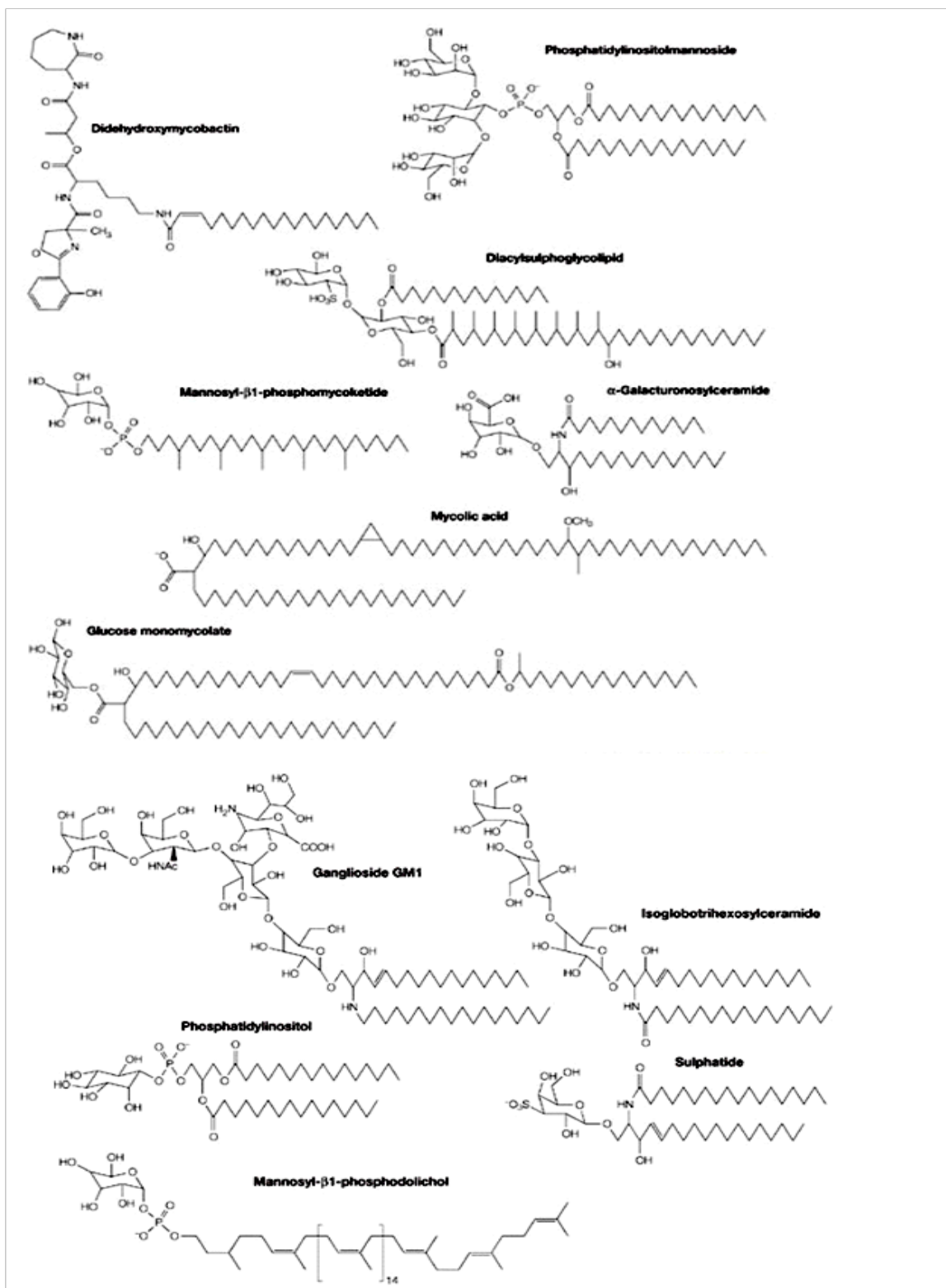
Figure 1.9: Microbial and Self glycolipid antigens: Structures of Microbial and self gylcolipids described in the literature; including the iNKT agonist iGb3.

Subsequently Bendelac *et al* showed that the antigen presentation molecule for these T cells was CD1 not MHC class II (Bendelac *et al*, 1995). Murine Vα14 NKT cells make up ~0.5% of circulating T cells, 2.5% of T cells in the spleen and up to 30% of T cells in the liver (Bendelac *et al*, 2007).

In 1993, while screening for anti cancer agents, Kirin Pharmaceuticals reported the first isolation of alpha galactosylceramide (αGalCer) from marine sponge *Agelas mauritianus* (Natori et al, 1993) (Figure 1.10). αGalCer was shown to have anti tumour properties. This resulted in the synthesis of KRN7000, an αGalCer analogue (Morita et al, 1995). KRN7000 demonstrated the ability to bind murine and human CD1d molecules. In 1997 it was shown that CD1d molecules present this glycolipid, to NKT cells, which resulted in their subsequent activation (Kawano *et al*, 1997). αGalCer and the related studies will be discussed in detail in section 1.7.5.

1.8.2   NKT cells development

Studies into the development of murine NKT cells revealed that they appear to be selected by bone marrow derived cells expressing β_2M and CD1d, and are most probably CD4+CD8+ thymocytes. These thymocytes express NK1.1 and a restricted set of αβ TCRs. These cells were CD4+ and CD4-8- but never CD8+, because the presence of CD8 caused them to be negatively selected. Thus, neither CD4 nor CD8 contributes signals that direct their maturation into the CD4+ and CD4-8- lineages (Bendelac *et al*, 1994, Taniguchi *et al*, 1995). This population of NKT cells in mice is characterised by its expression of the invariant Vα14 Jα18 TCR and NK markers.

Vα14+ iNKT cells arise in the thymus in the perinatal period and do not reach significant numbers until 3 weeks after birth. These cells are absent from nude mice and from thymectomized mice. (Gapin *et al,* 2001, Benlagha *et al*, 2002 and Matsuda *et al,* 2008).

Several factors are essential for NKT development. Targeted disruptions in the NFκB subunits showed NFκB p50 activation to be required for the maturation of NK1.1 positive precursors to mature Vα14+ NK1.1+ NKT cells (Sivakumar *et al,* 2003, Stanic *et al,* 2004). The expression of the gene which inhibits IKK2 kinase is also required for the development of iNKT cells (Schmidt-Supprian *et al,* 2004). Vα14 iNKT cells also require the expression of the transcrition factor Tbet (originally found to be required for the induction of IFN-γ synthesis and Th1 immunity by T cells), and mice with induced deletions of T-bet, showed a stem cell intrinsic defect in Vα14+ NKT generation (Townsend *et al*, 2004). Vα14 cell precursors express the GM-CSF receptor which is critical for the Vα gene rearrangement necessary for generating the Vα14 TCR. Mice negative for the GM-CSF receptor showed a significant reduction in mature Vα14+ NKT cells (Sato H *et al*, 1999, Taniguchi *et al*, 2003).

1.8.3   Human iNKT cells

Following on from the identification of murine populations of invariant NKT cells, CD1d reactivity and potent cytokine secretion by T cells which express the homologous Vα24 Jα18 co-expressed with Vβ11 TCR have been described (Porcelli *et al* J Exp Med 1993) (Dellabona *et al* J Exp Med 1994). Other subsets of CD1 restricted T cells which can be positive or negative for the invariant Vα24 Jα18 TCR chain and

CD161 have been identified in humans and are commonly referred as NKT cells. Some NKT cells have even been shown to express γδ TCR (Cardell *et al* Exp Med 1995). Advances in the development of CD1 tetramers have also lead to the discovery of a population of Vα24 Jα18 NKT cells that do not express CD161 and are CD1d independent (Hammond *et al*, 1999, Matsuda *et al*, 2000).

To classify these various NKT and NKT-like subsets, the nomenclatures type 1 and type 2 are used. Type 1 NKT cells are classical invariant Vα24 Jα18 TCR positive cells that are reactive to CD1d in the absence or presence of αGalCer. These cells can be double negative for CD4/CD8, CD4+ and CD8+ and are classically potent secretors of the cytokines IFN-γ and IL-4. Mature type I NKT cells express CD161, but this is downregulated after activation. Type II NKT cells are CD1d reactive but not αGalCer reactive T cells, expressing semi invariant TCRs, can be CD161 positive or negative and are potent secretors of cytokines (IFN-γ and IL-4) (Godfrey *et al*, 2004).

1.8.4   Antigens recognised by iNKT cells

The lack of a known natural ligand for human iNKT cells led to a search resulting in some controversy, and the identification of an agreed natural ligand is still awaited. In 2004 Zhou *et al* reported the identification of the mammalian lysosomal glycospingolipid, termed isoglobotrihexosylceramide (iGb3), which was recognized by iNKT cells and resulted in their activation, expansion and the secretion of both IFN-γ and IL-4. The authors also reported that mice deficient in β hexosaminidase b (necessary for iGb3 synthesis) showed a significant reduction in Vα14+ NKT numbers (Zhou *et al* 2004). The eligibility of iGb3 as the natural ligand was refuted in a series of

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publications in which it was reported that iGb3 could not be detected in mammals. Using highly sensitive HPLC, the only iGb3 detected was in the dorsal root ganglion (DRG) of mice. This study was backed up with the finding of normal iNKT development in iGb3 deficient mice, indicating that iGb3 is not necessary for iNKT selection (Speak *et al* 2007, Porubsky *et al* 2007).

1.8.5 α-GalCer

Currently the gold standard of iNKT ligands is αGalCer. αGalCer bound to murine and human CD1d molecules stimulate type I NKT cells to secrete large amounts of Th1 and Th2 cytokines (IFN-γ and IL-4 respectively) along with a milieu of other immune modulating cytokines. Therapeutic activation of iNKT cells in mice using α-GalCer can be used to treat many viral, bacterial and parasitic infections, and promote tumour reduction/protection in many tumour models such as B16 melanoma model (Kawano *et al* 1997).
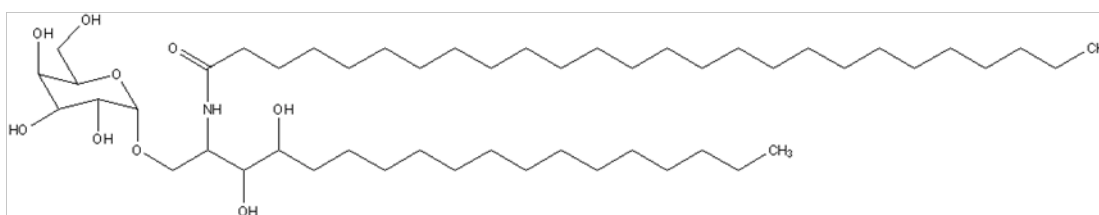
Figure 1.10: Structure of Alpha **galactosylceramide***: Diagram showing the structure of Alpha galactosylceramide. Reproduced from* **Kawano** *et al* **1997.**

1.9 iNKT cell function

As previously described the innate immune system responds rapidly to infection. The effectors functions of iNKT cells, cytotoxicity and cytokine production, are thought to be of particular importance in containing pathogen spread during initial stages of infection and while recruiting other cells in the development of an adaptive immune response. As the name suggest, iNKT cells are efficient killers, however natural cytotoxicity does not seem to be their main effector function (Godfrey *et al* 2002).

1.9.1 Cytotoxicity mediated by iNKT cells

Exley *et al* (J Exp Med 1998) reported that cytotoxicity by human iNKT cells is CD1d restricted and enhanced *in vitro* in the presence of αGalCer. Later studies confirmed that iNKT cells mediate CD1d restricted lysis of several tumour cell types. Tumour cells negative for CD1d were not killed by iNKT cells but after transfection with CD1d, the same lines where rendered susceptible (Metelitsa *et al* 2001). Kawano *et al* (PNAS 1998) reported αGalCer treated mice exhibited iNKT cell-mediated tumour target lysis in a CD1d and NK1.1 independent mechanism. Cytotoxicity was reported to be non-specific NK cell like killing, mediated by perforin. Concanamycin A (CMA) which inhibits perforin abrogated killing but blocking antibodies against CD1d, NK1.1 and FAS-L did not (Kawano *et al* 1998). Nicol *et al* reported that CD1d expression by target cells increased their susceptibility to lysis by human Vα24+ iNKT cells but was not essential as several CD1d negative targets were killed by Vα24+ iNKT cell lines. CD1d independent killing by human iNKT cells, suggested the lack of CD94

expression and co-stimulation as part of the CD1d independent cytotoxicity mechanism. (Nicol *et al* 2000), and indeed the subsets reported in CD1d dependant studies by Exley *et al* and Metelitsa *et al* both expressed CD94. This indicates different effector functions for different iNKT subsets, defined by surface marker expression.

Perforin is a cytolytic protein found in granules in the cytoplasm of cytotoxic cells such as iNKT, NK and cytotoxic T cells (CTL). Upon degranulation it inserts into the target cell membrane creating tubules or "pores" leading to cell death. Perforin is found in the cytoplasm of iNKT subsets and experiments by Metelitsa *et al* (2001) and Nicol *et al* (2000) demonstrated a reduction in iNKT cytotoxicity through the addition of concanamycin A, an inhibitor of perforin. Invariant NKT cells also express FAS ligand (FAS-L) which upon binding to FAS on a target cell, a death inducing complex is formed leading to apoptosis induced cell death. Stenger *et al* reported CD4–CD8– CD1 restricted T cell cytotoxicity against bacteria infected targets that was mediated by Fas-FasL interaction, whereas CD8+ cells killed targets via FAS-L independent mechanism (Stenger *et al*, 1997), these findings indicate that different iNKT cell subsets have different effector functions.

1.9.2   iNKT cell cytokine production

Functionally iNKT cells are best known for their ability to rapidly produce and secrete large amounts of cytokines (Yoshimoto *et al* 1994, Matsuda *et al* 2000, Kronenberg, 2005). The cytokine profile varies in different iNKT subsets but can include cytokines varying from the classical Th1, Th2 and Th17 cytokines IFN-γ and IL-4 to IL-2, IL-5,

IL-6, IL-10, IL-13, IL-17, IL-21 tumour necrosis factor (TNF) and GM-CSF (Kronenberg, 2005).

Inavriant NKT cells are most notable for their ability to produce both IFN-γ and IL-4. This ability is expressed by both human Vα24+ iNKT cells and murine Vα14+ iNKT cells (Exley et al, 1997, Chen & Paul 1997). Studies on pre activated iNKT cells have found mRNA for IL-4 and IFN-γ, demonstrating iNKT innate configuration to rapidly produce both cytokines (Stetson *et al* 2003, Matsuda *et al* 2003). Crowe *et al* reported that iNKT cells can produce IL-4 two hours after stimulation, and production lasts for up to 16 hours. IFN-γ was also produced within hours and was produced for 72 hours post stimulation (Crowe *et al* 2003). Gumperz *et al* reported two subsets of CD1d restricted T cells which differ in cytokine production. One is a CD4 negative iNKT cell which produced Th1 cytokines (IFN-γ and TNF-α), the second was a CD4 positive subset which produced both Th1 and Th2 (IL-4) cytokines (Gumprez *et al* 2002).

This milieu of cytokines allows the iNKT cells modulate an immune response through activation of other innate cells such as NK cells and DCs and, T cells and B cells of the adaptive immune response (Metelitsa *et al* 2001). TCR signalling influences the cytokine profile, the best evidence for this is provided by the studies using profile biasing αGalCer analogues such as OCH, C20:2 and α-C-GalCer (discussed in detail in section 1.10.1). Currently it is not known how a specific response is elicited after TCR activation, many hypotheses have been provided, the most probable are based around TCR signal strength based on the interaction between the TCR and CD1 molecule.

These depend on the bound lipid structure, avidity and association with lipid rafts which will be discussed in detail later. Another possible explanation is that the cytokine response depends on the subset and phenotype of the iNKT cell involved (Park *et al* 2005, Crowe *et al* 2005).

Reciprocal interactions between murine iNKT cells and DCs have shown that IL-12 production by DCs is essential for triggering NKT cell activation (Kitamura *et al* 1999). The same investigations showed αGalCer activation of iNKT cells resulted in the expression of IL-12R the IL-12 receptor on iNKT cells. In a series of blocking experiments these studies showed that anti-IL12, anti-CD40 and anti-CD40L mAb treatment resulted in the loss of IFN-γ production by iNKT cells.

1.9.3   iNKT cell transactivation of other immune cells

Many studies have demonstrated that iNKT cells interact with and can activate/recruit many immune cells such as NK cells, macrophages, DCs, B cells and cytotoxic T cells. Recently the transactivation of neutrophils, regulatory T cells and γδ T cells have also been reported (Matsuda *et al* 2008) (Figure 1.11).

Mouse iNKT cells have been reported to have NK like cytolytic activity against tumour cells in several early studies (Cui *et al* 1997, Kawano *et al* 1998). Subsequently it was shown that murine iNKT cells can activate NK cell proliferation and cytotoxicity in an IFN-γ mediated manner (Nakagawa *et al* 1998, Carnaud *et al* 1999, Eberl & Mac

Donald 2000. Metelitsa *et al* showed that purified human iNKT cells could also activate NK cell cytotoxicity. In a transwell system they showed that iNKT cells activated with α-GalCer secreted IFN-γ and IL-2 which mediated the NK activation and this was abrogated by the depletion of these cytokines from the system (Metelista *et al*, 2001).

iNKT cells can also interact with B cells and can influence antibody responses. Experiments which used α-GalCer as adjuvant to protein antigen showed 1-2 log higher levels of antibody and also increased the frequency of memory B cells in mice lacking MHC class II. This was mediated through cytokine and CD40-CD40L interactions, and suggests that iNKT can substitute CD4+ Th cell interactions with B cells (Galli *et al* 2007). The expression of CD1d on B cells is also required to stimulate iNKT cell enhanced antibody production (Lang *et al* 2008).

Recently cross talk between iNKT cells and CD4+ CD25+ regulatory T cells was investigated and it was reported that iNKT cells can modulate regulatory T cells function in an IL-2 mediated manner. In experiments inducing nickel tolerance, CD4+ NKT cells were required to induce the generation of regulatory T cells. NKT cell deficent mice did not induce tolerance and failed to generate regulatory T cells. In a reciprocal interaction regulatory T cells can suppress iNKT activation in a cell contact depentant mechanism, as shown by blocking of ICAM-1 or separation of cells into transwell system. (La Cava *et al* 2006).

In 2007 Jin *et al* reported that iNKT cells and γδ T cells interacted in a synergistic manner in an airway hyper-responsiveness (AHR) model but the mechanisms were not elucidated. AHR was induced in T cell-deficient mice by small numbers of co-transferred γδ T cells and invariant NKT cells, whereas either cell type alone was not effective (Jin *et al*, 2007).

1.9.4 iNKT cell interactions with dendritic cells

Several studies have reported extensive cross talk between dendritic cells and NKT cells (Brigl *et al*, 2003, Fujii *et* al, 2004, Munz *et al*, 2005). Murine iNKT cells have the ability to fully mature myeloid iDCs into MHC, CD80/CD86 expressing DCs, which can produce bioactive cytokines (IL-12 and IL-10) (Fujii *et al*, 2005). iNKT cytokines can induce DC maturation but CD40-CD40L interactions via iNKT-DC crosstalk is crucial for full activation of the DC and subsequent linking of innate and adaptive immunity (Fujii *et al,* 2004, Sporri & Reise-Sousa, 2005). Studies by Tomura *et al* demonstrated dendritic cell maturation and IL-12 production in response to αGalCer activated CD40L expressing CD4+ murine iNKT cells. Anti CD40L mAb inhibited the production of IL-12 indicating the necessity of CD40L. The same study reported that IL-12 release preceded IFN-γ and that IL-12 was required for IFN-γ but not IL-4 production, suggesting IL-12 favours Th1 type response (Tomura *et al* 1999). Yang *et al* reported that human Vα24+ iNKT cells exhibited the same cross talk and these cells select for a Th1 response through the production of IL-12. The study also showed that DC were susceptible to lysis by iNKT cells and the authors suggested that this was a feedback mechanism Yang *et al* 2001).

In 2002 Vincent *et al* reported that human self reactive CD1 restricted T cells can promote dendritic cell maturation by recognizing CD1 in the absence of foreign antigen. T cells reactive against all CD1 isoforms; CD1a, CD1b, CD1c and CD1d, stimulated DC maturation but with distinct mechanisms of co-stimulation (CD1a, CD1b CD1c required LPS, whilst CD1d required lipid antigen and CD40L), all resulting in IL-12 production by the DC. (Vincent *et al* 2002). Following up on this knowledge, a mechanism reported for iNKT activation by dendritic cells showed weak responses to CD1d self antigen which was amplified by DC produced IL-12 in response to microbial infection resulting in potent IFN-γ secretion (Brigl *et al* 2003).

In 2005 Yue *et al* reported that mAb ligation of CD1d in the absence of iNKT cells could rapidly stimulate monocytes and DCs to produce IL-12. Blocking of IFN-γ produced by iNKT cells and the transcription factor NFκB using mAbs, showed that IFN-γ from iNKT enhanced IL-12 production by APC but wasn't essential but that the transcription factor NFκB was essential for IL-12 production (Yue *et al* 2005). The iNKT/DC interaction has the potential to skew subsequent immune responses and reiterates the powerful potential of this interaction for immunotherapy.
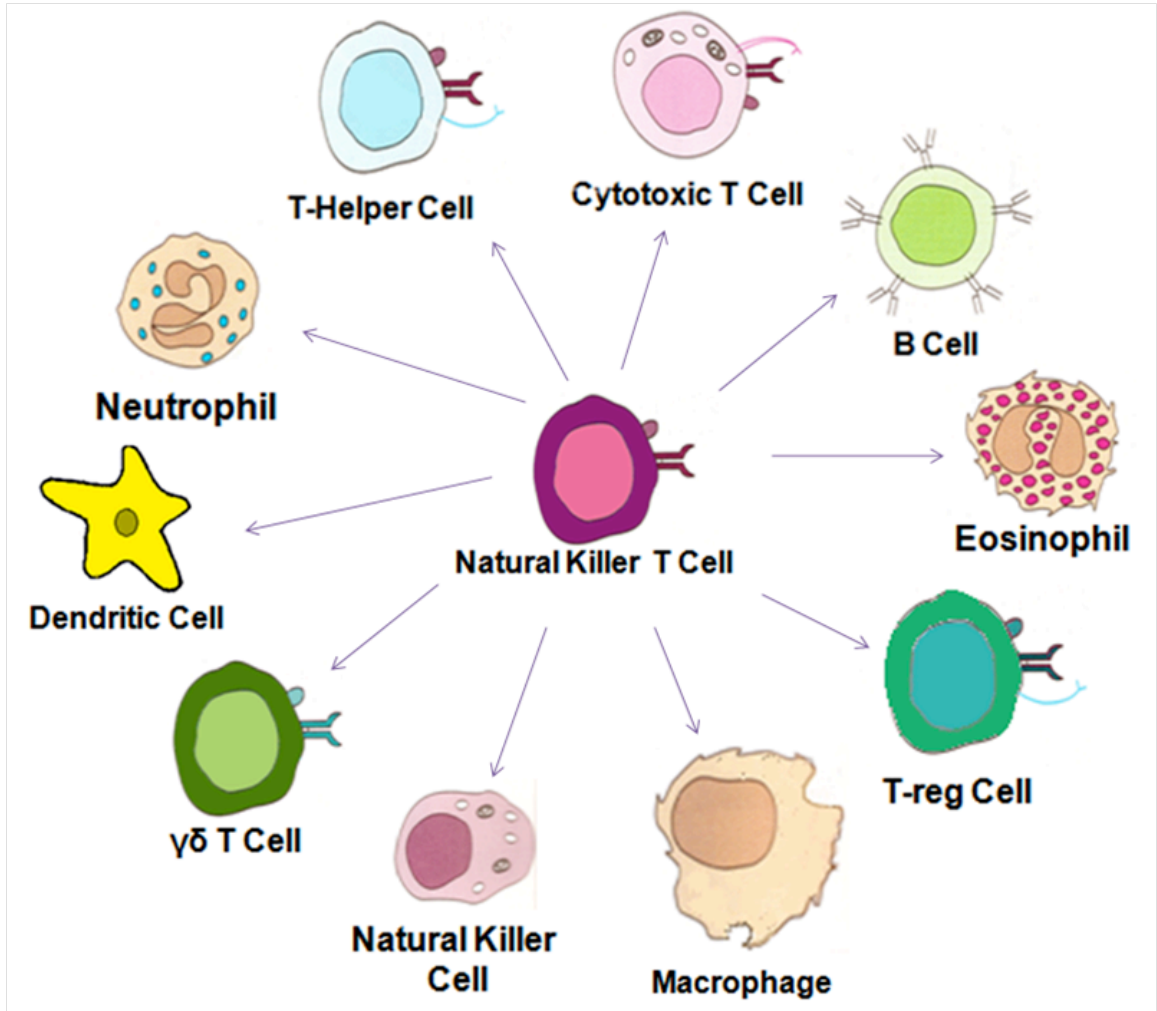
Figure 1.11: NKT cell transactivation: Diagrammatic representation of immune cells that NKT cells activate or interact with directly or by cytokine factors. Adapted from Matsuda et al 2008.

1.10 NKT cells in disease

After the identification of Vα14 iNKT cells in mice and their human Vα24 iNKT counterparts, studies into the function and role of this T cell subset revealed their involvement in numerous diseases including infectious and autoimmune disease and tumour surveillance and rejection.

1.10.1 Infectious Disease

The involvement of Vα14 iNKT cells in immunity against bacterial infection was examined in several infection models. Control and clearance of *Mycobacterium tuberculosis* (mTB) involves the formation of a granuloma, a vital step in the control of the infection. Studies into the control of mTB showed IFN-γ is involved in the formation of the granuloma (Altare *et al* 1998). A murine model of mTB granuloma showed the presence of Vα14 iNKT cells within the granuloma and the recruitment of iNKT was attributed to the presence of iNKT ligands in the bacterial cell walls. iNKT cell knockout mice did not form the granuloma so the formation was attributed to iNKT cells and IFN-γ (Apostolou *et al* 1999). However the protection against mTB is not primarily due to iNKT cells. This was shown in studies using CD1d and β_2 microglobulin knockout mice, the KO mice showed no difference in infection compared to wild type mice (Behar *et al* 1999, Sousa *et al*, 2000).

Kawakami *et al* showed that protection against the fungal pathogen *Cryptococcus neoforman* was due to IFN-γ from Vα14 iNKT cells. In Vα14 iNKT knockout mice the infection was sustained longer due to the limited early IFN-γ attributed to iNKT cells (Kawakami *et al* 2001, Kawakami *et al* 2001).

Gonzalez-Aseguinolala *et al* reported that αGalCer activated Vα14 iNKT cells display potent anti-malarial activity. Sporozoite inoculated mice were treated with αGalCer during the intrahepatic stages of infection and this inhibited disease development (Gonzalez-Aseguinolala *et al* 2000). The timing of NKT cell activation was critical in infection clearance, the iNKT cell target stage of the malarial life cycle was restricted to the intrahepatic stage of infection. Anti-malarial activity was due to IFN-γ and the activity was elicited in the absence of conventional T cells, NK cells and B cells.

Vα14 iNKT cells have also been reported to play a role in viral infection. In 2000 Kakimi *et al* reported a protective function of iNKT cell in a hepatitis B virus transgenic mouse model. 24 hours after a single injection of αGalCer, IFN-γ and IFN-α/β release by intrahepatic iNKT cells was detected and hepatitis B virus (HBV) replication was abolished. The recruitment of NK cells was also reported. In IFN knockout mice the abolishment of HBV replication was lost (Kakimi *et al*, 2000). αGalCer injection as an adjuvant for vaccination with HBV antigen resulted in the induction and proliferation of HBV specific CTLs. Blocking of CD40-CD40L by antibody showed that this mechanism of HBV specific responses are dependant on this interaction and also IL-2 (Ito *et al* 2008)

Several publications have reported that NKT cells are targeted by HIV-1 (Van der Vliet *et al*, 2002, Motsinger *et al* 2002, Sandberg *et al*, 2002). NKT subsets which express the CD4 receptor (targeted by HIV-1) and the co-receptor CCR5 are highly susceptible to infection (Motsinger *et al*, 2002).

1.10.2 Autoimmune disease

The link between iNKT cells and autoimmunity was first observed with the reduction of iNKT numbers in a murine lupus model. It was shown the transfer of iNKT cells delayed the disease progression (Mieza *et al* 1996). In the non obese diabetic (NOD) murine model, a reduction in $V\alpha14$ cell numbers was associated with progression to type 1 diabetes (Gombert *et al*, 1996). Following up on this study, several groups using CD1d knockout NOD mice, showed that the transfer of $V\alpha14$ iNKT cells into NOD mice prevented disease development (Baxter *et al* 1997, Hammond *et al* 1998). Over-expression of $V\alpha14+$ iNKT cells only partially corrected the disease so it was noted that the protection was due to iNKT functionality and not cell quantity (Lehuon *et al* 1998). Laloux *et al* reported that the loss of iNKT cell derieved IL-4 is an important factor in diabetes development in NOD mice (Laloux *et al*, 2001). Naumov *et al* (2001) reported that in the NOD murine model iNKT cell numbers are decreased in the pancreatic islets of NOD mice at the time of progression from peri-insulitis to invasive insulitis and diabetes. Administration of αGalCer ameliorated the development of diabetes in NOD female mice, and resulted in an accumulation of iNKT cells and myeloid DCs in the pancreatic lymph nodes (PLNs) but not the inguinal lymph nodes. Transfer of DCs from the PLN but not the inguinal lymph nodes to NOD female mice completely prevented diabetes. The authors concluded the immunoregulatory role of

iNKT cells is manifested by the recruitment of tolergenic DC to the PLN and inhibition of autoimmune inflammation. A later study by Chen *et al* showed that in response to soluble factor produced by the iNKT cells, DC accumulated in the PLN, and subsequently recruited pathogenic T cells and tolerized them thus preventing β cell destruction (Chen *et al,* 2005). In a study of human type 1 diabetes, the Th1 cell mediated damage of pancreatic β cells, was initially reported to be regulated by iNKT cells. The authors report a correlation between T1D and the loss of IL-4 produced by iNKT cells, suggesting that iNKT cell derived cytokine has a role in T1D (Wilson *et al* 1999).

1.10.3 Cancer

The ability of iNKT cells to respond rapidly, to secrete IFN-γ and to transactive NK cells and DCs has led to the hypothesis that iNKT cells could be important in cancer therapy. In 1997 Kawano *et al* and Cui *et al* investigated the role of iNKT cells in a murine B16 melanoma model which metalizes to the liver. Using NKT knockout mice, it was shown the activation of iNKT resulted in the clearance of the tumour in an IL-12 dependant manner. The clearance of tumour in Vα14 transgenic RAG knockout mice (which have iNKT cells but do not have conventional T cells) identified iNKT cells to be the effector cell population responsible. The studies also showed iNKT cell cytotoxicity was dependant on direct contact with CD1d, and that this activity was abrogated with the addition of the perforin blocking agent CMA (Kawano *et al* 1997).

The anti-metastastic effect of αGalCer in mice was further investigated by Smyth *et al* in 2002. Using gene targeted mice and blocking antibody treatments these authors showed that IFN-γ from NKT cells and NK cells was essential for the anti-metastastic effect of αGalCer. The authors also showed that IL-12 and IL-18 were required for optimal IFN-γ production but IL-4 was not needed for the anti-metastastic activity.

In 2002 Crowe *et al* reported that adoptive transfer of NKT cells from wild type mice into Jα281 KO mice with methylcholanthrene induced fibrosarcoma inhibited tumour growth in the absence of αGalCer stimulation. The NKT cells were reported to have an immunosurveillance role, and recruited NK and CD8+ T cells. The mechanism of protection was CD1d dependant and IFN-γ was found to be the protective cytokine, since IFN-γ KO mice failed to inhibit tumour growth. The IFN-γ was produced by NKT cells as well as NK and CD8+ T cells. Perforin produced by non-NKT cells was also critical in inhibition of tumour growth (Crowe *et al*, 2002).

NKT cells are also implicated in immunity against tumours in humans. CD1d/αGalCer dependent anti-tumour cytotoxicity by human Vα24 iNKT has been reported (Brossay *et al* 1998, Spada *et al* 1998). In 1999 Kawano *et al* reported that Vα24 iNKT kill tumour cells *in vitro* and that Vα24 iNKT cell numbers are reduced in cancers. iNKT cells from patients with malignant melanoma killed several tumour cell lines in a perforin dependant manner when activated with αGalCer. It was thus speculated that the NKT cells, although reduced in number, where functionally normal in patients with malignant melanoma (Kawano *et al*, 1999).

Several studies followed reporting significant reductions of iNKT numbers in human cancers. Circulating iNKT numbers in prostate cancer were reduced 100 fold from 0.21% in healthy controls to 0.02% in patients (Tahir *et al*, 2001). In contrast to the melanoma study from Kawano *et al*, the iNKT cells from prostate cancer patients showed a reduced in the production of IFN-γ, but normal levels of IL-4. Kenna *et al* reported that iNKT cell numbers are reduced in livers of patients with metastatic liver disease (Kenna *et al* 2001). Kenna *et al* later reported that CD1d expression and the level of CD1d T cell reactivity in tumour bearing livers to be unchanged in comparison to histologically normal livers (Kenna *et al*, 2007). The success in murine models coupled with reports of losses of Vα24 iNKT cell numbers and function in some human cancers, place iNKT cells as a potential targets for cancer therapy.

1.11 NKT cells based immunotherapy for cancer

Several approaches to iNKT based immunotherapy have entered clinical trial (Figure 1.12). In 2002 Giaccone *et al* enrolled 24 patients with solid tumors into a phase I clinical trial. Patients received intravenous injection of αGalCer (50-4800 μg/m^2) on days 1, 8 and 15 of a 4 weekly cycle. 24 hours post injection Vα24+ Vβ11+ iNKT cell numbers decreased in periphery, possibly due to activation induced down-regulation of TCR (Crowe *et al* 2003) and migration to the tissues. Increased serum levels of IFN-γ, IL-12 and GM-CSF was detected in several patients. The therapy was well tolerated with no adverse side effects, but no tumour regression was noted, although seven

patients displayed a prolonged period of stable disease with a median of 123 days (Giaccone *et al* 2002).

Another approach to iNKT based cancer therapy was the intravenous injection of *in vitro* expanded and activated Vα24 iNKT cells. 6 patients with recurrent non small cell lung cancer were enrolled. Patients received 2 injections of up to $5x10^7/m^3$ autologous iNKT cells, which were expanded from PBMC using αGalCer and IL-2 *in vitro* (mean numbers of Vα24 iNKT cells increased from 0.06% up to 25%). The expanded iNKT cells showed good cytotoxic activity *in vitro* against K562 and PC-13 tumor cell lines. No adverse side effects were reported and increases in circulating iNKT number were reported in 2 cases. Increases in serum IFN-γ levels were also reported in 3 patients with NK cells identified as the main source. No regression or stabilization of disease was reported in any patients (Motohashi *et al* 2006).

A third approach which has been the most widely investigated with several variations, uses infusions of dendritic cells pulsed with αGalCer. In 2004 Nieda *et al* enrolled 12 patients with metastatic tumors. Patients received 2 intravenous and 2 intradermal injections of $5x10^6$ autologus α-GalCer pulsed immature dendritic cells. The number of iNKT cells decreased 24 hours post injection again probably due to down-regulation of TCR and migration to the tissues. IFN-γ and IL-12 levels increased and activation of NK cells and T cells was also reported. The therapy was well received with reduction of tumor markers in 2 patients (Nieda *et al* 2004).

In 2005 Ishikawa *et al* enrolled 11 patients with non small cell lung cancer, and immunized intravenously up to 4 times with $>5\times10^7$ immature dendritic cells/m^2 of body mass pulsed with αGalCer. iNKT numbers increased in 1 patient after 2 injections. Increases in mRNA encoding IFN-γ was reported in circulating iNKT cells. Again the therapy was well received and no adverse side effects were reported, but no regression was reported but disease progression stabilized in 2 patients (Ishikawa *et al* 2005).

Chang *et al* immunized myeloma patients 3 times with mature DCs; the first injection used unloaded DC and subsequent injections used dendritic cell pulsed with αGalCer. The injection of pulsed but not unpulsed dendritic cells led to the >100 fold increase in circulating iNKT numbers in all 5 patients, and these could be detected for up to 6 months. IFN-γ and IL-12p40 levels increased and antigen specific CD8+ T cells were also reported. The therapy was well received and no adverse side effects were reported with one patients showing disease stabilization (Chang *et al* 2005).

Recently Uchida *et al* enrolled 9 patients with head and neck cancer into a phase I clinical trial. Two injections of autologus αGalCer pulsed antigen presenting cells (APCs), were administered into the nasal submucosa. Each injection of 1×10^8 cells was separated by 1 week and consisted of PBMC stimulated with GM-CSF and IL-2 for 7 days, then pulsed with αGalCer. The levels of DCs in the infusions ranged from 30-55%. After the second injection increased iNKT numbers were observed in 4 patients and enhanced NK cell activity was detected in eight patients. The therapy was well

received and no adverse side effects were reported. No tumour regression was reported but disease progression stabilised in 5 patients (Uchida *et al* 2008).

Motohashi *et al* conducted a follow up phase II clinical trial, enrolling 23 non small cell lung cancer patients (17 patients completed the trial). Patients received 4 intravenous injections of $1 \times 10^9/m^2$ PBMC cultured with GM-CSF and IL-2 then pulsed with αGalCer, before administration. IFN-γ producing cells increased in 10 patients and this correlated with an increased survival time. 5 patients were reported to have stabilized disease (Motohashi *et al* 2009).

Immunotherapy utilizing iNKT cells although well tolerated have been disappointing after the results observed in the murine models (Kawano *et al* 1997, Smyth *et al* 2002). There are several reasons for these disappointing results, (1) humans have different frequencies of iNKT cells compared to mice (2) the patients in the outlined human studies had advanced staged cancers, previous studies have reported the reduction in iNKT cells in cancers (Section 1.9.3). (3) The current therapies for human cancers are based on chemotherapy, radiation and surgery. The effects of these therapies on iNKT cell function are not well understood and could possibly effect immunotherapy. (4) The ligand for iNKT cells is unknown, currently αGalCer is the best know stimulator iNKT cells, however maybe an alternative glycolipid will result in optimal human iNKT cell activation.
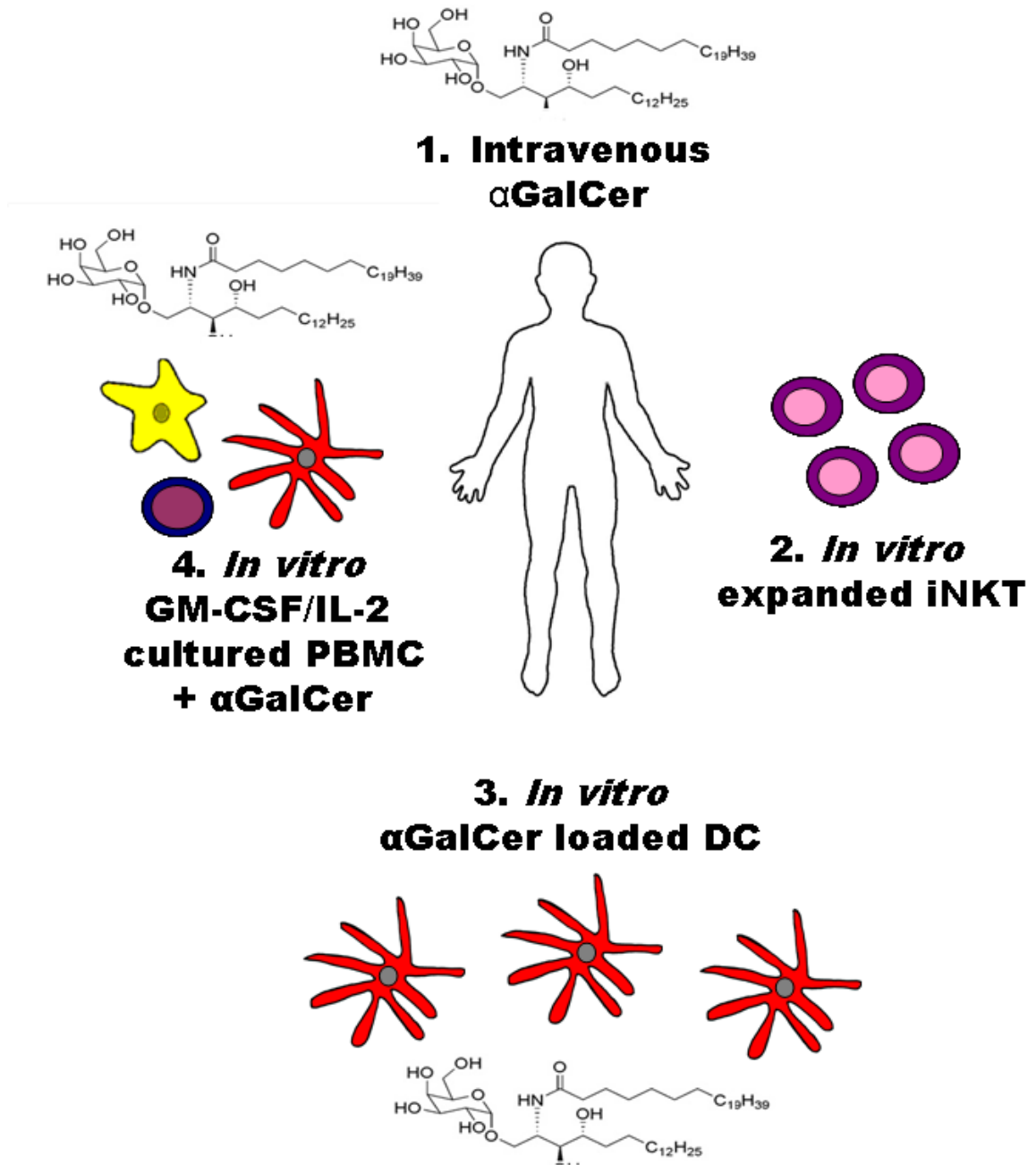
Figure 1.12: NKT cell based immunotherapy: *Schematics detailing the main approaches used in iNKT cell based immunotherapy. The intravenous injection of αGalCer (1), the infusion of αGalCer expanded iNKT (2), the infusion of monocytes derived DCs loaded with αGalCer (3), the infusion of GM-CSF and IL-2 cultured PBMC loaded with αGalCer (4).*

1.11.1 iNKT cell activation by αGalCer analogues

Miyamoto et al (Nature 2001) synthesised an αGalCer analogue termed OCH (Figure 1.13). OCH was an altered ligand with shortened acyl chains and resulted in a Th2 bias with predominant IL-4 production when injected in to experimental autoimmune encephalomyelitis (EAE) mice. Injection of OCH resulted in increased serum IL-4 levels and this was confirmed in an *in-vitro* assay using splenocytes. OCH activation of iNKT cells resulted in specific protection against EAE after a single intraperitoneal injection, whereas αGalCer stimulation of iNKT cells did not confer the same protection. OCH injection did not confer protection in Vα14+ knockout mice, IL-4 was speculated to be the protective cytokine, as EAE is thought to be a Th1/Th17 driven condition. OCH stimulation resulted in 5 fold lower proliferations of iNKT cells compared to αGalCer. Schmieg *et al* (2003) synthesised another αGalCer analogue with a single change replacing the glycosidic *O* link of αGalCer with a CH_2 link, named α-C-GalCer (Figure 1.13). Following up on previous studies showing that iNKT protected against hepatic stage malaria in mice (Gonzalez-Aseguinolasa, 2000) the authors reported 1000 fold more anti-malarial and 100 fold greater anti-metastatic activities than with αGalCer in an iNKT/CD1d dependant manner. To investigate the anti malarial effect of α-C-GalCer, the ability of either glycolipid to prevent blood stage malaria infection in mice challenged with live sporozoites was assessed. When given 3 days before challenge, α-C-GalCer completely protected 9 out of 10 mice from blood-stage malaria, whereas an identical dose of α-GalCer protected 0 out of 10 mice, the same as untreated controls. The results showed α-C-GalCer activation with 1 ng/ml led to a Th1 bias with increased IFN-γ and IL-12 and decreased IL-4 compared to

αGalCer stimulation with 100 ng/ml. The response to α-C-GalCer was also sustained longer and was detectable for 3 days longer than αGalCer.

Porcelli *et al* reported that substituting the N-acyl chain of αGalCer with a shorter unsaturated fatty acid modifies the response by iNKT (Yu *et al*, 2005). C20:2 (Figure 1.13) potently induced a Th2 biased cytokine response in serum. This was associated with diminished IFN-γ protection and Vα14+ NKT cell proliferation in splenocyte culture assay. In studies following up on this information Bittman *et al* reported a truncated nonisosteric C glycoside, termed α-1C-GalCer. These investigations used human DCs as antigen presenting cells for Vα24+ NKT cells and found that stimulation with α-1C-GalCer resulted in less IFN-γ than stimulation with αGalCer but higher Th1:Th2 ratios typical for Th1 response. (Lu *et al*, 2006).

Recently Trappeniers *et al* reported another series of Th1 biased analogues, with structural modifications in the 6'OH group of the glycoyl moiety, after structural studies by Borg *et al* (2007). The Gal 6'OH sugar alcohol was identified as the only non involved moiety in the H-bond formation. The rational was that induction of changes at this point could induce further interactions between the ligand with CD1d. Several analogues were synthesized and tested. The immunoproperties of the resulting 6'-derivatised αGalCer analogues, were investigated by examination of murine serum levels after treatment with the ligand. It was found that the 6' derivatised αGalCer analogues resulted in a Th1 bias (Trappeniers, 2008).

α-GalCer   : X=O,   n=13, R= $CO(CH_2)_{24}CH_3$
OCH   : X=O,   n=4, R= $CO(CH_2)_{22}CH_3$
C20:2   : X=O,   n=13, R= Linoleoyl
α-C-GalCer: X=CH_2, n=13, R= $CO(CH_2)_{24}CH_3$
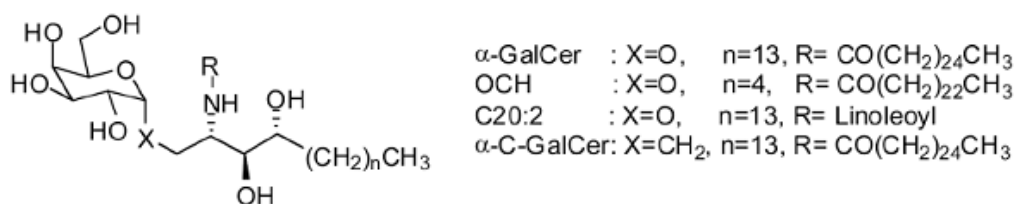
Figure 1.13: αGalCer analogues: *Schematic detailing the modifications made to KRN-7000 in the derivation of OCH, C20:2 and α-C-GalCer (Reproduced from Wu et al 2008)*

1.12 Hypothesis

The above studies arguably show that two of the most important cellular links between the innate and adaptive immune responses are iNKT cells and the dendritic cell (DCs). As described iNKT cell activation using αGalCer can prevent and cure cancer in several murine models, but therapies using the same in humans have been disappointing (Section 1.9.3). Possible reasons for these results could be; (1) Lower NKT cell numbers in humans than mice both in the periphery and the liver, (2) Human NKT cells are functionally different to or have different subsets their murine counterparts, (3) Human iNKT cells react different to αGalCer than murine iNKT cells. The potential to manipulate the interaction between the iNKT cells and DCs is one that provides attractive potential for immunotherapy.

The main aim of the proposed study was to investigate the reciprocal interactions between iNKT cells and DCs, and establish a system to test novel αGalCer analogues with proposed beneficial modifications for improved iNKT activation with the ultimate aim of therapeutic use.

1.12.2 Aims of present investigation

- To investigate the extent of human CD1a-d reactivity in human peripheral blood

- To test reactivity of human iNKT cells to αGalCer and other lipids *in vitro*

- To optimize a method for the isolation and expansion of iNKT cells

- To analyse the phenotype and function of iNKT cells

- To establish a system to study DC-iNKT cell interactions *in vitro*

- To test αGalCer analogue biological functionality

Chapter 2: Materials & Methods

2.1 Materials

2.1.1 Human Peripheral Blood Samples

Human Peripheral blood mononuclear cells were obtained with permission from healthy donors in EDTA coated tubes or obtained from buffy coat packs supplied by the Irish Blood Transfusion Service, St James Hospital, Dublin 8, with the approval of the NUI Maynooth Ethical Committee.

2.1.2 Reagents

Reagents used in this project are shown in Table 1 below with the exception of monoclonal antibodies (mAb) which have a dedicated sub-section and table.

Table 1: Reagents used in this thesis and their sources

Product	Company
15 ml centrifuge tubes	Sarstedt, Germany
20 μm nylon mesh filter	Falcon 2340, USA
24-well tissue culture plate	Nunc, Denmark
3,3',5,5' tetramethylbenzidine (TMB)	Sigma Aldrich, UK
40 μm nylon mesh filter	Falcon, USA
50 ml centrifuge tubes	Sarstedt, Germany
6-well tissue culture plate	Nunc, Denmark
96-well flat bottomed tissue culture plate	Nunc, Denmark
96-well Maxisorp ELISA plates	Nunc, Denmark
96-well round bottomed tissue culture plate	Nunc, Denmark
α-galactosylceramide	Kirin, Japan
Anti-phycoerythrin microbeads	Miltenyi Biotech, GmbH

Bovine serum albumin	Sigma Aldrich, UK
Brefeldin A	Sigma Aldrich, UK
Cell Trace CFSE proliferation kit	Invitrogen, UK
CD3 Microbeads	Miltenyi Biotech, GmbH
CD14 Microbeads	Miltenyi Biotech, GmbH
CD56 Microbeads	Miltenyi Biotech, GmbH
Cryovials	Nagene, Denmark
Dimethyl-sulfoxide (DMSO)	BDH, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, UK
Ethidium Bromide (EtBr)	Sigma Aldrich, UK
Flow Cytometry Tubes	Falcon, USA
Foetal Bovine Serum (FBS)	Gibco, NZ
Foetal Calf Serum (FCS)	Gibco, NZ
Fungizone	Gibco, NZ
Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)	R&D Systems, UK
H_2SO_4	Sigma Aldrich, UK
Hanks Buffered Salt Solutions	Gibco, NZ
HEPES Buffer	Gibco, NZ
HEK-Blue LPS detection kit	InvivoGen, France
Human IFN-γ DuoSet ELISA	R&D Systems, UK
Human IL-2 DuoSet ELISA	R&D Systems, UK
Human IL-4 DuoSet ELISA	R&D Systems, UK
Human IL-10 DuoSet ELISA	R&D Systems, UK
Human IL-12 DuoSet ELISA	R&D Systems, UK
Human IL-13 DuoSet ELISA	R&D Systems, UK
Hyclone Foetal Calf Defined Serum	Hylclone, USA
Isoglobotrihexosylceramide (iGb3)	Alexis, Switzerland
Interleukin 2 (IL-2)	R&D Systems, UK

Interleukin 4 (IL-4)	R&D Systems, UK
Ionomycin	Sigma Aldrich, UK
Lipopolysaccharide (LPS)	Alexis, Switzerland
LS Separation Columns	Miltenyi Biotech, GmbH
Lymphoprep	Nycomed, Norway
Normal Goat Serum	Sigma Aldrich, UK
Normal Mouse Serum	Sigma Aldrich, UK
Parafilm	Fisher Scientific
Paraformaldehyde (PFA)	Sigma Aldrich, UK
Pasteur Pipettes	Sarstedt, Germany
Penicillin Streptomycin	Gibco, NZ
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich, UK
Phosphate Buffered Saline Tablets	Sigma Aldrich, UK
Phytohaemagglutin (PHA)	Sigma Aldrich, UK
Poly I:C	Invitrogen, UK
Preseparation Filters	Miltenyi Biotech, GmbH
Roswell Park Memorial Institute (RPMI)	Gibco, NZ
S-Monovette Vacutainers	Sarstedt, Germany
Saponin	Sigma Aldrich, UK
Sodium Azide	Sigma Aldrich, UK
T25 Tissue Culture Flasks	Sarstedt, Germany
T75 Tissue Culture Flasks	Sarstedt, Germany
Total cytotoxicity & apoptosis detection kit	Immunochemistry Technologies, USA
Tween 20	Sigma Aldrich, UK

2.1.3 Monoclonal Flow Cytometry Antibodies

The monoclonal antibodies (mAb) used in this project are listed in table 2. The conjugated fluorochrome, supplier and catalogue number are also listed. Unconjugated CD1a, CD1b, CD1c, CD1d are non commercial and a kind gift from Prof. Steven Porcelli, Albert Einstein School of Medicine, NY, USA.

Table 2: Monoclonal antibodies used in this thesis and their sources

Antibody	Fluorochrome	Company	Catalogue Number
IFN-γ	PE	BD Biosciences	554552
IL-4	PE	BD Biosciences	554516
IL-10	PE	BD Biosciences	554498
IL-13	PE	BD Biosciences	559328
CCR7	PE	BD Biosciences	552176
CD1a	PE	BD Biosciences	555807
CD1a	Unconjugated	Non commercial	
CD1b	FITC	BD Biosciences	555969
CD1b	Unconjugated	Non commercial	
CD1c	FITC	Miltenyi Biotech	130-090-507
CD1c	Unconjugated	Non commercial	
CD1d	PE	BD Biosciences	550255
CD1d	Unconjugated	Non commercial	
CD3	PE-Cy5	BD Bisosciences	555334

CD4	FITC	BD Bisosciences	555346
CD8	PE	BD Bisosciences	555635
CD11c	PE	BD Bisosciences	555392
CD14	PE-Cy5	BD Bisosciences	550787
CD19	APC	BD Bisosciences	555415
CD25	PE	BD Bisosciences	555432
CD28	FITC	BD Bisosciences	555728
CD40	PE-Cy5	BD Bisosciences	555590
CD54	APC	BD Bisosciences	559771
CD56	FITC	BD Bisosciences	345811
CD69	PE-Cy5	BD Bisosciences	555532
CD80	APC	Immunotools	21270806
CD83	PE	BD Bisosciences	556855
CD86	FITC	BD Bisosciences	555657
CD94	FITC	BD Bisosciences	555888
CD161	FITC	BD Bisosciences	556080
Goat Anti Mouse	FITC	BD Bisosciences	553443
Goat Anti Mouse	PE	BD Bisosciences	550083
HLA-DR	FITC	BD Bisosciences	555811
Invariant NKT (6B11)	PE	BD Bisosciences	552825
NKG2D	APC	BD Bisosciences	558071

| Vα24 | FITC | Immunotech | IM-1589 |
| Vβ11 | PE | Immunotech | IM-2290 |

2.1.4 Glycolipids

The glycolipids used in this project are listed in table 4. The supplier and catalogue

number are also listed.

Glycolipid	Supplier	Cat Number
αGalCer	Enzo Life Sciences	BML-SL232-0100
iGb3	Enzo Life Sciences	ALX-306-028-M001

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2.2 Cellular Preparation

2.2.1 Generation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells were isolated from whole venous blood by lymphoprep gradient density centrifugation. Venous whole blood was taken into EDTA coated vaccutainers as an anticoagulant and diluted 1:1 with Hanks Buffered Salt Solution (HBSS) supplemented with 1% FCS. Buffy coat packs where mixed with 5 ml of 0.5% w/v EDTA and diluted 1:4 with HBSS supplemented with 1% v/v FCS. Carefully, 35 ml of diluted blood was layered onto 15 mls of Lymphoprep in a 50 ml centrifuge tube and centrifuged for 25 minutes at 400 g without accelerator or brake. The plasma layer was aspirated and discarded. The buffy coat was transferred into a clean centrifugation tube. The remaining Lymphoprep and red blood cell pellet were discarded. The buffy coats were diluted with 20 ml of HBSS containing 1% v/v FCS and centrifuged for 5 minutes at 800 g with accelerator and brake applied. The supernatants were aspirated and discarded; the cell pellet was resuspended by vortexing. The cells were again resuspended in 20 ml of HBSS supplemented with 1% v/v FCS and centrifuged for 10 minutes at 300 g. The supernatant was discarded and the cell pellet resuspended by vortexing and diluted in complete RPMI (RPMI 1640 with Glutamax medium supplemented with 10% v/v FCS, 1% v/v penicillin-streptomycin, 1% v/v fungizone and 25mM HEPES).

2.2.2 Enumeration of cells

Cells in suspension were enumerated by UV microscopy using a haemocytometer. Briefly 20 µl of cell suspension was diluted with 380 µl of ethidum bromide acridine orange (EBAO) (0.8ml of 4mg/ml EB and 2 ml of 1mg/ml AO in 200 ml of 0.85% w/v NaCl) in a 0.5 ml tube and mixed by vortexing. Carefully 10 µl of the final dilution was applied to the haemocytometer and cells were counted in two opposing 1.0 mm x 1.0 mm sections. Each of these squares accommodates 0.1 mm^3 of liquid; therefore the number of cells in 1 ml is calculated and multiplied by the dilution factor. Live cells fluoresce green whilst dead cells appear orange under UV light after EBAO staining.
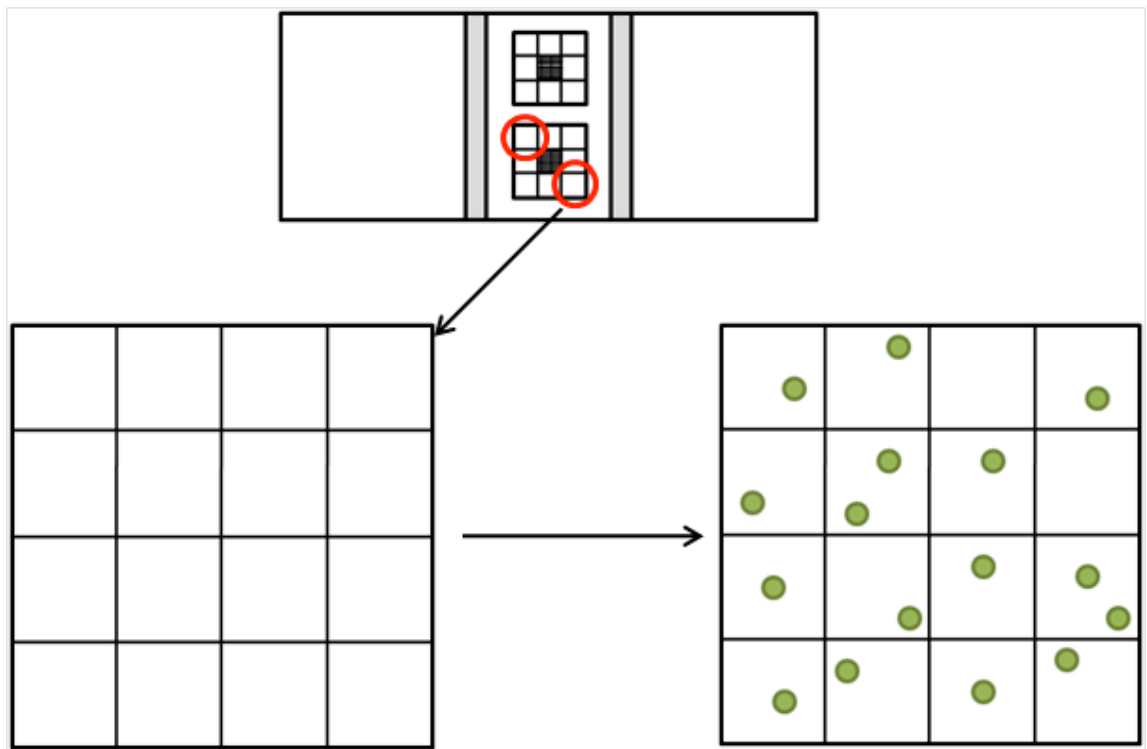
Figure 2.1: Enumeration of cells using the haemocytometer. 2 grids of 16 squares are chosen and the number of live cells counted (green.) Each of these squares accommodates 0.1 mm^3 of liquid; therefore the number of cells in 1 ml is calculated and multiplied by the dilution factor to give the final concentration of cells.

2.2.3 Cryopreservation and reconstitution of cells

For long term storage, cells were cryopreserved in liquid nitrogen. Cells were pelleted by centrifugation for 7 mins at 400 g, the supernatant was discarded and the cells resuspended in freezing mixture (90% FBS and 10% DMSO, made freshly but in advance to allow exothermic heat produced from mixing to subside). Cells were immediately transferred to labelled cryovials and frozen at -80ºC for 24 hours before being transferred to liquid nitrogen stores. Cryopreserved cells were reconstituted by rapid thawing in a 37ºC water bath and transferring into universal tube containing pre-warmed complete RPMI. Cells are centrifuged for 10 mins at 300 g, the supernatants are discarded and cell pellets resuspended in complete RPMI. Recently recovered cells were incubated at concentration of $1X10^6$ cells/ml for 24 hours at 37ºC with 5% CO_2 before use.

2.2.4 Cell lines

Several cell lines were used in the studies within this thesis. The C1R lymphoblastoid cells which where stably transfected with CD1a, CD1b, CD1c or CD1d and a mock transfected C1R cell (A kind gift from Prof Steven Porcelli, Albert Einsten School of Medicine NY, USA). The NK sensitive tumour cell line K562 was also used as target cell for cytotoxicity. All cell lines were cultured in complete RPMI in T175 vented tissue culture flasks and maintained at a density of $5X10^5$ cells/ml 37ºC with 5% CO_2.

2.2.5 Magnetic bead separation of cells

PBMC were separated into purified populations of monocytes (CD14+), T cells (CD3+) and iNKT cells (6B11+). PBMC were resuspended in 80 µl per $1X10^7$ cells of cold Miltenyi Buffer (PBS supplemented with 0.5% BSA) by vortexing. Next 20 µl of magnetic anti-human marker Microbeads were added to each tube and incubated at 4ºC for 15 mins. Cells were diluted with 10 ml of buffer and centrifuged for 10 mins at 300 g in a 4ºC centrifuge to remove excess Microbeads. Supernatants were removed by pipetting and discarded. Cell pellets were resuspended in 500 µl /$1X10^7$ total cells of cold Miltenyi Buffer and applied to pre-washed LS separation column and magnet. The cell suspension was washed through with five 3 ml washes of cold buffer into waste collection tube. The column was removed from the magnet and 5 ml of cold buffer was forcefully pushed through into a clean universal tube. Cells were centrifuged for 10 mins at 300 g in a 4ºC centrifuge and resuspended in complete RPMI at concentration of $1X10^6$ cells/ml. Cell purities were examined by flow cytometry and cell yields were determined by EBAO enumeration.

2.3 Flow Cytometry

Flow cytometry is a technique for rapidly analysing multiple parameters on large numbers of living (or dead) cells. Single cells suspended in a stream of fluid pass through a laser beam and interact with the light. Biochemical and biophysical characteristics of individual cells are quantified by measurement of visible and fluorescent light emissions.

A number of photodetectors are strategically placed inside the machine to detect transmitted and scattered light. One detector in line with the laser beam detects Forward Scatter (FSC) whereas another placed at 90º to it detects Side Scatter (SSC). There are also usually one or more fluorescent detectors (FL-1, 2, 3, 4) (Figure 2.2). Cell populations are distinguished on the basis of morphological differences as indicated by differences in their FSC and SSC. On this principle, larger and more granular cells such as macrophages are easily distinguished from smaller less granular lymphocytes. Flow cytometry also allows the detection of multiple fluorochrome-conjugated mAbs bound to cells. Each fluorochrome when excited by the laser emits a different wavelength. The intensity of the emission is converted into a value, mean fluorescence intensity (MFI).

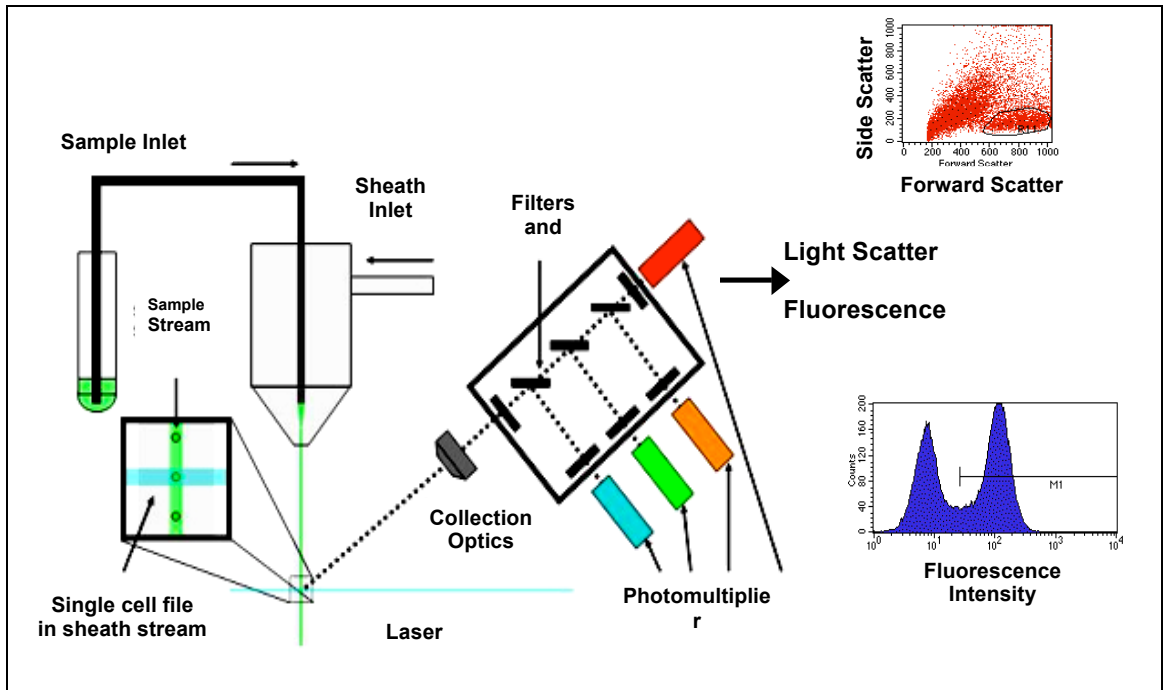*Figure 2.2: Schematic representing the principles of flow cytometry. Sample is taken into the sample inlet by suction or reverse pressure and forced into a single cell stream in sheath fluid. Cells pass through laser and the light emitted passes through filters and collected by photo multipliers which change signal from light into displayable signal. (Adapted from images at La Jolla Bioenginering Institute)*

2.3.1 Direct cell surface staining for Flow Cytometry

Cells at a concentration of $1X10^5$- $2X10^5$ in single cell suspension were prepared for each control and sample tube. Cells were washed using 2 ml per $2X10^5$ cells of PBA buffer (PBS with 1% v/v BSA and 0.02% w/v sodium azide), then centrifuged at 300g for 10 mins. Cells were resuspended in 80 μl of PBA per sample and stained with the appropriate amount of each required fluorochrome labelled mAb, cells were simultaneously stained with up to 4 mAbs, each with fluorochromes that emit light at a different wavelength, or relevant IgG isotype controls. Cell samples were also stained with single colour antibodies and stained as fluorescence minus one (FMO) controls (1 colour is omitted, i.e PE FMO is FITC, PER-CP and APC). Samples were vortexed and incubated away from light for 15 mins at room temperature to allow binding. Cells are then washed as before to remove any unbound mAb. Fluorochrome labelled cells were resuspended in 500 μl of PBA or 1% PFA, incubated at 4ºC and acquired within 1 hour or 24 hours respectively.

2.3.2 Indirect cell surface staining for Flow Cytometry

As with direct staining $1X10^5$- $2X10^5$ cells in single cell suspension were prepared for each control and sample tube in 80 μl of PBA per sample and stained with 0.05-0.2 μg per $2X10^5$ cells with primary unlabelled mAb. Optimal amounts of mAb were tested for each new lot of unlabelled mAb by comparing staining intensities of varying amounts (0.01-1μg) of mAb. Cells were incubated for 10 min at room temperature in the dark. Excess mAb was washed as previously described. Cells were resuspended in

80 µls of PBA and stained with 5 µl per $2X10^5$ of fluorochrome labelled secondary mAb (anti-primary mAb) and incubated for 15 min at room temperature and washed to remove excess mAb. Cells were resuspended in 80 µl of PBA with 4% normal mouse serum to block non-specific binding sites for 10 min at room temperature. Cells were washed and resuspended. The cells were then stained with mAbs that were directly conjugated with fluorochrome as described above.

2.3.3 Intracellular staining for Flow Cytometry

$1X10^6$- $1.5X10^6$ cells were prepared for each control and sample tube, and incubated for 24 hours with or without specific stimuli (i.e. αGalCer). Cells were stimulated for 4 hours with medium alone, αGalCer (100 ng/ml), PMA (10 ng/ml) and ionomycin (1 µg/ml). Brefeldin A (BFA prevents cytokine secretion) was added to each sample at concentration of 10 µg/ml. Cells were then washed using 2 ml per $1X10^6$ cells of cold PBA, then centrifuged at 300 g for 10 mins. Cells were resuspened in 80 µl of PBA per sample. The appropriate amount of each surface staining mAb was added and incubated at room temperature for 15 min. Excess antibody was diluted with PBA and removed by centrifugation. Cells were resuspended in 80 µl of cold PBA. Next 500 µl of 4% paraformaldehyde was added to each sample for 10 mins at room temperature to fix cells (stops internalisation of mAb). Cells were washed with 2 ml per $1X10^6$ cells of cold PBS with 1% v/v BSA (PBA), and then centrifuged at 300 g for 10 mins. Cells were permeabilised by adding 1 ml of 0.2% w/v saponin in PBA to each sample and incubating for 10 mins at room temperature in the dark. Excess saponin was removed by dilution and centrifugation. Cells were stained for the presence of intracellular

cytokine by adding the approriate amount of anti-human intracellular cytokine diluted in 50 µl of 0.2% w/v saponin or appropriate control antibody to each and sample and incubated for 20 mins at room temperature in the dark. Excess antibody was diluted and removed by centrifugation. Samples were resuspended in 500 µl of 1% PFA and acquired by flow cytometry.

2.3.4 Flow Cytometry Analysis

Post acquisition analysis was carried out using Cell Quest (BD). Briefly, analysis was based on the FSC and SSC of the total cell population, a single or series of electronic gates determining the population of cells analysed were set (Figure 2.3). Isotype controls are used to set the detectors and settings, allowing visualisation of a negative population. Isotype controls used in this study were all IgG (different isotypes) and matched to the corresponding specific labelling antibody; this allows detection and compensation for non specific binding (Figure 2.3). Flow cytometric histograms are used when analysing single staining, markers set according to isotype control determine positivity (Figure 2.3). Single stained cell and FMO sample dot plots were used for flourochrome compensation settings when analysing 2 or more staining mAb, quadrants determining positivity were set according to isotype controls (Figure 2.3). For analysis of lymphocytes, the following combinations of mAb were used; T-helper cells (CD3+, CD4+), CTLs (CD3+, CD8+), NK cells (CD56+, CD3-), CD56+ T cells (CD56+, CD3+), iNKT cells (CD3+, 6B11+) (Analyse sample: Figure 2.4). Cell Quests inbuilt statistical program determined % of total cell, % of gated cells and the mean fluorescence intensity values.
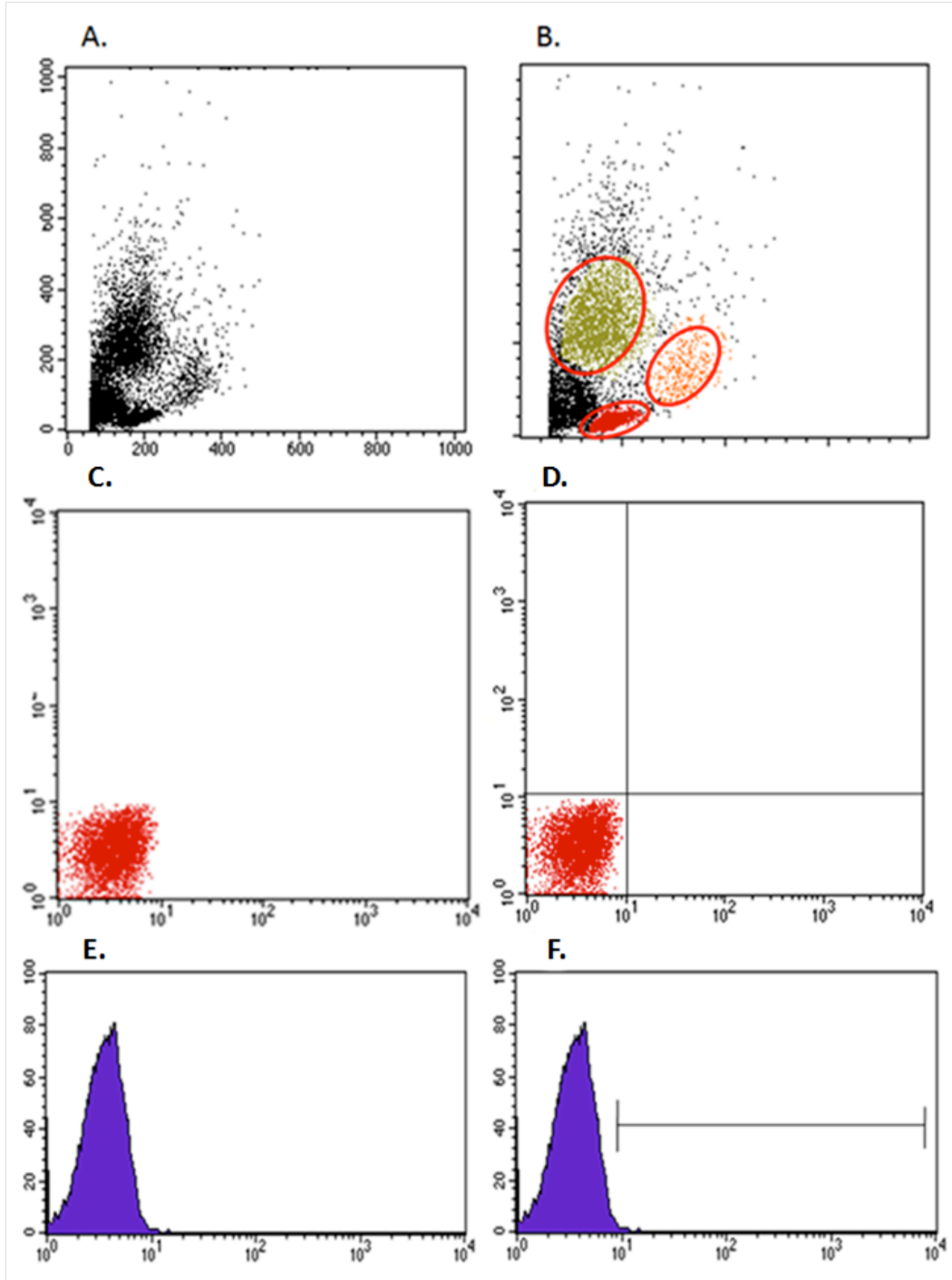
Figure 2.3: Flow cytometry analysis and electronic population gating: A, Sample flow cytometric dot plots showing total PBMC. B, PBMC electronically gated into separate populations of lymphocytes (red), monocytes (orange) and residual granulocytes (green). C&D, Sample flow cytometric dot plots showing IgG isotype control staining and same plot showing relevant quadrant. E&F, Flow cytometric histogram showing IgG isotype and same histogram showing the relevant marker.
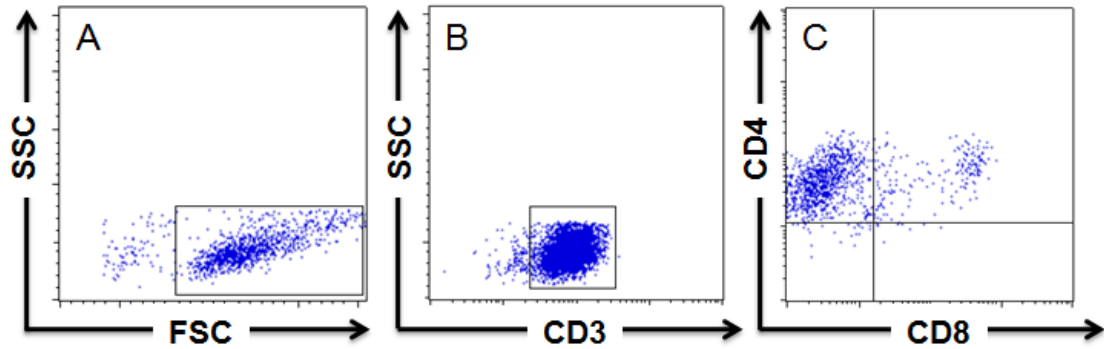
Figure 2.4: Flow cytometry dot plots showing, A, total electronically gated T cell lines. B, Parent population from (A) electronically gated into CD3+ cells. C, Dot plot showing CD4+ and CD8+ cells from the CD3+ parent population selected in (B).

2.4 Enzyme linked immunosorbent assay (ELISA)

ELISA paired antibody kits from R&D systems were used for identification of secreted cytokine in assay supernatants. Firstly, 96 well plates were coated with 100 µl per well of specific capture antibody against the target protein (e.g. IFN-γ, IL-4, IL-10, IL-12 and IL-13) at concentration outlined in manufacturer's protocol in PBS buffer overnight at room temperature, covered in film and kept away from direct light. Excess antibody was removed by washing 5 times with phosphate buffered saline and detergent and the plate was thoroughly dried. Non specific binding sites were blocked by adding 200 µl per well of PBS containing 1% v/v BSA (BSA acting as a non specific protein) and incubated at room temperature for 2 hours covered and kept away from light. The wash step outlined was repeated and the plate was dried by blotting. 80 µl per well of sample supernatant was added to triplicate wells. A high standard was added in triplicate to each plate using recombinant cytokine (at given concentration) and serially diluted row by row to give a 7 point standard curve and an 8th triplicate was left blank. Samples and standards were incubated at 4ºC overnight covered in film and

away from direct light. Plates were washed as before and 100 µl of specific detection antibody (at manufacturers recommended concentration) was added to each well. Plates were incubated at room temperature for 2 hours covered in film and away from light. Excess detection antibody was washed away and 100 µl of Streptavidin/Horse Radish Peroxidise (HRP) was added to each well at concentration outlined by manufacturer's protocol. Plates were incubated for 20 minutes at room temperature covered in foil. Plates were again washed and 100 µl of 1:1 mix of substrate solutions A and B was added to each well and incubated at room temperature for 20 mins covered in foil. The enzyme reaction was stopped by adding 50 µl per well of 2N H_2SO_4. Plates were immediately read at 450nm using Tecan Sunrise plate reader (Figure 2.5).
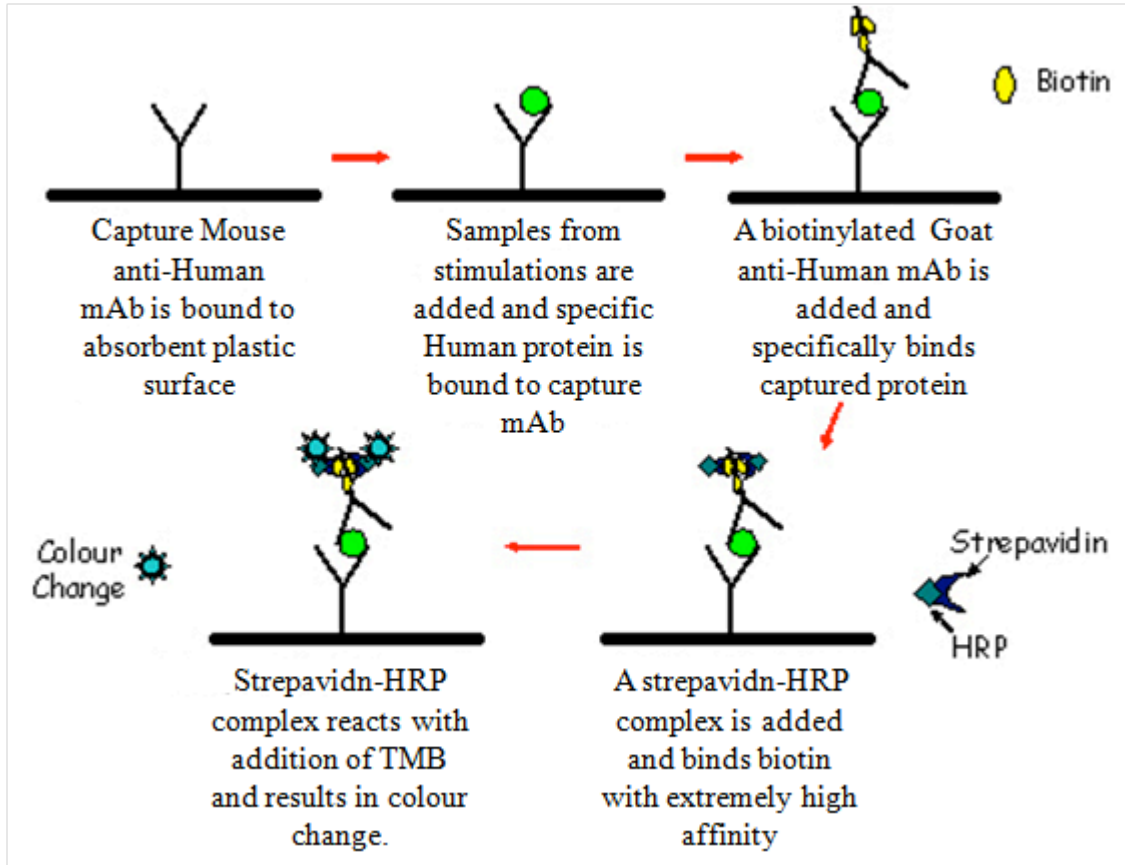
Figure 2.5: Schematic representation of Enzyme Linked Immunosorbent Assay (ELISA) showing the major steps; from coating with capture anitbody, addition of samples, detection using biotin coated antibody, addition of streptavidin-HRP complex and final colourimetric step.

2.5 Human CD1 restricted T cells

2.5.1 Preparation of iNKT cell ligands

Glycolipid antigens; αGalCer, iGb3, SαGalCer, SαGalCer14 and Analogues W1-4 (analogues described in chapter 6) were suspended in DMSO and vigorously vortexed for 10 minutes. Glycolipid stocks were then diluted to a concentration of 1 mg/ml in PBS containing 10% DMSO in PBS. The solution was split into 10 µl aliquots and frozen at -80°C. Several other solvents are recommended by manufactures for lipid solubility including; pyridine and 2:1 chloroform/methanol, however these solvents proved toxic to cells in culture and were not used.

2.5.2 Isolation of 6B11 positive iNKT cells

PBMC were isolated from whole blood by lymphoprep density gradient centrifugation and enumerated as described in previous sections. Cells were diluted with PBS to a density of 1×10^8 cells per 100 µl in a 15 ml Falcon centrifugation tube. Next 100 µl of mouse anti-human 6B11 mAb conjugated with PE was added to cell aliquots and incubated at room temperature, away from direct light for 15 minutes. Cells were diluted with 10 ml of PBS containing 1% v/v BSA and centrifuged for 10 minutes at 300g to remove excess mAb. 6B11 positive cells were then magnetically separated using anti-PE Microbeads as previously described in section 2.2.5. The magnetically purified iNKT cells where subsequently expanded using three methods outlined in section 4.1. Each method is detailed in a dedicated sub-section below. Briefly the iNKT

cells where stimulated using; a mitogen, antigen or glycolipid and maintained in culture media containing IL-2.

2.5.3 Generation of 6B11 positive iNKT cell lines: Method 1

Magnetically isolated 6B11+ cells were plated in 24 well plates in 1 ml of cRPMI medium per well. Cells were stimulated with (1:1 cell ratio) irradiated (4000 RADS for 11 minutes) PBMC feeder mix (2 different donors at concentration of $5X10^5$ per 500 μl) in cRPMI and 100 ng/ml of αGalCer. 24 hours after isolation, cells were supplemented with 50 U/ml of recombinant IL-2. Cell cultures are examined every 3 days and supplemented with fresh medium containing 50 U/ml IL-2. When cells reached ~50% confluency, each well was split into 2 wells with the addition of 500 μl per well cRPMI and 50 U/ml IL-2.

2.5.4 Generation of 6B11+ iNKT cell lines: Method 2

As outlined in section 2.5.3, 6B11+ cells were plated with (1:1 cell ratio) irradiated PBMC feeder mix (2 different donors at concentration of $5X10^5$ per 500 μl) in cRPMI and stimulated with 5 μg/ml of PHA. 24 hours after isolation cells were supplemented with 50 U/ml of recombinant IL-2. Cell cultures were examined every 3 days and supplemented with fresh medium containing 50 U/ml IL-2. When cells reached ~50% confluency, each well was split into 2 wells with the addition of 500 μl per well of complete RPMI and 50 U/ml IL-2.

2.5.5 Generation of 6B11+ iNKT cell lines: Method 3

Isolated 6B11+ cells were plated at concentration of $1X10^6$ cells/ml on 24 well plates coated with 10 µg/ml OKT-3 (wells coated with 10 µg in 1ml of PBS for 4 hours at room temperature or 4°C overnight). Irradiated feeder cells were not added. 24 hours post stimulation; medium was supplemented with 100 U/ml of IL-2. Cells were cultured for 7 days after which spent medium was replaced with cRPMI supplemented with 100 U/ml of IL-2. When cells confluency exceeded 50%, each well was split into 2 wells (new well not coated with OKT-3) as described previously.

2.5.6 Characterisation of 6B11+ iNKT cell lines

After several rounds of stimulations, cell line purities were examined by flow cytometry. Briefly $2X10^5$ cells were triple stained with Vα24 FITC, Vβ11 PE and CD3 PE-Cy5 or CD161 FITC, 6B11 PE and CD3 PE-Cy5, CD8 FITC, CD4 PE and CD3 PE-Cy5 or CD94 FITC, CD56 PE and CD3 PE-Cy5. Also $2X10^5$ cells from 2 cell lines (due to budget restrictions) were stained with a CD1d PE tetramer (previously loaded overnight with αGalCer) and CD3 PE-Cy5. Cells were stained also for relevant IgG controls. Cells were acquired and analysed as described in section 2.3.4.

2.5.7 Analysis of T cell reactivity against CD1 transfected C1R cells

PBMC or purified CD3+ T cells were prepared as described previously and cultured at a concentration of $0.2X10^6$ cells per 100 µl of cRPMI. C1R cells expressing transfected CD1 were also cultured at $0.2X10^6$ cells per 100 µl. PBMC or CD3+ cells (100 µl per well) were co-cultured with 100 µl per well of each CD1 isotype transfectant C1R cell line including the mock transfected C1R cell, as a negative control for 3 days at 37ºC with 5% CO_2. PBMC or CD3+ T cells stimulated with 10 µg/ml of PHA served as a positive control. On day 3 supernatants were harvested and assayed for IFN-γ or IL-13 by ELISA. C1R-CD1d cells were either unloaded or loaded for 24 hours with 100 ng/ml of αGalCer, iGb3 or synthetic glycolipids.

2.5.8 Analysis of iNKT cell reactivity against CD1d transfected C1R cells

6B11+ iNKT cell line reactivity was examined against either mock transfected C1R cells or CD1d transfected C1R cells loaded or unloaded (2 hours prior to culture) with 100 ng/ml of αGalCer, iGb3 or synthetic glycolipid. $2X10^5$ 6B11+ iNKT cells were co-cultured with equal amounts of CD1d-transfectant cells, in the absence or presence of glycolipid, in round bottomed 96 well plates for 3 days at 37ºC with 5% CO_2. On day 1 & 3 supernatants were harvested and assayed for secreted IFN-γ and IL-4 by ELISA (Individual assay procedures are outlined within results chapters).

2.5.9 Analysis of iNKT cell cytotoxicity

The total cytotoxicity and apoptosis detection kit provides a flow cytometry based method for the detection of cell lysis. Cytotoxicity by iNKT cell lines was examined against either K562 or C1R-CD1d target cells. Briefly target cells were incubated with 1:250 dilution of stock carboxyfluorescein succinimidyl ester (CFSE) at 37ºC with 5% CO_2 for 30 mins. The reaction was quenched by adding 10 mls of cold cRPMI. Target cells were pelleted by centrifugation for 10 minutes at 300g with no accelerator or break. Target cells were incubated with effector cells (6B11+ NKT cells) at ratios of 1:1, 10:1 and 20:1 for 4 hours at 37ºC with 5% CO_2. After incubation, cells were harvested and incubated on ice. 7-aminoactinomycin D (7-AAD) (1:10 dilution) stain was added immediately before acquisition by flow cytometry. An electronic gate selected the CFSE positive cells. CFSE positive cells which stain double positive for 7-AAD have recently undergone apoptosis.

2.6. Dendritic Cells

2.6.1 Generation of monocyte derived dendritic cells

PBMC were isolated from whole blood and enumerated as described in previous sections. Cells were diluted with PBS containing 1% v/v BSA to a volume of $1X10^8$ cells per 100 µl in a 50 ml centrifugation tube and incubated with 100 µl of anti human CD14 Microbeads for 30 minutes at 4°C. Excess microbeads were removed by dilution with PBA and centrifugation at 300 g for 10 minutes. Cells were magnetically separated as described in section 2.5.2, washed and resuspended at a concentration of $1X10^6$ cells per ml in cRPMI containing defined Hyclone Foetal Calf Serum (dcRPMI). Hyclone defined FCS was used to prevent maturation of the DC due to endogenous endotoxins such as LPS (Figure 2.6). The endotoxin levels in batches of sera were tested using the invitrogen HEK-blue assay. CD14+ cells were plated on 6 well tissue culture plates, to give final concentration of $3X10^6$ cells in 3 ml per well. Immature DC were generated from CD14+ monocytes by culture with 50 ng/ml of GM-GSF and 70 ng/ml of IL-4. Cultures were incubated for 3 days at 37ºC with 5% CO_2. On day 3 medium was replenished by removing 1.5 ml per well of spent media and adding 2 ml per well of dcRPMI containing 50 ng/ml of GM-GSF and 70 ng/ml of IL-4. Cultures were incubated for a further 3 days at 37ºC with 5% CO_2. On day 6, immature DC were carefully harvested from wells by gentle agitation and flushing using dcRPMI and a pasteur pipette. iDC were enumerated and fixed to a final concentration of $1X10^6$ cells per ml in dcRPMI.
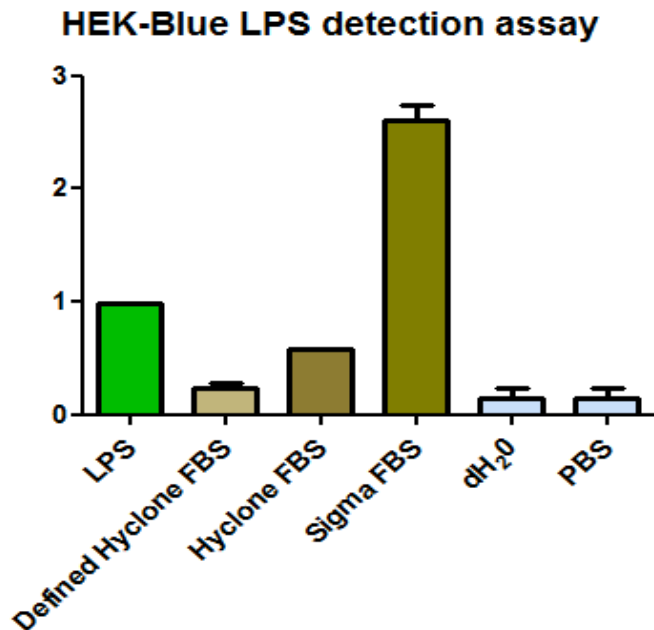
Figure 2.6: Detection of LPS using HEK Blue reporter cells. Bar graphs showing the levels of LPS in batches of FBS. LPS was used as positive control and PBS as negative control. HEK-Blue cells cultured in the presence of HEK-Blue selection mix are cultured in the presence of 20 μl of FBS for 24 hours. In the presence of LPS the reporter cells turn medium blue. Graphs are representative of 3 separate experiments

2.6.2 Stimulation of dendritic cells with LPS and poly I:C

Freshly isolated iDC were plated in triplicate in 96 well round bottom tissue culture plates at $1X10^5$ cells per 100 μl in dcRPMI. 100 μl of dcRPMI was added to the first triplicate (medium alone), 100 μl of dcRPMI and 1 μg/ml of LPS (final concentration) was added to the second triplicate (LPS) and 100 μl of dcRPMI and 7.5μg/ml of poly I:C was added (Poly I:C) to the final triplicate. Cultures were incubated for 24 hours at 37ºC with 5% CO_2.

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2.6.3 Stimulation of dendritic cells with iNKT cells

Freshly isolated iDC were plated in triplicate in 96-well round bottom tissue culture plates at $1X10^5$ cells per 100 µl in dcRPMI. 100 µl of dcRPMI was added to the first triplicate (medium alone). iNKT cells; $1X10^5$ cells in 100 µl of dcRPMI were added to the second triplicate (iNKT), the same amount of iNKT cells were added to the third triplicate of iDC, along with 100 ng/ml of αGalCer or synthetic analogues (iNKT + αGalCer, analogues). PBMC were substituted as a non iNKT cell control into the 2nd and 3rd triplicates. The final triplicate was a lipid control to which 100 µl of dcRPMI was added with 100 ng/ml of αGalCer. Cultures were incubated for 24 hours at 37ºC with 5% CO_2. In some α-S-GalCer experiments, S-linked αGalCer replaced αGalCer.

2.6.4 Analysis of dendritic cell maturation

After 24 hours stimulation, 180 μl of supernatant were removed from the plate for analysis of cytokine secretion by ELISA. 120 μl of PBA buffer was added to each well and cell triplicates were harvested into 3 tubes A, B and C. Five micro-litre of mAb was added to each as out lined in table 6 below. Tubes were incubated for 15 minutes at room temperature in the dark to allow binding. Cells were then washed with 2 ml per tube of PBA to remove any unbound mAb and centrifuged at 300g for 10 minutes. Flourochrome labelled cells were resuspended in 500 μl of 1% PFA, incubated at 4ºC for 15 minutes and acquired on flow cytometer. As described in flow cytometry section an electronic gate was used to select the monocyte derived DC population, and the isotype controls (Figure 2.7) used to compensation for non specific binding before samples are acquired.

Table 6: DC maturation staining panel of mAb

Tube A1 or A2	Tube B	Tube C
IgG1 or IgG 2a FITC	HLA-DR FITC	CD86 FITC
IgG1 or IgG 2a PE	CD83 PE	CCR7 PE
IgG1 or IgG 2a PE-Cy5	CD40 PE-Cy5	CD14 PE-Cy5
IgG1 or IgG 2a APC	CD54 APC	CD80 APC
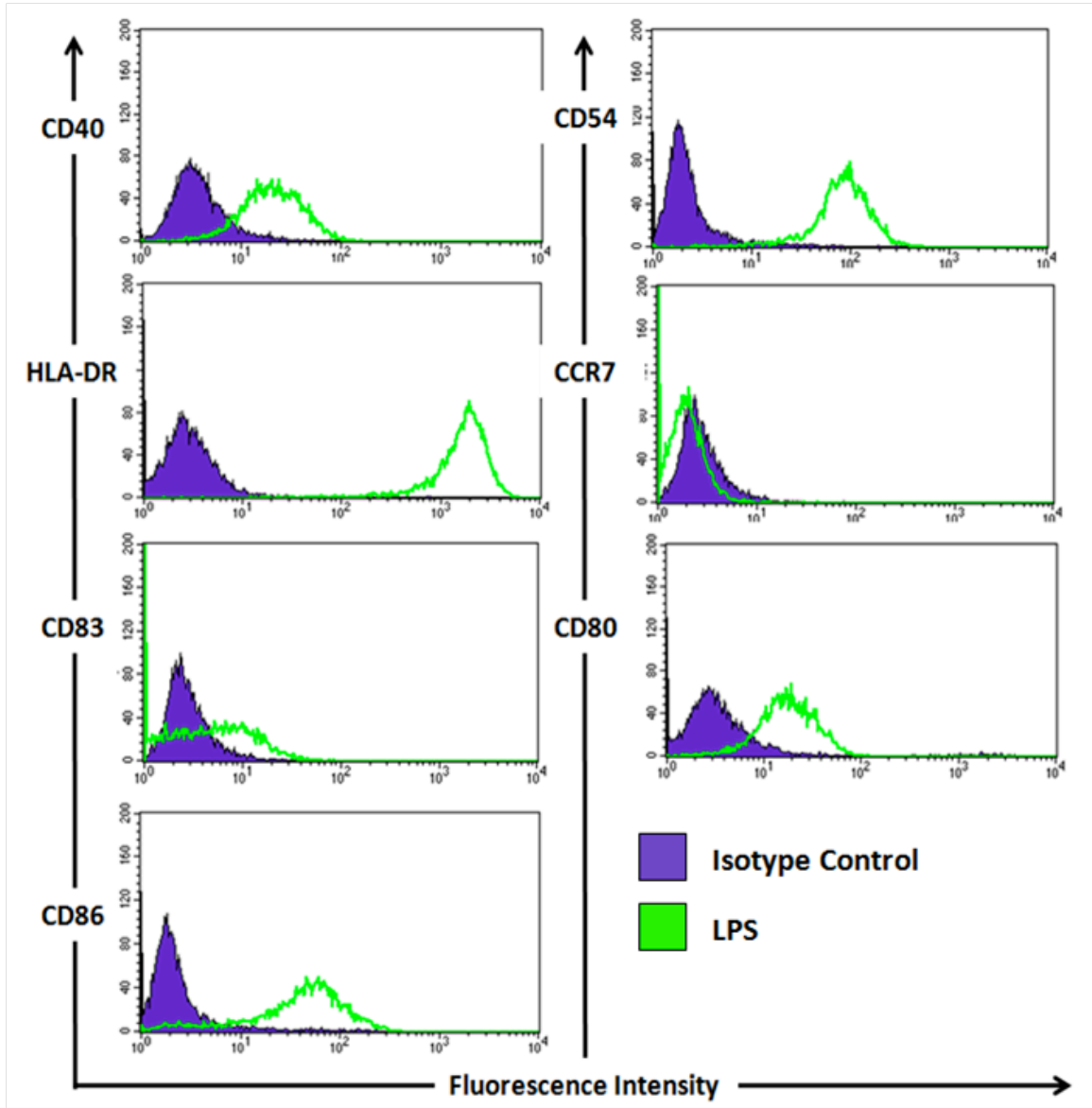
Figure 2.7: Flow cytometric analysis of DC: Flow cytometric histograms showing isotype controls used in DC maturation experiments. Purple histogram represents specific IgG isotype mAb compared to DC stained with specific mAb after stimulation with LPS. Plots are representative of 5 separate experiments.

2.6.5 Dendritic cell stimulation of allogeneic T cells

After 24 hours stimulation with medium alone, LPS, iNKT cells or iNKT cells in the presence of 100 ng/ml of lipid antigen. $1X10^4$ DC were incubated with $1X10^5$ allogeneic CD3+ T cells freshly isolated and labelled with CFSE (as described 2.5.9) in 96 round bottomed plates for 3 and 5 days. Allogeneic T cell proliferation was analysed by flow cytometry, an electronic gate selected the CFSE positive cells, proliferative divisions where calculated by examining the fold reduction in CFSE intensity. Supernatant from the co-cultures were examined for cytokine production by ELISA

2.7 Statistical Analysis

Data was tested for normality using Graphpad Prism® statistical package. Normal data was analysed by Students T test (Graphpad Prism®). Data with non-normal distribution was analysed using a Mann Whitney U test (Graphpad Prism®). P values < 0.05 were considered as significant with 95% confidence.

Chapter 3: Investigation into CD1 reactivity in human blood

3.1 Introduction

The human immune system comprises of the innate immune system and the adaptive immune system. The innate immune system recognises conserved PAMPs and endogenous molecules, and responses rapidly. The adaptive response is antigen-specific and requires time for the clonal expansion of cells before a response is elicited. Conventional T cells recognise peptide antigen presented by MHC molecules. T cells have the ability to generate a huge number of TCR structures to recognise the diverse antigen repertoire presented by MHC. A subset of T cells recognise conserved antigens, and are not restricted by MHC. These T cells are termed innate T cells. Some innate T cells recognise glycolipid antigen presented by CD1. CD1 molecules are a family of glycoproteins resembling major histocompatibility complex class 1 (MHC I) in overall structure (Gumperz 2006). Humans have 5 isoforms of CD1, CD1a-e (Martin *et al* 1986). CD1a, b, c and d function as antigen presenting molecules for T cells (Angenieux *et al* 2000). Mice have lost their group 1 CD1 molecules (CD1a, CD1b, CD1c) but have duplicated CD1d genes, CD1d1 and CD1d2 (Dascher & Brenner 2003). CD1 molecules are expressed in several tissues including intestine, thymus, liver, spleen, lung (Bradbury *et al* 1990, Bleicher *et al* 1990, Lacasse & Martin 1992, Bendelac *et al* 1997). CD1 molecules are also expressed on many cell types, most notably monocyte, DCs and B cells (Bendelac *et al* 1997, Bendelac *et al* 2007).

CD1 molecules present glycolipids to CD1 restricted T cells. In murine studies, the activation of CD1d restricted T cells resulted in the prevention and clearance of B16 melanoma tumours (Kawano 1997, Cui *et al* 1997). In the succeeding years, activation of CD1d restricted T cells in mice was found to not only clear tumours

but prevent and clear multiple bacterial, viral and parasitic infections as well as ameliorate autoimmune disease (Smyth *et al* 2002, Apostolou *et al* 1999, Kawakami *et al* 2001, Gonzalez-Aseguinolala *et al* 2000 and Baxter *et al* 1997). Kawano *et al* (1999) subsequently investigated the role of human CD1d restricted T cells in cancer, and showed the *in vitro* cytotoxicity abilities of CD1d restricted T cells. However immunotherapy in human cancer using the CD1d ligand αGalCer based on the murine studies proved disappointing with no tumour regression or clearance (Giaccone *et al* 2002). It was noted that humans have significantly lower numbers of CD1d restricted T cells than mice (Wingender & Kronenberg 2008). In contrast to mice, humans have T cells restricted by CD1a, CD1b and CD1c (Gumprez & Brenner 2001). These CD1 restricted T cell subsets are not as well defined as their CD1d counterparts due to the lack of murine models.

The majority of studies on human CD1a, b and c restricted T cells show they respond to exogenous mycobacterial glycolipids or endogenous glycolipids synthesised in response to bacterial infection (Gumprez & Brenner 2001, De Libero *et al* 2005). These studies outline the glycolipids from various microbes which are loaded onto group 1 CD1 molecules and elicit CD1 restricted T cells responses (Section 1.6.6). De Libero *et al* reported the APC upregulate endogenous GSL synthesis in the presence of bacterial infection and stimulate GSL specific T cells in and CD1 dependant manner (De Libero *et al* 2005, Muindi *et al*, 2010).

The aim of this chapter was to investigate the expression of CD1 by PBMC subsets (monocytes, DCs, NK cells and T cells) to determine the cellular expression of CD1a, CD1b, C1Dc and CD1d in healthy human peripheral blood. Expression was

investigated by flow cytometry. We also aimed to establish a system to identify and quantify the levels of CD1 restricted T cells reactivity in human peripheral blood in the absence of exogenous or microbial antigen as previous studies had examined CD1 restricted T cells in the context of infection. Briefly CD1 transfected C1R cells expressing single isoforms of CD1 were co-cultured with freshly isolated human PBMC or enriched T cells. A mock transfected C1R cell was used as a negative control and the T cell mitogen PHA was used as a positive control. T cell reactivity against the four CD1 isotypes was measured using IFN-γ and IL-13 as readouts (Figure 3.1). The CD1d binding lipids αGalCer and iGb3 were used in the iNKT reactivity studies. Finally, we used flow cytometry to investigate the frequencies of iNKT cells in human blood.

3.2 Objectives

1. To examine CD1a, CD1b, CD1c, CD1d expression on circulating lymphocyte and monocyte populations

2. To identify reactivity to CD1 isotypes a-d by human PBMC and enriched T cells

3. To identify and characterise CD1 restricted T cell populations

4. To enumerate human iNKT cells
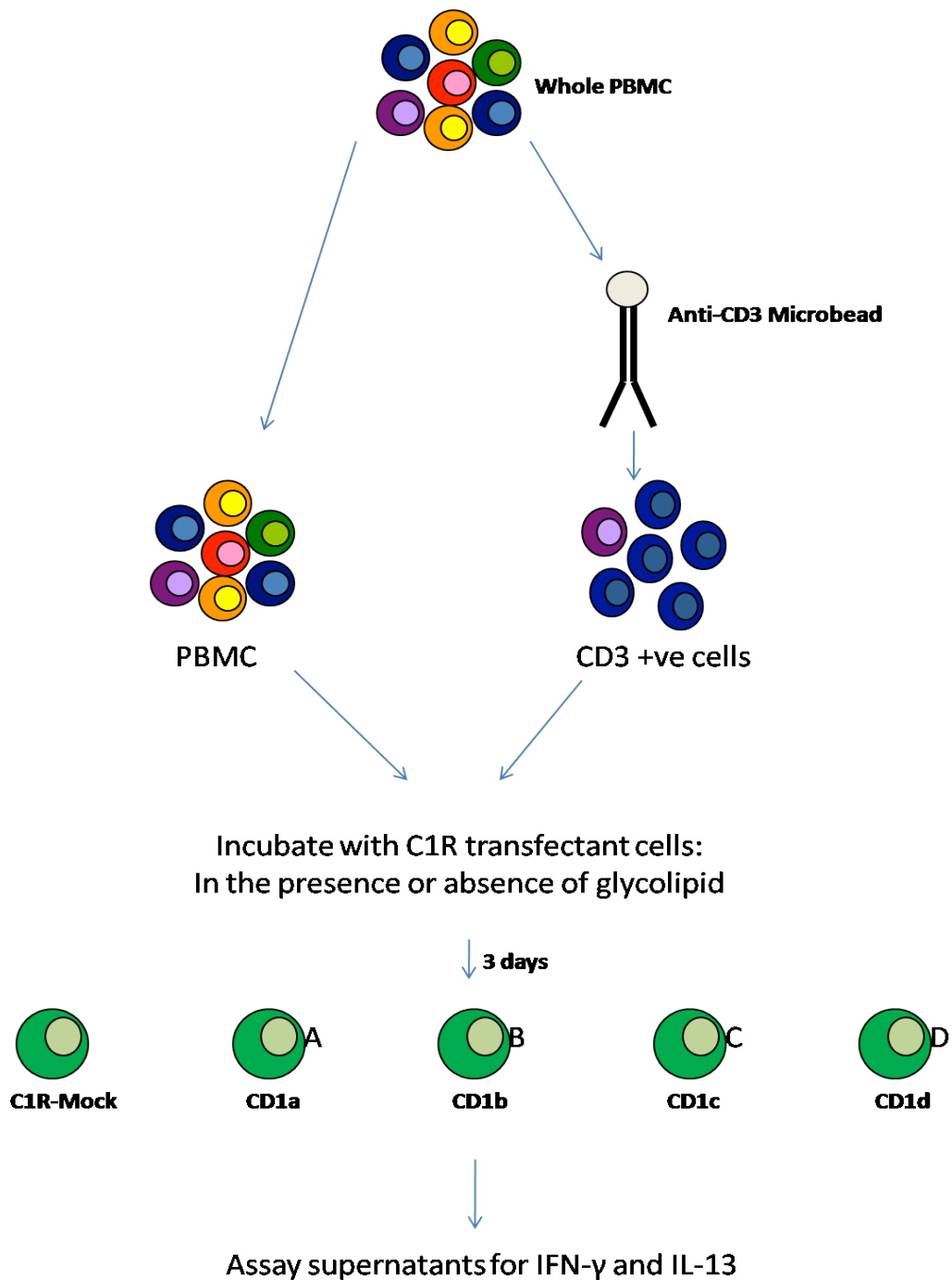
Figure 3.1: Strategy for identification of human CD1 cell reactivity in human PBMC. Peripheral Blood Mononuclear Cells (PBMC) or CD3 +ve cells isolated from PBMC were incubated with mock transfected C1R cell or transfectant C1R cells expressing either CD1a, CD1b, CD1c or CD1d, loaded or unloaded with glycolipid. The mock transfectant C1R cell served as negative control. Reactivity was measured as amount of cytokine released on day 3.

3.3.1 CD1 expression by PBMC subsets

The expression levels of four CD1 isotypes; CD1a, CD1b, CD1c & CD1d, on PBMC subsets were investigated by flow cytometry, by co-staining PBMC for the four CD1 isotypes CD1a-d with either CD19 (B cells), CD14 (monoctyes), CD3 (T cells) or CD56 (NK and CD56+ T cells). PBMC were isolated using the lymphocyte density gradient centrifugation method described in chapter 2. The cells were co-stained with the above mAbs and analysed by flow cytometry.

Cell populations which stained positive for CD19 and a single CD1 isotype were deemed to be CD1 expressing B cells (Figure 3.2A). Of the four CD1 isotypes examined, CD1d was the most abundantly expressed with a mean of 70% expressed on B cells (Figure 3.2B), CD1c was expressed on 15% of B cells, while CD1a and CD1b were expressed by mean of 2% and 1% respectively (Figure 3.2B). Cells which stained positive for both CD14 and CD1 were identified as CD1 expressing monocytes (Figure 3.3A). CD1d was the most abundantly expressed, with a mean of 90% of CD14 + cells expressing CD1d (Figure 3.3B). 15% of CD14 positive monocytes expressed CD1c (Figure 3.3B), while CD1a and CD1b was expressed on 10-12% of monocytes respectively (Figure 3.3B). Cells which stained positive for CD56 were identified as CD1 expressing NK cells or NK-like T cells (Kelly-Rogers *et al,* 2006*)* (Figure 3.4A). Cells positive for CD1 and CD3 were identified as CD1 expressing T cells (Figure 3.4B). CD1 isotype expression on NK cells and T cells was very sparse with less than 1% expressing each isotype.
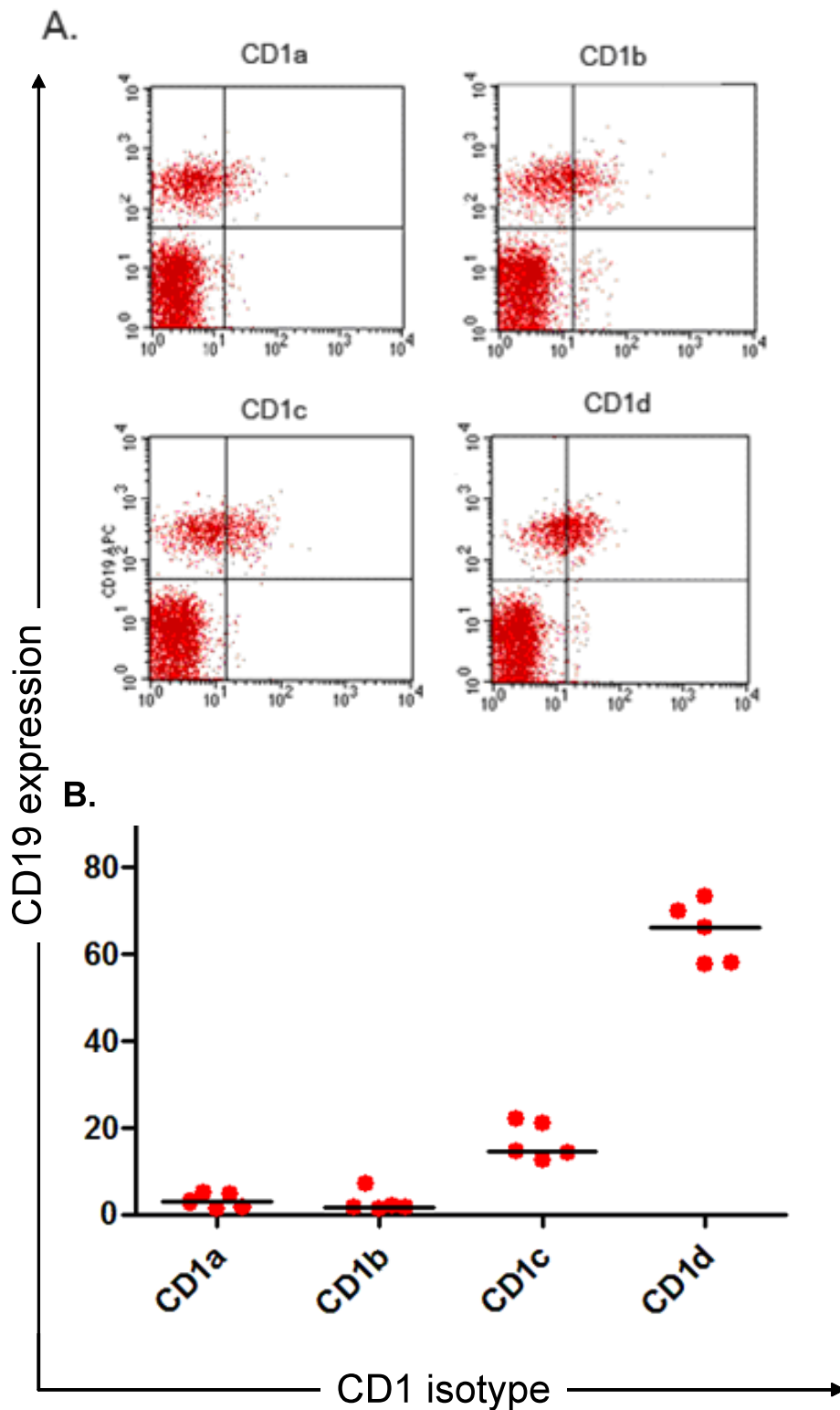
Figure 3.2: Expression of CD1 isotypes by B cells. (A) Representative flow cytometric dot plots of PBMC co-stained for B cells (CD19) and CD1 isotypes a-d. Cells in the upper right quadrant are CD1 expressing B cells. (B) Scatter plot showing the percentage of B cells expressing individual CD1 isotypes. Black lines denote the means of 5 experiments.
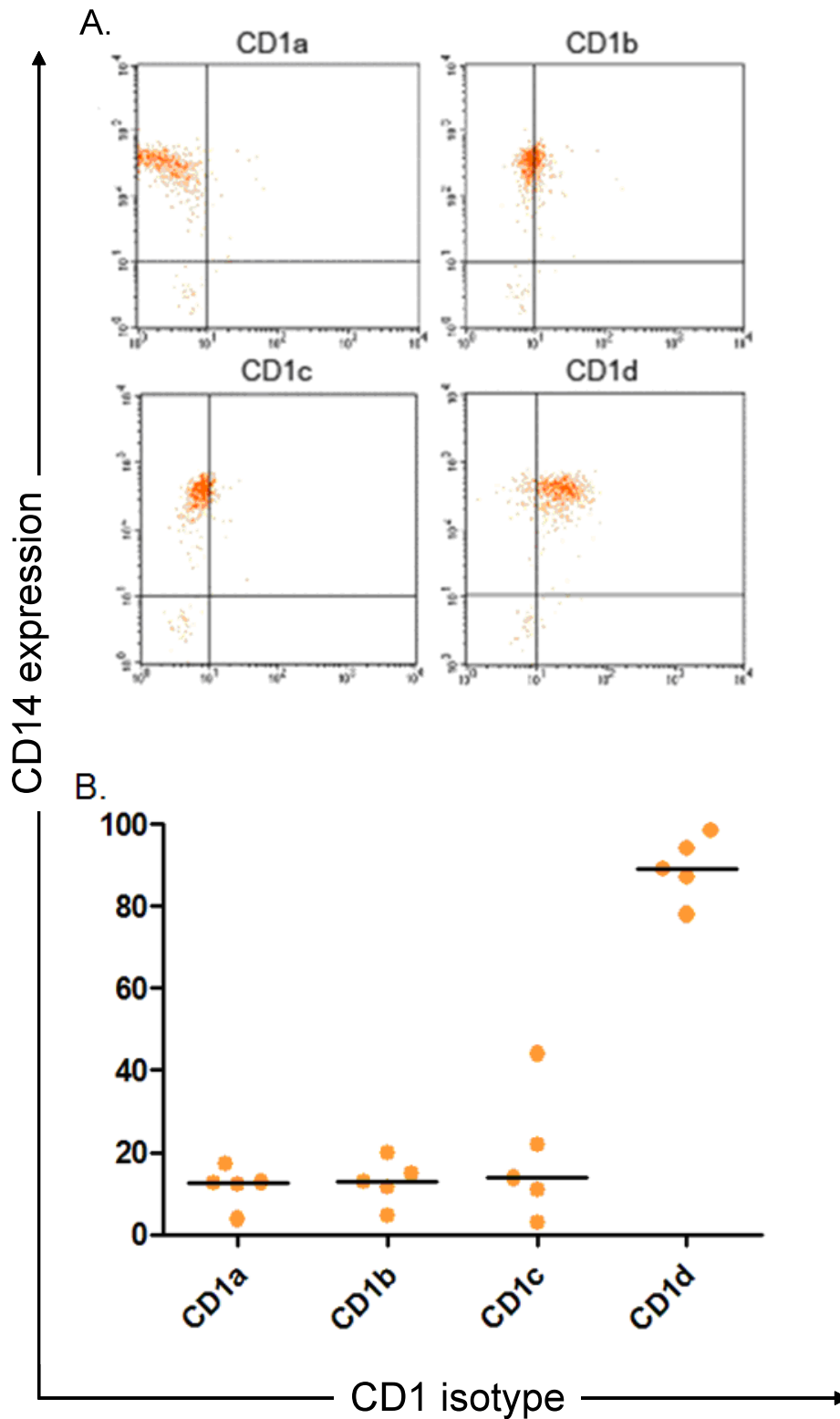
Figure 3.3: Expression of CD1 isotypes by CD14+ cells: (A) Representative flow cytometric dot plots of PBMC co-stained for monocytes (CD14) and CD1 isotypes a-d. Cells in the upper right quadrant are CD1 expressing monocytes. (B) Scatter plot showing the percentage of monocytes expressing individual CD1 isotypes. Black lines denote the means of 5 experiments.
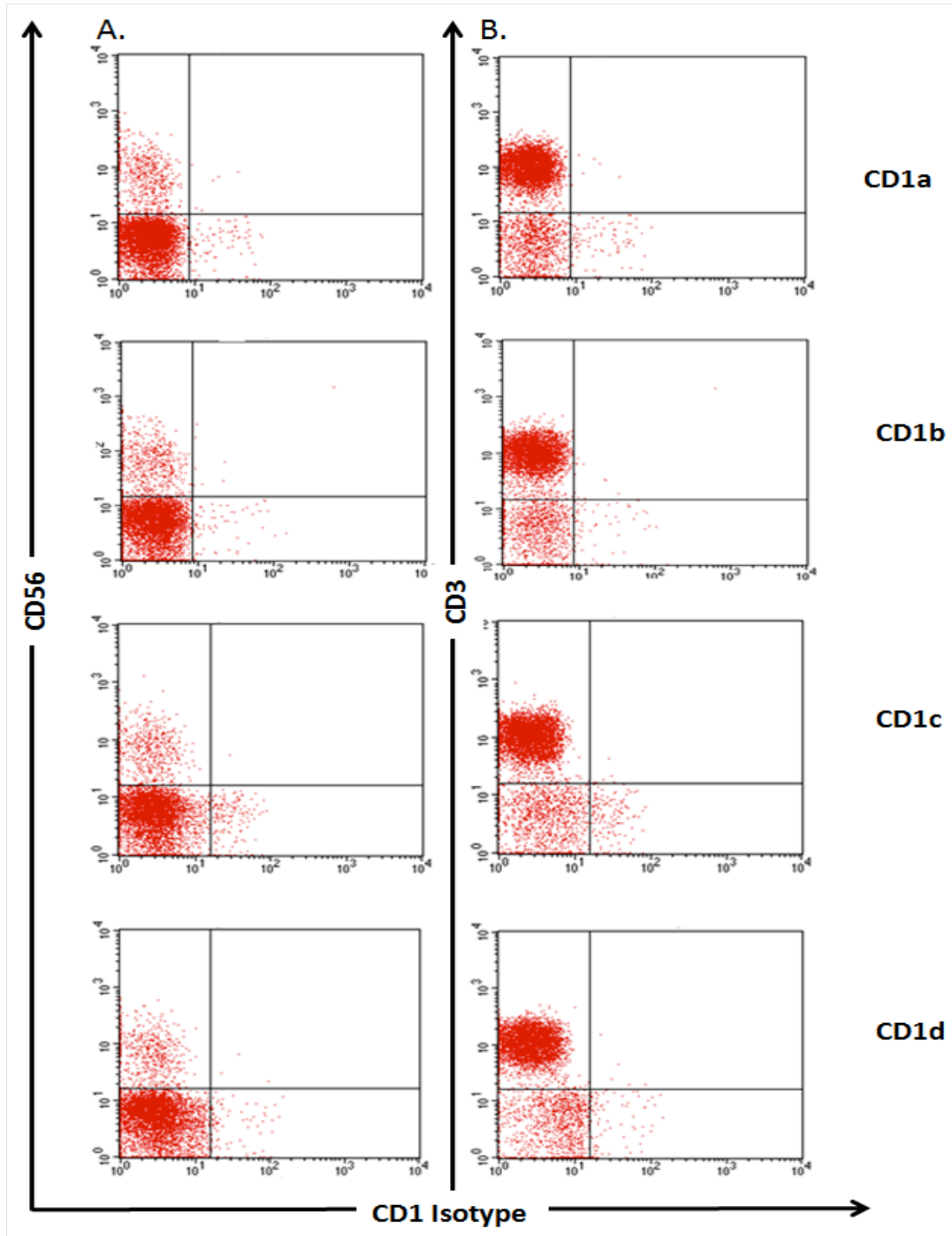
Figure 3.4: Expression of CD1 isotypes by CD56+ cells (NK cells and CD56+ T cells) and CD3+ cells (T cells). (A) Flow cytometry dot plots of PBMC co-stained for CD56 and CD1 isotypes a-d. (B) PBMC co-stained for CD3 and CD1 isotypes a-d. Cells in the upper right quadrant are CD1 positive cells.

3.3.2 Verification of CD1 expression by CD1 transfected C1R cell lines

C1R cell lines stably transfected with a single CD1 isotype (Exley *et al* 1997) were used to test for reactivity by CD1 restricted T-cells. Each C1R CD1 cell lines expressed a specific CD1 isotype, CD1a, CD1b, CD1c or CD1d and a mock transfected cell line served as a negative control. Flow cytometry was used to verify the expression of each CD1 isotype expression on each C1R CD1 cell line and the lack of any isotype expression on the mock transfected C1R cell line. The C1R mock cell line did not stain for the expression of any of the CD1 isotypes (Figure 3.5). Examination of the C1R CD1a-d cell lines showed the CD1 transgene was stable and expressed on ~99% of the cells (Figure 3.5). Some cross reactivity was noted for mAb specific for CD1b and the CD1c and CD1d cell lines, and the CD1a mAb and the CD1d cell line.
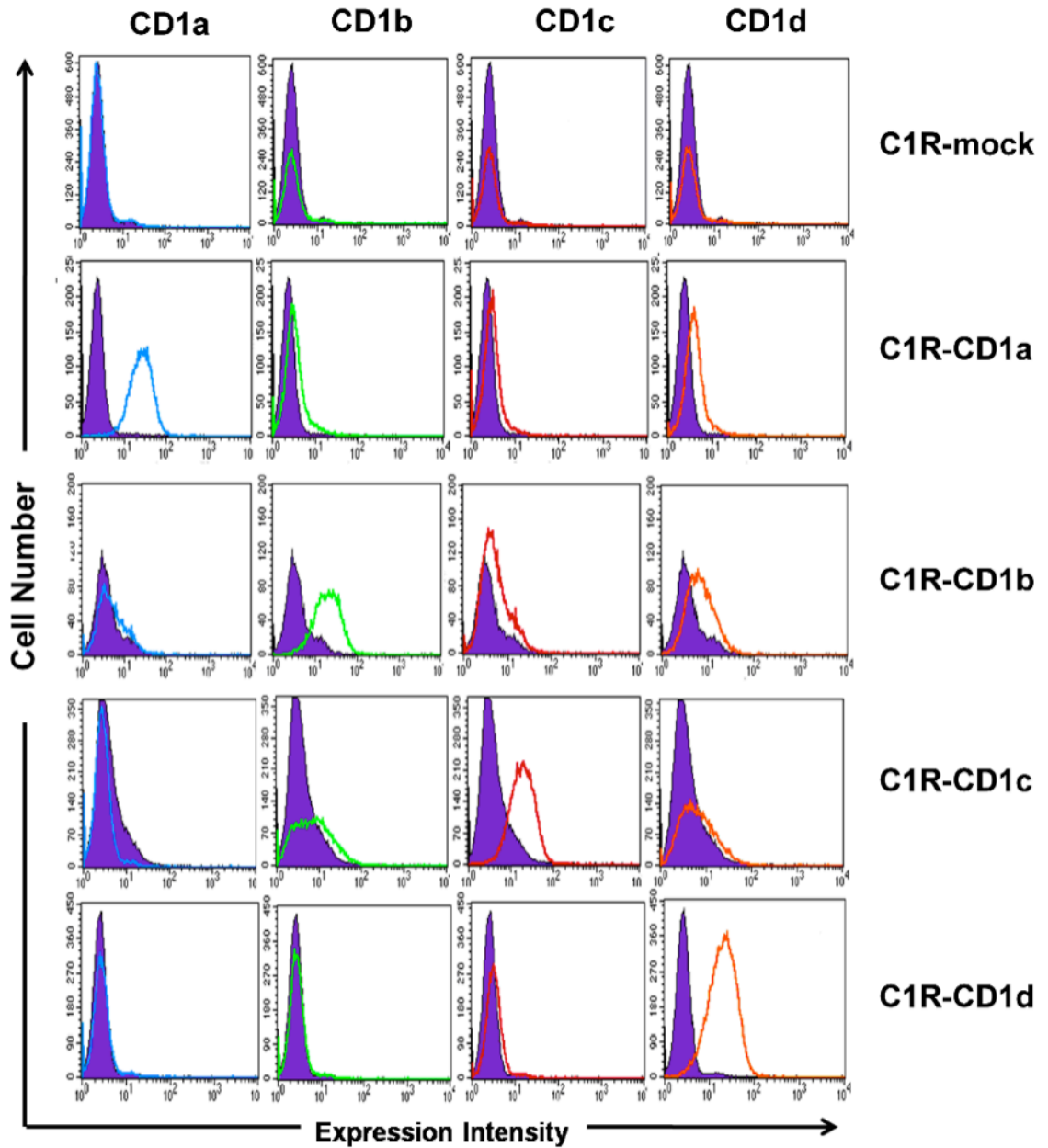
Figure 3.5: Verification of CD1 isotype expression by transfected C1R cell lines; Mock, CD1a, CD1b, CD1c and CD1d. Each line was examined for the expression of CD1a (Blue histogram, CD1b (Green histogram), CD1c (Red histogram) or CD1d (Orange histogram). Isotype control staining is shown as purple filled histogram. Graphs are representative of 3 separate experiments.

3.3.3 PBMC responses to CD1a-d transfected C1R cells in the absence of added antigen

In order to assess CD1 reactivity in human blood a functional assay was designed to measure responses of PBMC to the four CD1 isotypes CD1a, CD1b, CD1c and CD1d in the absence of added antigen. PBMC were co-cultured with mock transfected C1R cells (control) or C1R cells expressing single transfected CD1 isotypes (CD1a-d) at 1:1 ratios for 3 days. The resulting reactivity was determined as the fold increases of measured cytokine (IFN-γ and IL-13), over the amount released in response to the mock (IFN-γ range 84-342 pg/ml, IL-13 range 5-25 pg/ml). The mitogenic stimulator PHA was used as a positive control. Results from 5 separate assays show 2-3 fold increases in reactivity of PBMC against CD1a (p =0.04) and CD1b resulting in IFN-γ release, and an ~1.5 fold increase in IL-13 release in response to CD1a, CD1c (p =0.01) and d (Figure 3.6A and B respectively).

3.3.4 CD3+ T cell responses to CD1a-d transfected C1R cells in the absence of antigen

The experiments presented in section 3.3.3 used total PBMC as effector cells in the measurement of CD1 reactivity. However it is possible that the response observed includes non-T cell reactivity against C1R-CD1 cells and/or reactivity against CD1 expressed by B cells and monocytes present in the PBMC. To eliminate this possibility magnetic bead enriched CD3+ T cells were used as effector cells. The resulting reactivity was determined by the fold increase of measured cytokine (IFN-γ and IL-13) over the levels released in response to the C1R mock transfected control (IFN-γ range 265-622 pg/ml, IL-13 range 26-31 pg/ml). Results from 5 assays showed that peripheral blood T cells exhibit significant reactivity against CD1d with

3.8 fold increase in IFN-γ (p =0.008) (Figure 3.7A) and 6 fold increase in IL-13 (p =0.04) (Figure 3.7B). Low level reactivity against CD1a was noted, with 2 fold increase in IFN-γ but not IL-13 secretion (Figure 3.7A&B).
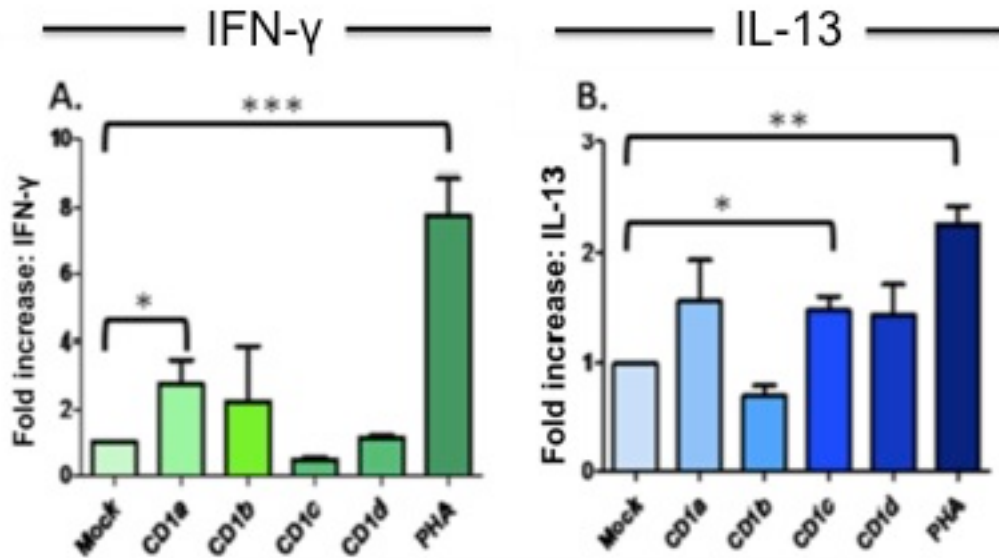
Figure 3.6: IFN-γ and IL-13 secretion by PBMC in response to CD1. (A) IFN-γ and (B) IL-13 release by PBMC in response to CD1a, b, c and d. PBMC were co-cultured with CD1 transfected C1R cells at 1:1 ratios, the mock transfected C1R cell served as a control and T cell mitogen PHA as positive control. Cytokine levels are expressed as fold-increases in response to CD1-transfected cells compared to those released in response to the mock transfected control C1R cells. (IFN-γ range 84-342 pg/ml), (IL-13 range 4.5-25 pg/ml) measured by ELISA. Results are mean of 5 separate experiments.
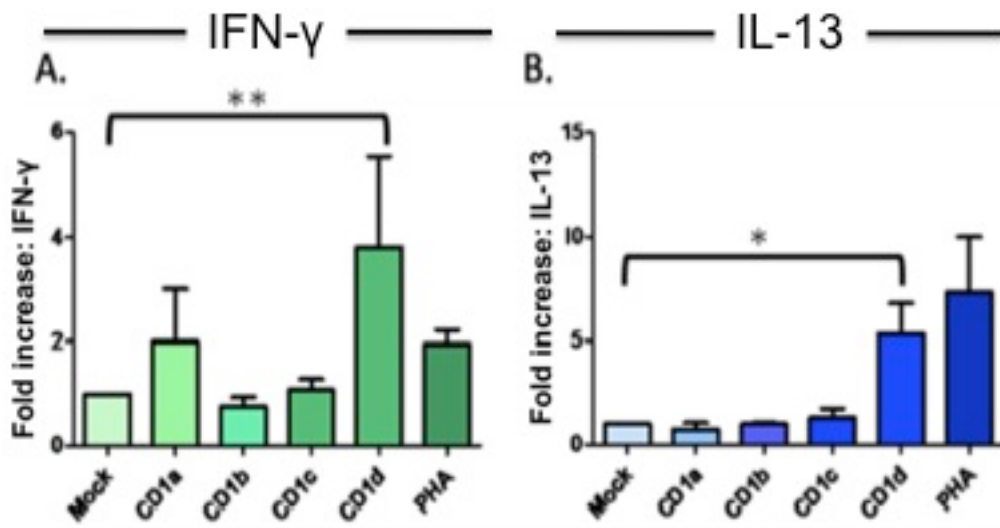
Figure 3.7 IFN-γ and IL-13 secretion by CD3+ T cells in response to CD1. (A) IFN-γ and (B) IL-4 released by CD3+ T cells in response to CD1a, b, c and d. Magnetic bead enriched CD3+ T cells were co-cultured with CD1 transfected C1R cells at 1:1 ratios, the mock transfected C1R cell served as a control and T cell mitogen PHA as positive control. Cytokine levels are expressed as fold-increases in response to CD1-transfected cells compared to those released in response to the mock transfected control C1R cells (IFN-γ range 265-622 pg/ml), (IL-13 range 26-31 pg/ml). Results are mean of 5 separate experiments.

3.3.5 PBMC responses to CD1a-d transfected C1R cells in the presence of exogenous glycolipid antigen

Since PBMC responses against CD1d were observed, we next examined the responses of PBMC to CD1d transfected C1R cells, in the absence and presence of 100 ng/ml of two human CD1d binding glycolipid ligands, αGalCer & iGb3 (Bendelac *et al* 2007). When PBMC were cultured with the mock transfected C1R cells, means of 220 ng/ml of IFN-γ and means of 14 ng/ml of IL-13 were detected in the supernatants 3 days after culture. These cytokine levels were 1.8 fold and 2 fold higher when CD1d transfected C1R cells were used as stimulators (Figure 3.8). In the presence of αGalCer loaded CD1d transfected C1R cells, a further increase in IFN-γ but not IL-13 secretion was observed. In the presence of iGb3 loaded CD1d transfected C1R cells, an increase in IFN-γ was observed but similar to αGalCer no increase in IL-13 release was observed compared to PBMC cultured with CD1d transfected cells (Figure 3.8). IFN-γ range 84-342 pg/ml, IL-13 range 5-25 pg/ml. These results suggest that a-GalCer and iGb3 promote Th1 but not Th2 cytokine secretion by PBMC.

3.3.6 CD3+ T cell responses to CD1a-d transfected C1R cells in the presence of exogenous glycolipid antigen

We also examined the responses of magnetic bead enriched CD3+ T cells to CD1d in the absence or presence of αGalCer and iGb3. When enriched T cells were cultured with the mock transfected C1R cells, means of 220 ng/ml of IFN-γ and means of 14 ng/ml of IL-13 were detected in the supernatants 3 days after culture. These cytokine levels were 4 fold higher for IFN-γ secretion when CD1d transfected C1R cells were used as stimulators, but no increase in IL-13 was observed (Figure

3.9). In the presence of αGalCer loaded onto CD1d transfected C1R cells, an 11.6 fold increase in IFN-γ (p =0.003) and 4.2 fold increase in IL-13 (p =0.03) secretion was observed. In the presence of iGb3 loaded onto CD1d transfected C1R cells, a 6.1 fold increase in IFN-γ secretion and a 2.6 fold increase in IL-13 was observed when compared to CD1d transfected cells alone (Figure 3.9).
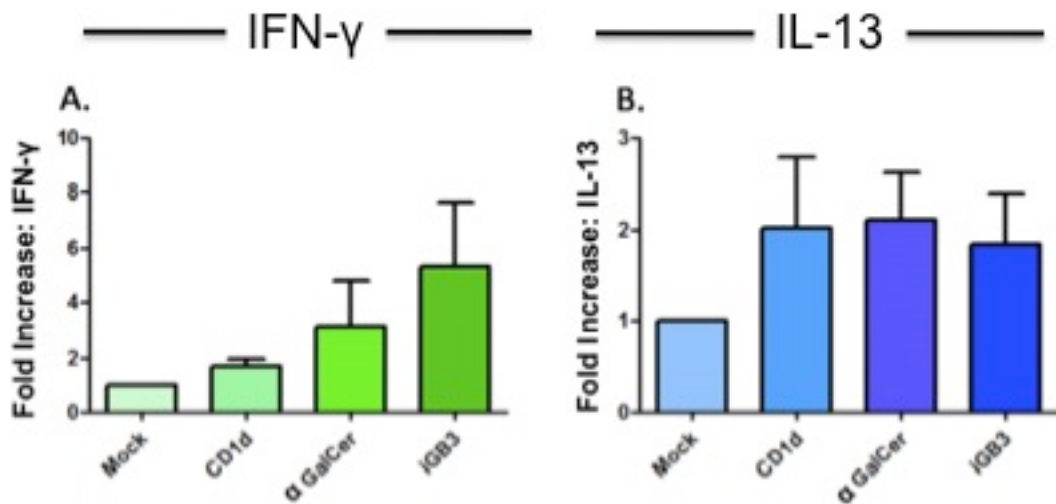
Figure 3.8: (A) IFN-γ and (B) IL-13 secretion by PBMC in response to CD1 loaded or unloaded with glycolipid. Graphs displaying CD1d reactivity by PBMC co-cultured at 1:1 ratios with CD1d transfected C1R cells in the absence or presence of 100 ng/ml of the exogenous lipid antigens αGalCer and iGb3. Cytokine levels are expressed as fold-increases in response to CD1-transfected cells compared to those released in response to the mock transfected control C1R cells (IFN-γ range 84-342 pg/ml, IL-13 range 5-25 pg/ml). Results are means of 5 separate experiments.
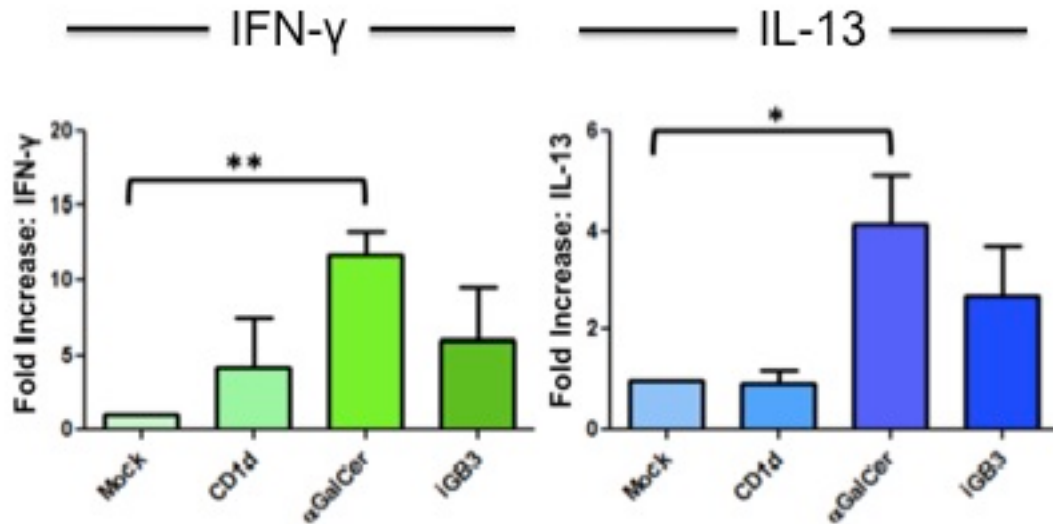
***Figure 3.9: CD1 reactivity by T cells in the presence of glycolipid: Graphs
displaying CD1d reactivity by magnetically enriched T cells co-cultured at 1:1
ratio with CD1d transfected C1R cells in the absence or presence of 100 ng/ml of
the exogenous lipid antigens αGalCer and iGb3. Cytokine levels are expressed as
fold-increases in response to CD1-transfected cells compared to those released in
response to the mock transfected control C1R cells (IFN-γ range 265-622 pg/ml,
IL-13 range 26-31 pg/ml). Results are mean of 5 separate experiments***

3.3.7 Invariant NKT cell populations in peripheral human blood

Due to high levels of CD1d reactivity observed and significant responses to iNKT ligands αGalCer and iGb3 identified in previous experiments, enumeration of iNKT populations in human peripheral blood was determined by flow cytometric analysis. PBMC were co-stained using the invariant NKT mAb 6B11 and CD3 (Figure 3.10A) or Vα24 and Vβ11 (Figure 3.10C). Using 6B11 co-stained with CD3 a mean of 0.05% cells stained positive for iNKT cells, from a cohort of 20 healthy donors with a range of 0.01% - 0.15% (Figure 3.10B). The co staining using Vα24 and Vβ11 resulted in a mean of 0.065% positive cells, from a smaller cohort of 7 healthy donors with a range of 0.02% - 0.1% (Figure 3.10D).
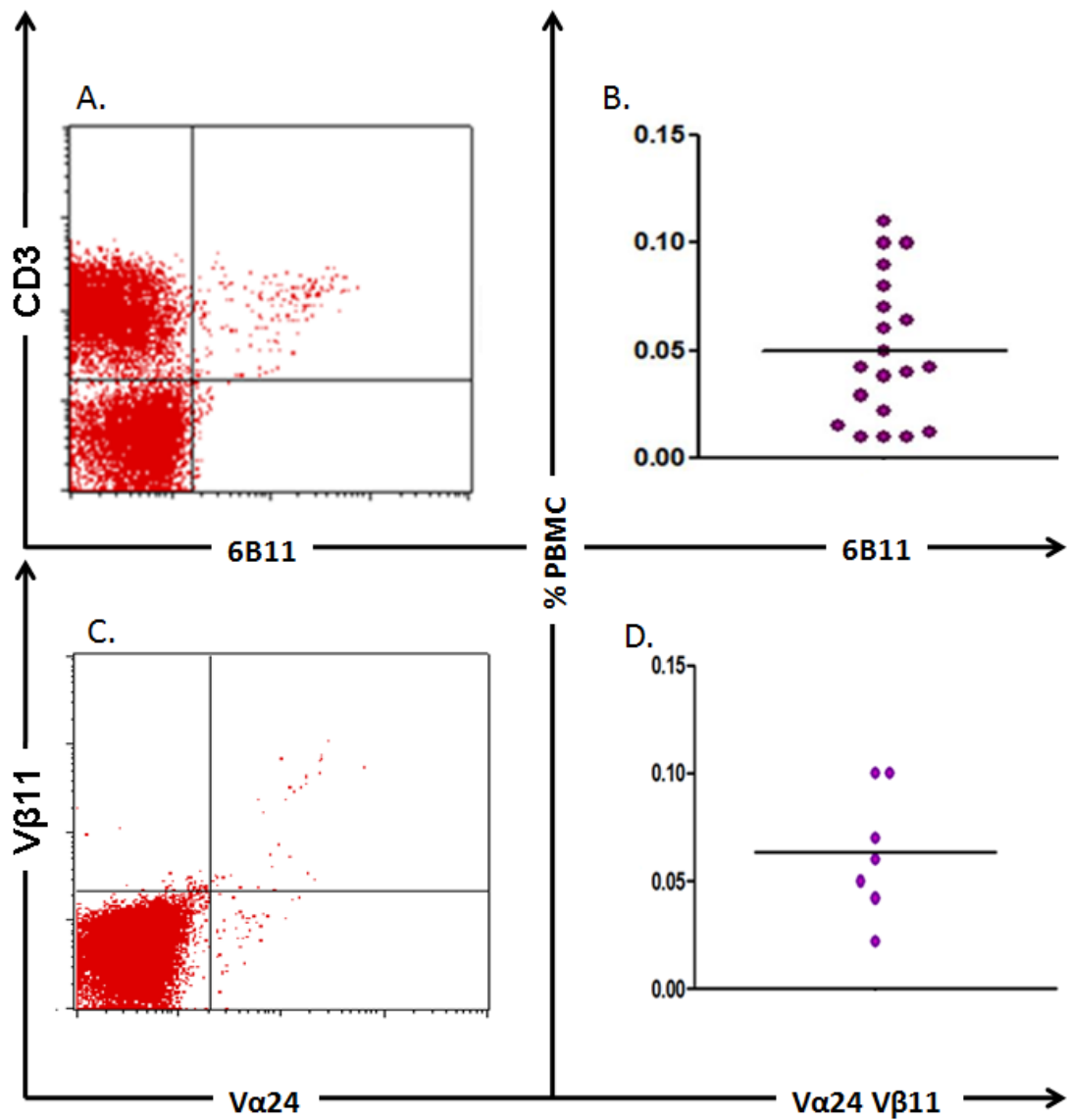
Figure 3.10: Enumeration of iNKT cells in human blood: (A) Representative dot plot of cells expressing 6B11 and CD3, the upper right quadrant shows the iNKT cell population. (B) Invariant NKT frequency plot using 6B11 from 20 donors shows mean percentage of 0.05%. (C) Representative dot plot of cells expressing Vα24, Vβ11. Upper right quadrant shows the iNKT cells. (D) Frequency plot from 7 donors using Vα24, Vβ11 shows mean of 0.06%.

3.4. Discussion

CD1 expression has been described in many tissues, such as intestine, liver, spleen, thymus and lungs and on several populations of cells including monocytes, DCs and B cells. We examined human peripheral lymphocytes and monocytes from healthy donors for expression of CD1. In the investigation of CD1 expression, 4 subsets were examined; Monocytes (CD14+), B cells (CD19+), NK and NT cells (CD56+, (Natural T cells) CD56+ CD3+) and T cells (CD3+). Our findings showed that monocytes and B cells can express all isoforms, with CD1d being the most abundant. Little or no CD1 expression was found on NK, NT cells or T cells (Figure 3.4). This study identifies CD1d as the most abundantly expressed isoform and monocytes as the major population of CD1 expressing cells in human peripheral blood. This is consistant with the CD1 expression levels described in previous work (Martin *et al* 1987, Porcelli 1995, Bendelac *et al,* 2007). CD1d is also expressed on epithelial cells and in several tissues including skin and adipose tissue.

Due to the lack of CD1a, CD1b and CD1c in murine models, the majority of CD1 restricted T cells described and studied are CD1d restricted T cells. T cells restricted by CD1a, CD1b or CD1c are reported in the context of microbial infection (Gumperz & Brenner 2001, De Libero *et al* 2005). Previously Kenna *et* al investigated CD1 group 1 and 2 reactivity by hepatic mononuclear cells and reported that reactivity was observed against CD1c and CD1d but not CD1a or CD1b. We aimed to test the CD1 reactivity to all 4 isotypes in healthy human peripheral blood with the aim of identifying populations of CD1 restricted T cells as this is not well defined in the literature.

In the investigation of CD1 reactivity in human blood in the absence of added antigen, low level reactivity was observed against CD1a, b and d in the form of IFN-γ secretion and CD1a, c and d in the form of IL-13 secretion (Figure 3.7). The cytokines detected might not be solely attributed to CD1 reactivity by T cells due to the mixed PBMC populations, so the study was repeated using magnetic bead enriched CD3+ T cells. Low level reactivity in the form of IFN-γ secretion was noted for CD1a but the majority of CD1 reactivity was observed against CD1d, in the form of both IFN-γ and IL-13 secretion (Figure 3.8). Reactivity to CD1a, b or c was not readily detected in healthy human peripheral blood. In discussion with Dr David Lammas, it was hypothesized that reactivity with CD1a, CD1b and CD1c is only observed in the presence of microbial infection (Personal communication with Dr David Lammas, University of Birmingham, UK). Previous studies describing CD1a, b and c reactivity was predominantly in the presence of microbial infection as described in section 1.6.6.

Due to the predominant CD1d reactivity by human T cells, two human CD1d binding glycolipids were included in the reactivity assay; αGalCer and iGb3. Several groups have reported CD1d restricted T cell reactivity to these ligands (Kawano *et al* 1997, Zhou *et al* 2004). In the PBMC reactivity assay, a response comparable to that of PHA was observed with the addition of both ligands (Figure 3.9), and as before CD1d reactivity in the form of cytokine secretion was increased with the substitution of PBMC with purified CD3+ T cells. The literature describes this subset of CD1d restricted T cells which respond to αGalCer and iGb3 as invariant iNKT cells (Bendelac *et al,* 1997).

In the final study of this section we investigated the frequency of iNKT cells in human peripheral blood. The range of peripheral human iNKT cells is reported in several studies as 0.01-1% of lymphocytes (Prussin & Foster 1997, Exley *et* al 1998, Kronenberg *et al* 2005). It has also been reported that iNKT cell numbers in the periphery is changed in cancer (Kawano 1999). We examined the range of iNKT cells in healthy human peripheral blood using 2 different staining patterns, the mAb pairings of Vα24, Vβ11 and 6B11, CD3 a mean of 0.05% iNKT cells was observed in human peripheral blood, this is within the ranges reported in the literature of 0.01%-1% (Prussin & Foster 1997, Exley *et* al 1998, Kronenberg *et al* 2005). It is well established that iNKT vary in frequency between peripheral blood and tissues in both humans and mice, with mice having significantly more iNKT cells than humans (Bendelac *et al* 1997, Kronenberg *et al* 2005). NKT cells comprise 0.5% of hepatic T cells compared to 50% in murine liver (Kenna *et* al 2007). Recently Lynch *et al* reported the presence of a significant population in the human omentum, the highest frequency reported in humans to date (Lynch *et al*, 2009). The presence of more significant populations of iNKT cells in humans, such as those in the omentum suggests new roles for iNKT cells in immunity and regulation. In this chapter we investigated the expression of CD1 on PBMC and found that monocytes and B cells but not T cells or NK cells express all isoforms with CD1d being the most abundantly expressed. We aimed to identify populations of CD1 restricted T cells in peripheral blood and found that the majority of CD1 restricted T cells in healthy human peripheral blood are CD1d restricted.

3.5 Conclusions

- CD1 is predominantly expressed in human peripheral blood by the professional antigen presenting cells, monocytes and B cells

- CD1d is most abundantly expressed CD1 molecule on human PBMC

- CD1a, b and c reactivity is very weak in healthy human peripheral blood

- CD1d restricted T cell activity is the most abundant in healthy human peripheral blood

- iNKT cells comprise ~0.05% of peripheral human PBMC and react to the CD1d antigens αGalCer and iGb3

4: Results: Expansion and Characterisation of Human NKT cells

Chapter 4: Results

4.1 Introduction

Invariant natural killer T (iNKT) cells are a rare subset of human innate T cells (0.01-1% of PBMC), which express an invariant TCR (Vα24 Jα18) and NK cell like surface molecules (e.g. CD56, CD94, CD161). The invariant TCR recognises glycolipid antigen presented by CD1d molecules. The natural ligand is currently unknown but several iNKT agonists have been identified. The glycolipid αGalCer, derived from marine sponge is the best defined agonist and the current "gold standard" for iNKT activation (Kawano *et al,* 1997, Kronenberg, 2005).

Invariant NKT cells vary in their surface phenotypes and functionally distinct subsets (CD4+ subsets, DN subsets, CD94+ iNKT cells) (Bendelac *et al*, Exley *et al,* 1998*,* Nicol *et al,* 2000). Some subsets of iNKT cells display potent cytotoxic ability but classically iNKT cells are best described as prolific cytokine producers (Yoshimoto *et al* 1994, Matsuda *et al,* 2000, Godfrey *et al* 2002, Kronenberg, 2005). The simultaneous secretion of both IFN-γ and IL-4 is a defining factor of some iNKT cells (Exley et al 1997, Chen & Paul, 1997). iNKT cells can however secrete a wide range of immunomodualtory cytokines, such as; GM-CSF, IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, TNF-α (Matsuda *et al*, 2008).

The small numbers of iNKT cells (~0.05%) present in human peripheral blood (Figure 3.11), provide a difficult obstacle in the functional study of iNKT cells. The most efficient way to study circulating human iNKT cells is to isolate and expand them *in vitro*. Several methods are described in the literature (Porcelli *et al* 1996, Exley *et al,* 1997, Tahir *et al* 2001, Exley *et al,* 2003). In this body of work we aimed to investigate and optimize 3 methods to obtain highly pure populations of

iNKT cells. We also aimed to test the functional capabilities of the resulting iNKT cell lines for cytokine production and cytotoxic activity in response to αGalCer. A method for the isolation and expansion of functional iNKT cells from human peripheral blood will provide a tool for use in a series of assays for testing novel αGalCer analogues (Chapter 6) and the reciprocal interactions between iNKT cell and peripheral blood DCs (Chapter 5).

Three methods were tested for the isolation and expansion of iNKT cells from fresh healthy human blood. Method 1 (Figure 4.1) was adapted from a method described in 1997 by Exley *et al*. PBMC were stained using mAb 6B11 PE as previously described in chapter 2, and positively stained cells were magnetically separated using anti-PE magnetic Microbeads. The purified iNKT population was cultured with irradiated PBMC from two donors acting as feeders and accessory cells. Glycolipid (αGalCer) was added at 100 ng/ml along with 50 U/ml of IL-2 on day 1. The cultures where then cultured in a CO_2 incubator and fresh IL-2 added every 3 days.

Method 2 was adapted from method described by Tahir *et al* (Tahir *et al,* 2001). The iNKT cell isolation procedure was repeated for Method 2 and isolated cells were again cultured with irradiated PBMC from 2 donors but PHA stimulation was substituted for glycolipid stimulation on day 1. Cells were cultured with IL-2 and the stimulations were repeated on day 14 and subsequently every 14 days.

The third method was adapted from a protocol outlined in 2003 by Exley *et al.* This method differed from method 1 and 2 only in the stimulation steps. The protocol

describes the stimulation of purified iNKT cells with irradiated feeders and αGalCer, then the transfer of expanding iNKT cells to anti-CD3 mAb coated plates. After personal communications with Prof Mark Exley, the purified iNKT cells where cultured on day 1 in wells coated (4 hours prior to use) with 1 µg/ml of the anti-CD3 monoclonal antibody OKT-3. No irradiated feeder cells were used. This simplified the protocol. On day 2, 50 U/ml of IL-2 was added to the culture. IL-2 medium was replenished every 7 days (Figure 4.1).

After the expansion and development of iNKT cell lines, we phenotyped expanded iNKT cell lines by flow cytometry to confirm their identity as iNKT cells, to determine iNKT cell purities and to determine their surface phenotypes. These lines were then used for functional studies.

4.2 Objectives

1. To optimize a method for the isolation and expansion of iNKT cells from human peripheral blood

2. To phenotypically characterise expanded iNKT cell lines

3. To functionally characterise iNKT cell lines
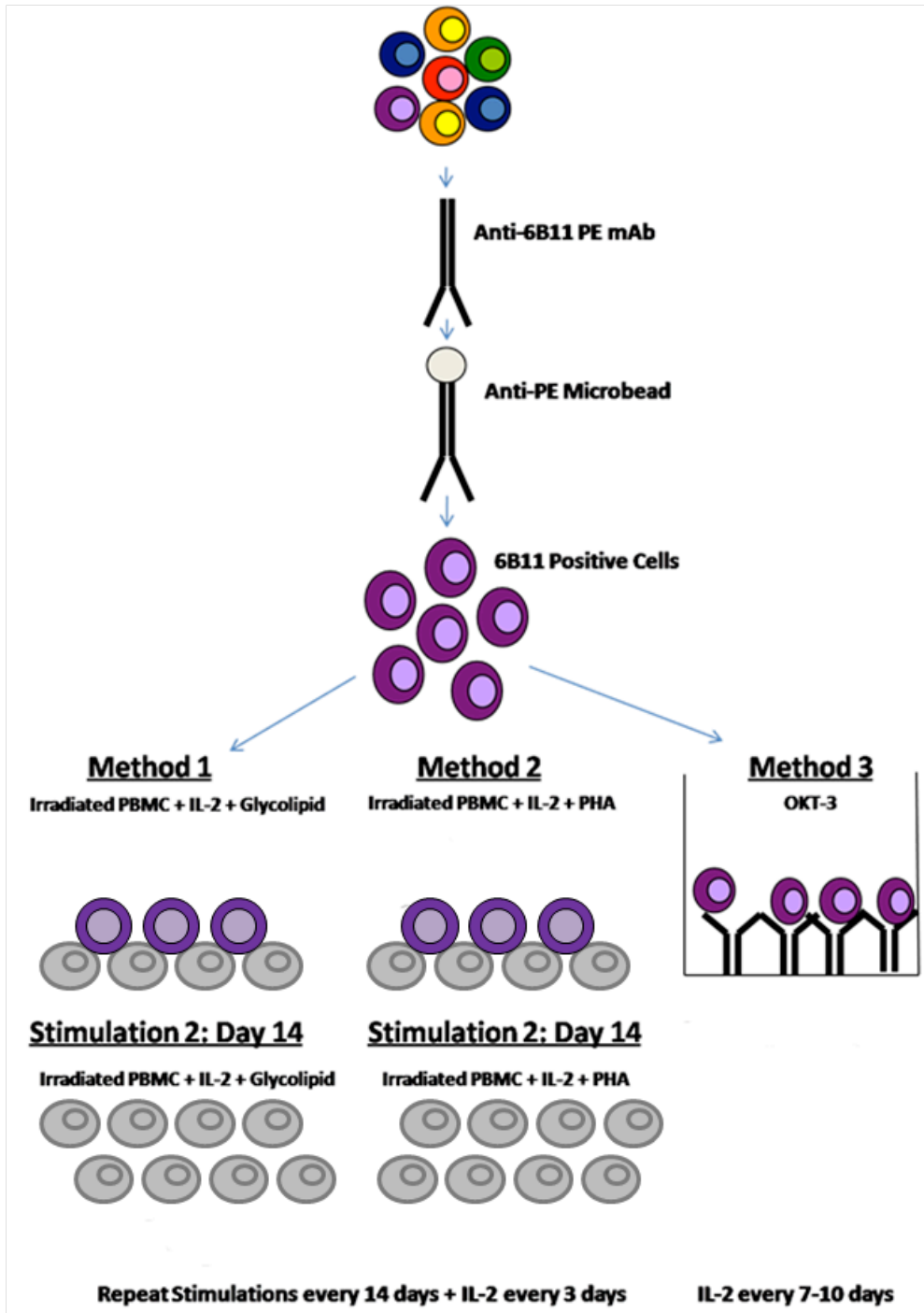
Figure 4.1: Strategy for the expansion and generation of NKT cell lines: Three methods (1-3) were employed. Each method was based on the same isolation method of staining with 6B11 PE mAb and the magnetic removal of PE stained cells using anti-PE Micro-bead. The 6B11+ cells were then stimulated by 1 of 3 methods; (1) culture with irradiated feeders and 100 ng/ml αGalCer, (2) culture with irradiated feeders and 10 μg/ml PHA or (3) incubation on anti-CD3 OKT-3 coated plates. All cultures were maintained in the presence of IL-2 from day 2 onwards.

Chapter 4: Results

4.3.1: Optimization of iNKT cell expansion

PBMC were prepared from a total of 35 buffy coat packs, before being used for magnetic bead separation of iNKT cells. Because of the low frequencies of iNKT cell in peripheral human blood the yields and purities were not determined in order to maximise the starting numbers of iNKT cells in the expansion phase. iNKT cells isolated from peripheral human blood were expanded *in* vitro using 3 methods. The yields and purities of iNKT cells were then determined by EBAO cell counting and flow cytometry. Ten lines were generated using Method 1. This method resulted in minimal expansion of iNKT cells, and low purities ranging from 0.8% to 2.1% with a mean of 1.5% of cells expressing the iNKT marker 6B11. Method 1 also yielded low numbers of cells (Figure 4.2A, 4.3A). Five lines were established using method 2 and yielded cell lines with purities of iNKT cells ranging from 1% to 25% of cells. Although significant expansions were achieved, numbers of iNKT cells remained modest with yields reaching $5x10^6$ of cells expressing the iNKT marker 6B11 (Figure 4.2B, 4.3B). 7 lines were established using method 3. The purities of iNKT cells in these lines ranged from 15 to 99% with the average purity of 57% (Fig 4.4, 4.5A-B). Numerically, yields were also high with total cell numbers reaching $<50x10^6$ cells total. iNKT cells were detected by staining aliquots of each cell line with 3 different combinations of mAb and analysis by flow cytometry. Interesting to note is the variation in iNKT cell frequencies determined when three different types of flow cytometry stainings were used. Staining of the same lines with Vα24 and Vβ11, 6B11 and CD3, or the CD1d tetramer with CD3 (Figure 4.5A-D) resulted in different estimates of iNKT cell purity. The CD1d tetramer loaded with αGalCer was used to verify several of the lines but was not routinely used due to budget and time restrictions.

Method 3 was the choice of method used for development of further iNKT lines. In total 20 iNKT cell lines were established using Method 3. Of the 20 lines, 7 lines successfully expanded to purities of over 30%, the remaining lines either failed to expand or never reached purities in excess of 30% (Figure 4.7).
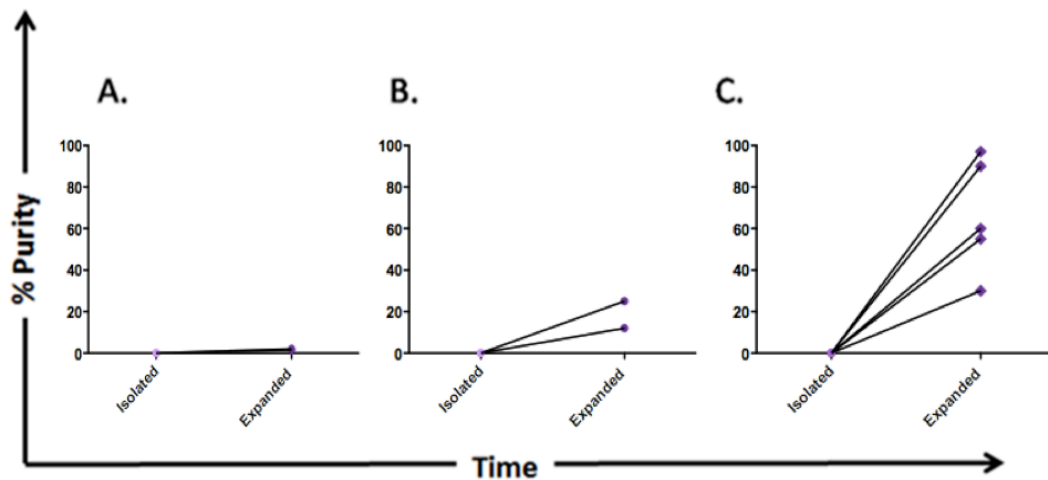
Figure 4.2: Expansion of iNKT from human blood: Line graphs displaying the percentage of iNKT cells over time from isolation (1) to post expansion (2). Time point 1 represents the % of iNKT in PBMC from each donor before isolation. (A) Method 1, (B) Method 2 and (C) Method 3. iNKT cells were isolated from peripheral blood using magnetic Microbead separation, and expanded using either 100 ng/ml αGalCer and irradiated feeders (Method 1), PHA and irradiated feeders (Method 2) or plate bound OKT3 anti CD3mAb in the absence of irradiated feeders (Method 3).
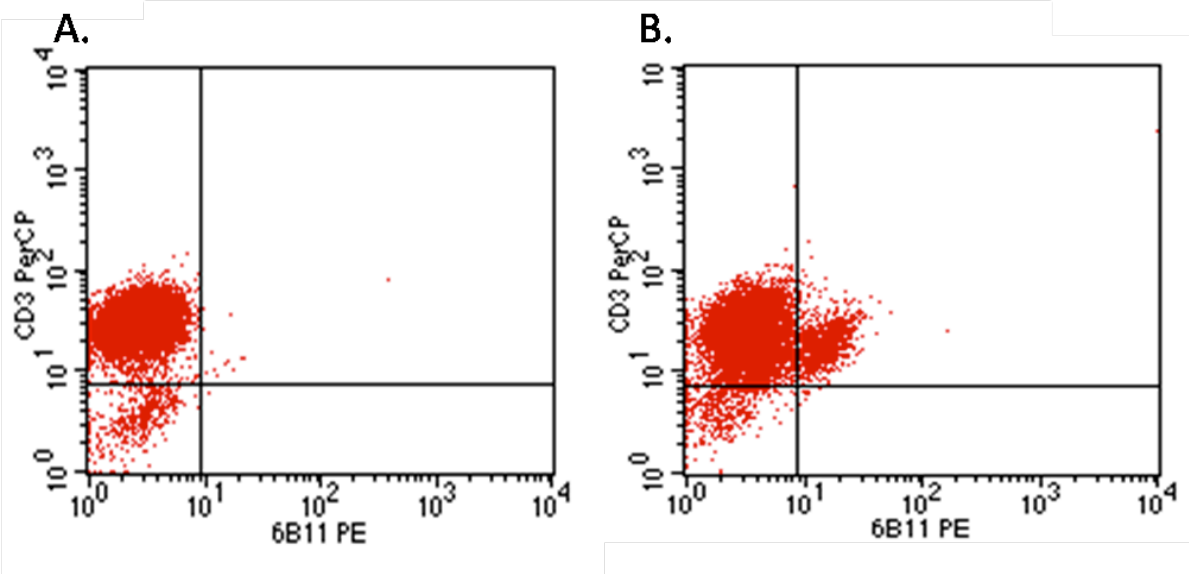
Figure 4.3: iNKT cell expansion. Representative flow cytometry dot plots of expanded iNKT populations using (A) Method 1 and (B) Method 2. Cells in the upper right quadrants are positive for 6B11 and CD3.
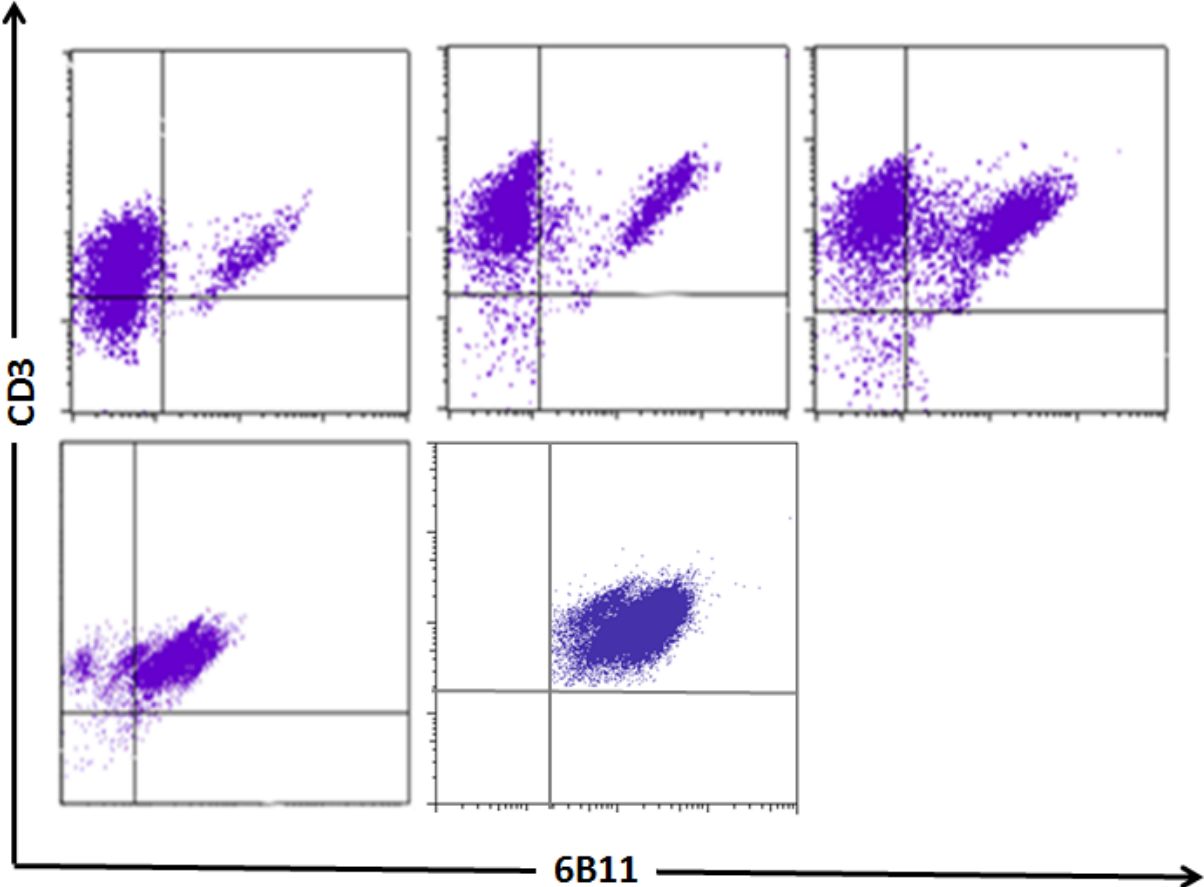
Figure 4.4: Expansion of iNKT cells. Flow cytometry dot plots of five different iNKT cell lines generated using Method 3. Cells in the upper right quadrant are positive for CD3 and 6B11 and deemed to be iNKT cells (dot plots are representative of 20 iNKT cell lines). Only lines with purities in excess of 30% purity were used in subsequent experiments.
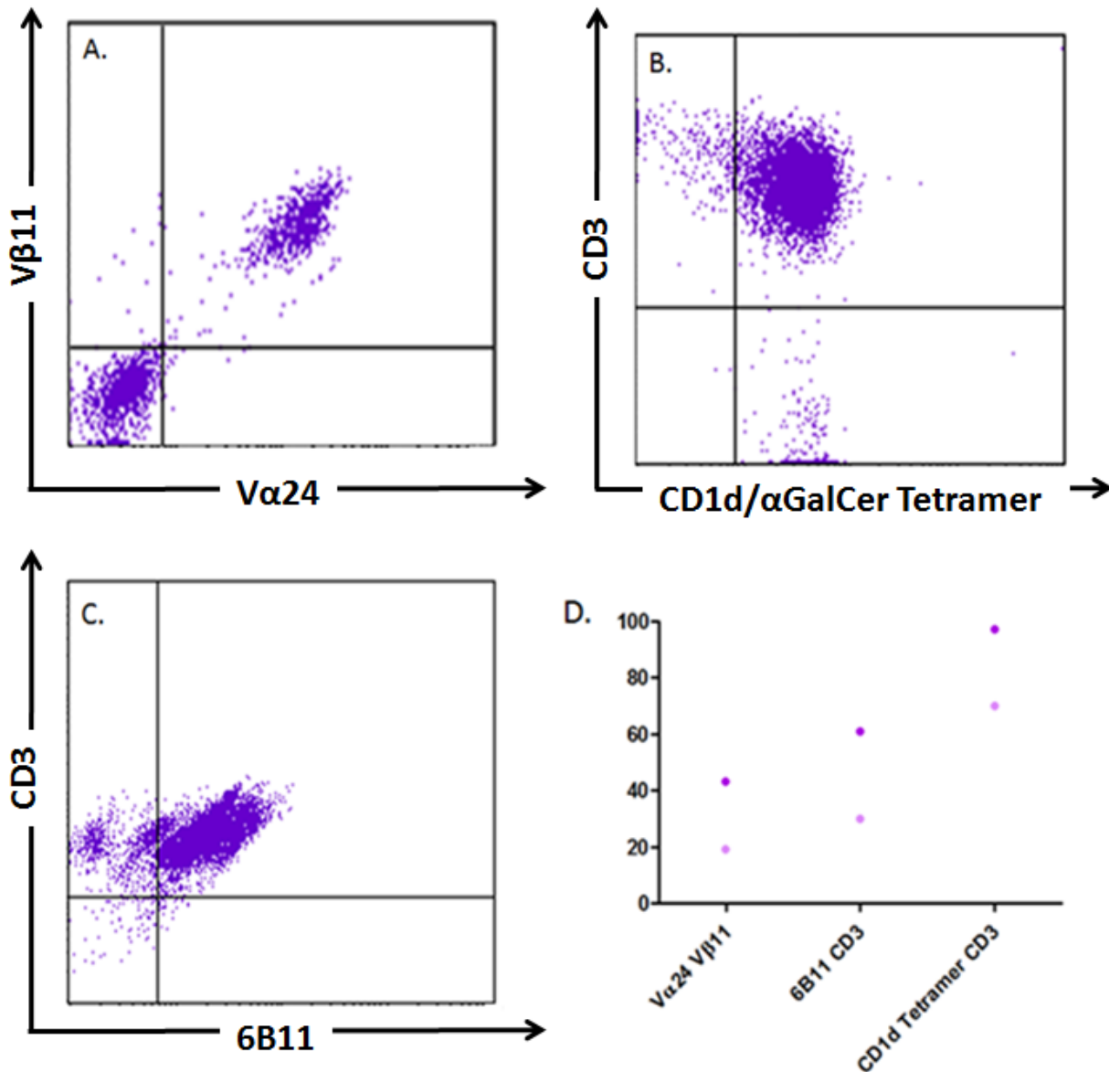
Figure 4.5: Flow cytometric detection of iNKT cells. Three different staining procedures were used to detect iNKT cells by flow cytometry. iNKT cell line number 3 was stained with mAbs specific for the Vα24 and Vβ11 TCR chains (A), CD3 and a CD1d tetramer loaded with αGalCer (B) or 6B11 and CD3 (C). Cells in the upper right quadrants are iNKT cells. (D) Scatter plot displaying the difference in % purity determined for 2 iNKT lines using the 3 staining methods.

4.3.2: iNKT cell phenotyping

Seven iNKT cell lines expanded from human blood with purities of over 30% were characterised by flow cytometric analysis of iNKT cell surface marker phenotypes. Electronically gated iNKT cells where analysed for the expression of CD4, CD8, CD94 and CD161 by co-staining with mAbs specific for these molecules and mAbs specific for CD3 and 6B11. Phenotypic profiles of two iNKT cell lines are shown in figure 4.6 and 4.7. The percentage of gated iNKT cells that expressed CD4, CD8, DN, CD94 and CD161 on iNKT cell line generated from 7 donors are shown in figure 4.8. CD4 was expressed by a mean of 48% of iNKT cells (Figure 4.8) with a range of expression from 31% to 75% (Figure 4.8). CD8 was expressed by a mean of 11% of iNKT cells (Figure 4.8) with a range of 5 to 15 % expression. iNKT cells double negative for CD4 and CD8 (DN) comprised 40% of iNKT cells examined with range varying from 14% to 63% (Figure 4.8). The co-stimulatory/inhibitory molecule CD94 was expressed on a mean of 13% of iNKT cells with a range of expression from 1% to 33% (Figure 4.8). CD161, another stimulatory/inhibitory molecule had mean expression of 40% on the lines examined with a range of 13.3% to 69% (Figure 4.8). These results show that iNKT cells are phenotypically heterogeneous.
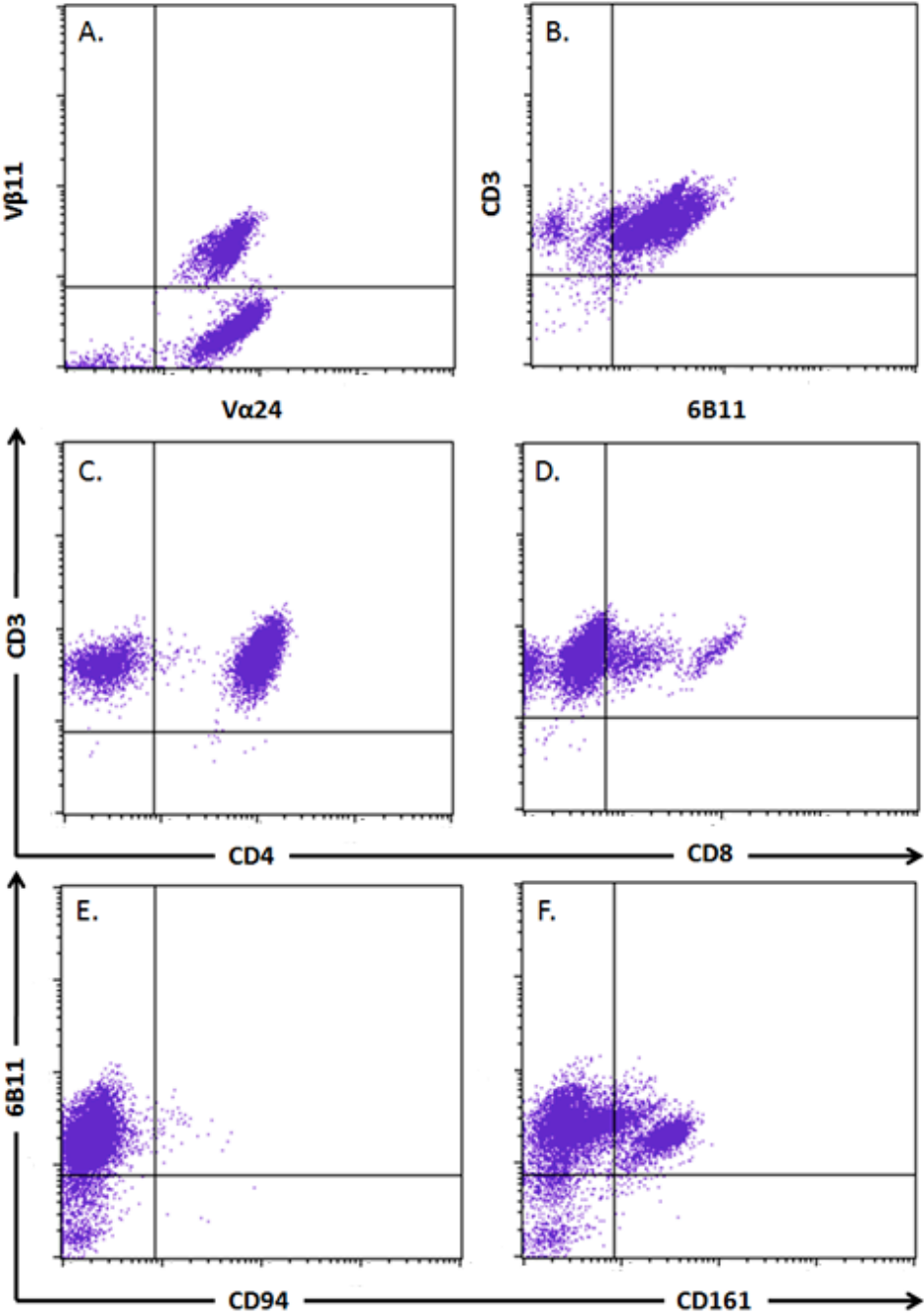
Figure 4.6: Phenotypic characterisation of iNKT cell line Number 3 by flow cytometry. Flow cytometric dot plot showing expression of Vα24 and Vβ11 TCR chains (A) and 6B11 and CD3 (B). C-F, flow cytometry dot plots showing the expression of CD4, CD8, CD94 and CD161 by electronically gated iNKT cells.
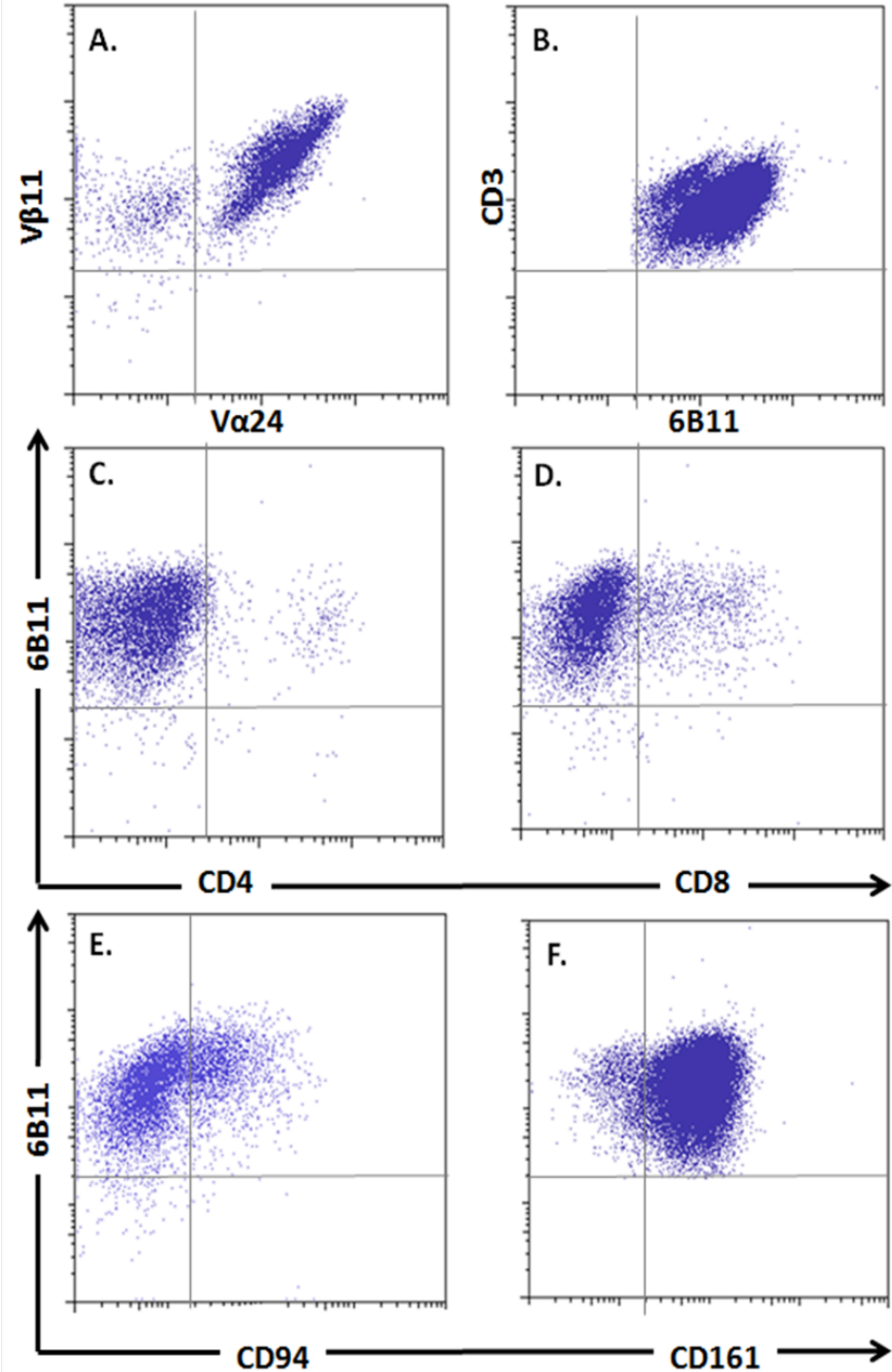
Figure 4.7: Phenotypic characterisation of iNKT cell line number 7 by flow cytometry. Representative flow cytometric dot plot showing expression of Vα24 and Vβ11 TCR chains (A), and 6B11 and CD3 (B). C-F, flow cytometry dot plots showing the expression of CD4, CD8, CD94 and CD161 by electronically gated iNKT cells
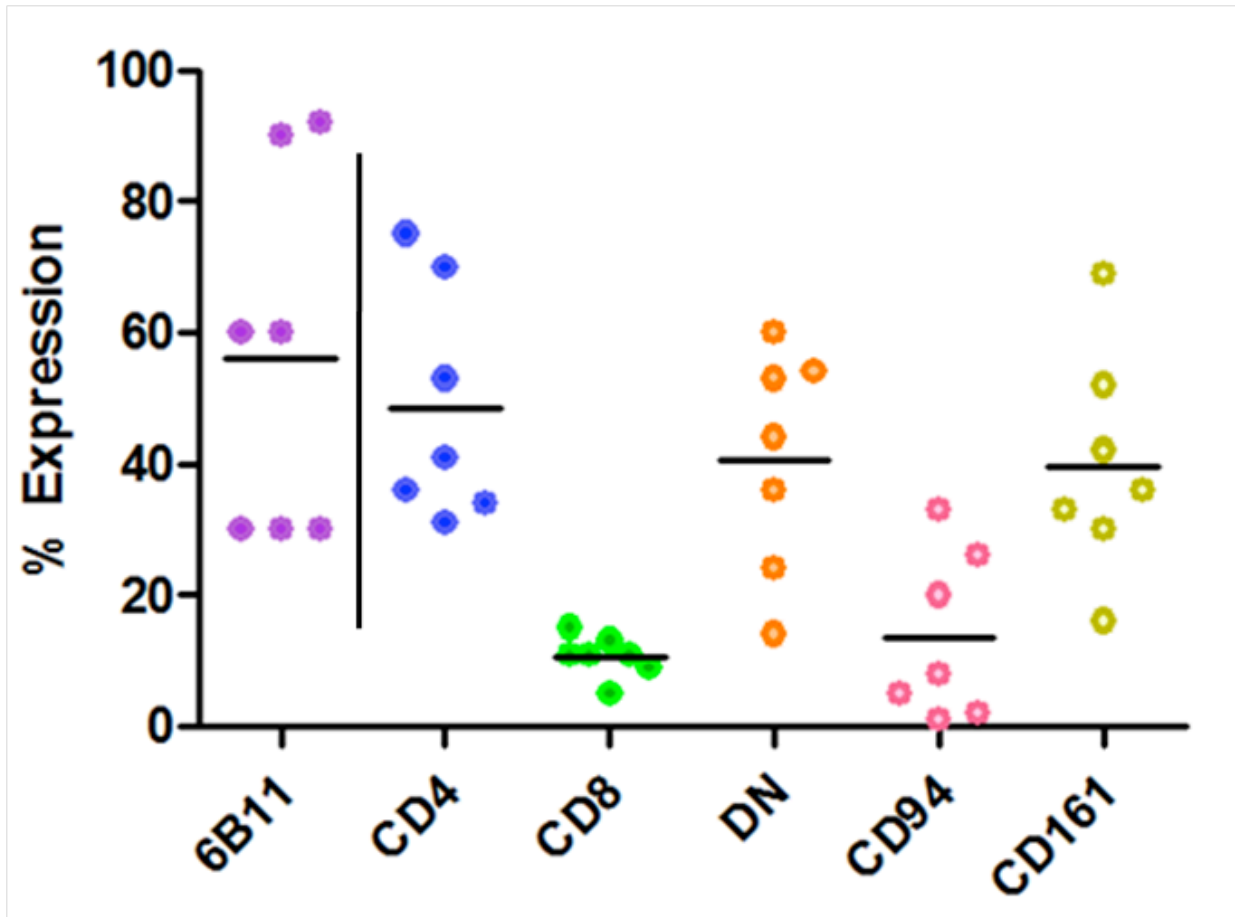
Figure 4.8: iNKT cell line phenotypes: Scatter plot showing the mean frequencies (horizontal line) of surface marker expression by iNKT cells in cell lines established using Method 3. Cell markers are shown as percentage of the 6B11 positive cells. Results are based on 7 iNKT cell lines from different donors.

4.3.3: Investigation into iNKT cell cytokine secretion in response to CD1d and exogenous glycolipid αGalCer

CD1d reactivity and glycolipid specificity of iNKT cell lines were assessed in functional assays. iNKT cells were expanded from human peripheral blood using Method 3 (described in section 4.3.1) and were co-cultured with the stably transfected C1R CD1d cell line, loaded or unloaded with 100 ng/ml of αGalCer, using mock transfected C1R cells as negative control and the T cell mitogen PHA as positive stimulus. Reactivity was measured by quantifying secreted IFN-γ and IL-4 in cell culture supernatants using ELISA. Small amounts of IFN-γ (112 pg/ml) and IL-4 (58 pg/ml) were released by iNKT cell lines cultured with mock-transfected C1R cells. iNKT cells co-cultured with CD1d transfected C1R cells released means of 4.5 and 3 fold more IFN-γ and IL-4 respectively. The levels of both cytokines were increased with the addition of iNKT ligand αGalCer with 8 fold more IFN-γ ($p = 0.01$) and a 7 fold increase in IL-4 secretion ($p = 0.04$) compared to the levels released in response to mock transfected C1R cells (Fig 4.9 A&B). To confirm that this cytokine secretion required CD1d, CD1d molecules on C1R CD1d cells were blocked using anti-CD1d mAb and iNKT cell reactivity against CD1d transfected C1R cells in the presence or absence of αGalCer was tested as before. This resulted in 4 fold reductions in both IFN-γ and IL-4 secretion in comparison to the CD1d loaded with αGalCer (Figure 4.9 A&B). These results indicate that the iNKT cell lines respond to αGalCer in a CD1d dependant manner, releasing IFN-γ and IL-4.
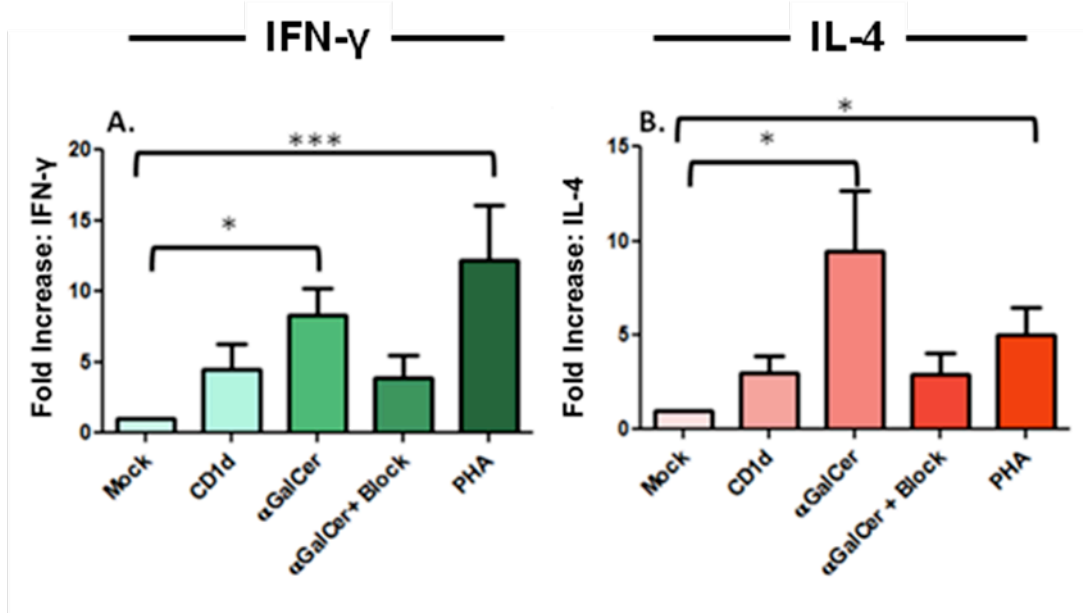
Figure 4.9: iNKT cell responses to CD1d: Bar graphs showing the fold increase in levels of secreted (A) IFN-γ and (B) IL-4 by iNKT cells in response to mock transfected C1R cells or CD1d transfected C1R cells in the presence or absence of αGalCer. Block denotes the blocking of loaded CD1d with an anti-CD1d mAb as a control for specificity. PHA was used as a positive stimulus. Results are a mean of 5 separate experiments.

4.3.4: Investigation into iNKT cell cytotoxicity against CD1d transfected C1R target cells

iNKT cell cytotoxic capabilities were assessed using the total cytotoxicity and apoptosis assay (Chapter 2). C1R cells stably transfected with CD1d were labelled with CFSE and were incubated with iNKT cells in the presence or absence of αGalCer for 4 hours at effector target ratios of 10 to 1. After incubation cells were co stained with 7-AAD, a marker of recent apoptosis and double positive cells staining for CFSE and 7-ADD represented apoptotic target cells. C1R CD1d target cells incubated with no effector cells resulted in a mean of 3% apoptotic target cells (Figure 4.10A, 4.11), Addition of iNKT cells resulted in a mean lysis of 9% of target C1R CD1d cells (p =0.03) (Figure 4.10B, 4.11). The further addition of

αGalCer resulted in an increase in lysis to 13% of the target cells (p =0.002) (Figure

4.10C, 4.11). These results indicate that the iNKT cell lines are capable of killing

CD1d expressing target tumour cells and this cytotoxicity is enhanced with

αGalCer.
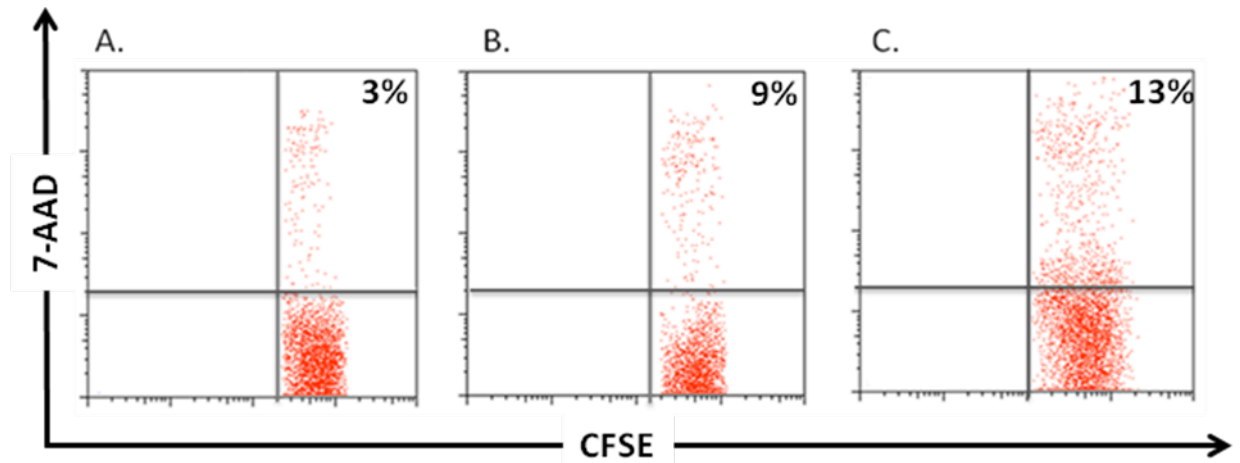
Figure 4.10: iNKT cell cytotoxicity against CD1d transfected C1R cells. Flow cytometric dot plots representative of iNKT target cell lysis. C1R-CD1 cells labelled with CFSE stain were cultured with iNKT cells in the absence or presence of αGalCer. A, Target cells alone. B, Target cells co cultured with iNKT cells. C, Targets co cultured with iNKT in the presence of 100ng/ml of αGalCer. Cells in the upper right quadrant represent apoptotic target cells. Results are representative of 3 different experiments.
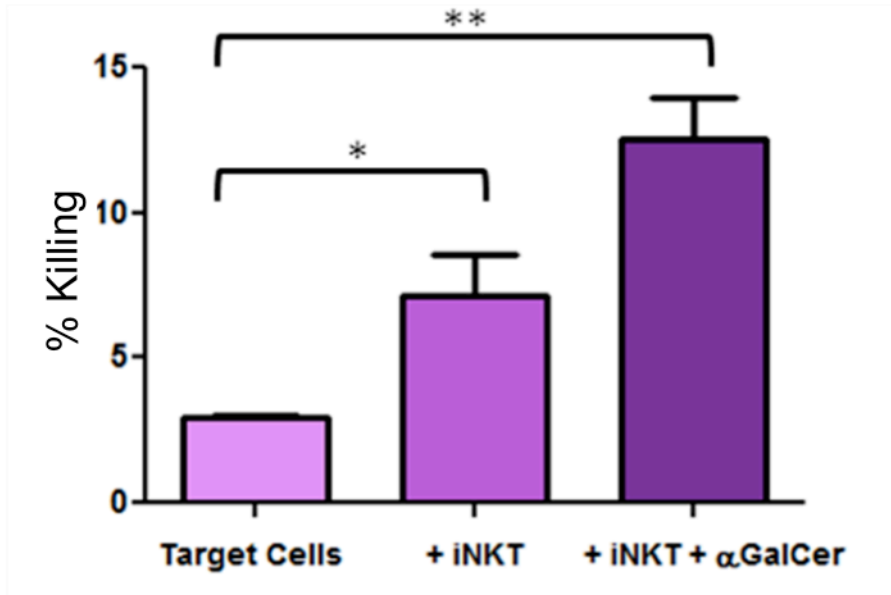
Figure 4.11: iNKT cell cytotoxicity against CD1d+ target cells. CFSE labelled CD1d transfected C1R target cells were incubated either alone, with iNKT cells in the presence or absence of 100 ng/ml of αGalCer for 4 hours at 10:1 ratios. The induction of apoptosis was identified using the apoptotic stain 7-AAD. Results are mean of 3 separate experiments.

4.3.5: Investigation into iNKT cell cytotoxicity against K562 targets

Natural cytotoxicity by iNKT cells against the CD1d-negative NK sensitive human cell line K562 was also assessed using the CFSE incorporation cytotoxicity assay (Figure 4.12). K562 target cells stained with CFSE were incubated with iNKT cells in the absence or presence of αGalCer for 4 hours at effector target ratios of 1:1, 10:1 and 20:1 (Figure 4.13). After incubation cells were co-stained with 7-AAD. Double positive cells (CFSE and 7-AAD) represented apoptotic target cells. K562 target cells incubated in the absence of effector cells resulted in 3% apoptotic cells (Figure 4.12A). Addition of iNKT cells resulted in mean lysis of 19% of target cells (p =0.0001) (Figure 4.12B & 4.13). Addition of αGalCer resulted in an increase in lysis to 26% of target cells (p =0.0035) (Figure 4.12C, 4.13 & 4.14). These results indicate that the iNKT cell lines are capable of killing CD1d negative target tumour cells and this cytotoxicity is enhanced with αGalCer. H&E staining of iNKT cell and K652 co-cultures show that cell to cell contact occurs in the co-cultures (Figure 4.15).
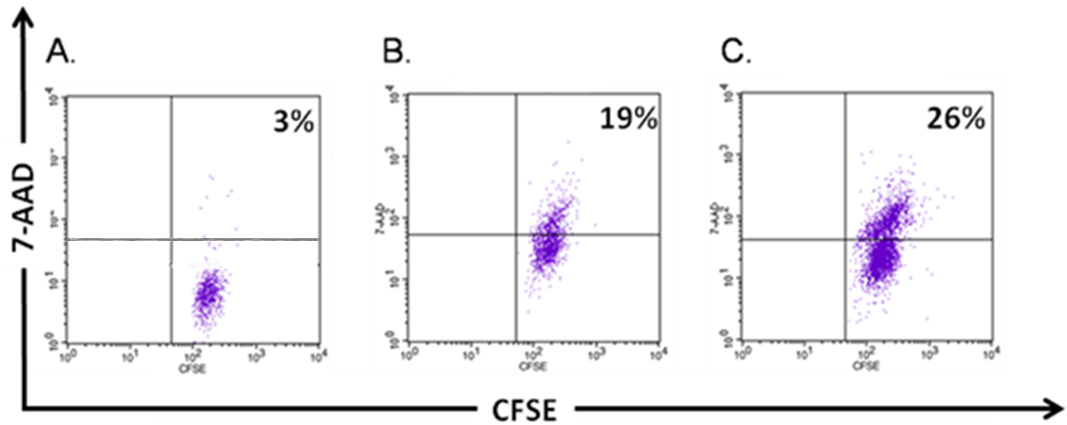
Figure 4.12: iNKT cell cytotoxicity against K562 cells. Flow cytometric dot plots representative of iNKT target cell lysis. K562 cells labelled with CFSE were cultured with iNKT cells in the absence or presence of αGalCer. (A) Target cells alone. (B) iNKT co cultured with targets . (C) iNKT co cultured with targets in the presence of 100 ng/ml of αGalCer. Cells in the upper right quadrant represent apoptotic target cells. Results are representative of 3 different experiments.
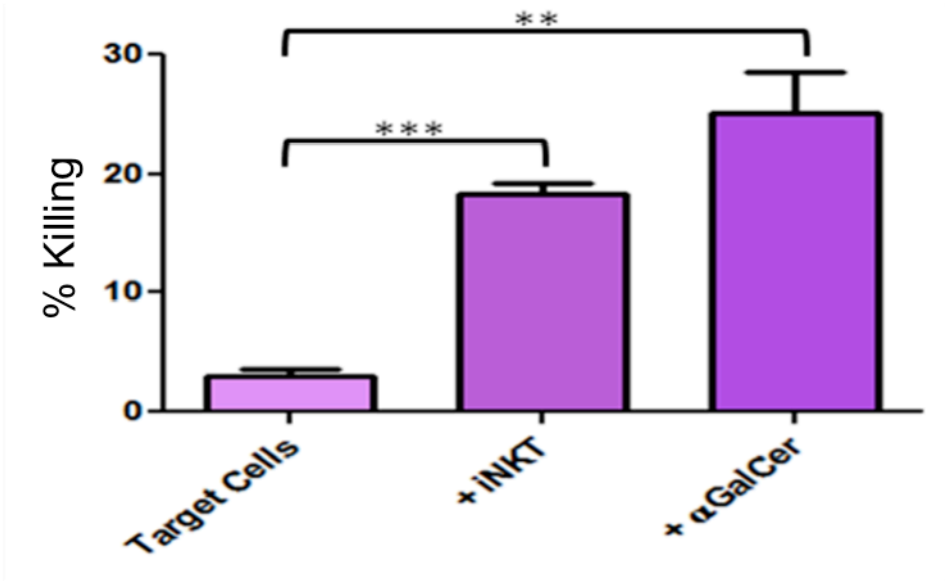
Figure 4.13: iNKT cell cytotoxicity against K562 target cells. CFSE labelled K562 target cells incubated either; alone, with iNKT in the presence or absence of 100ng/ml of αGalCer at 10:1 ratios. The induction of apoptosis was identified using the apoptotic stain 7-AAD. Results are the mean of 3 separate experiments
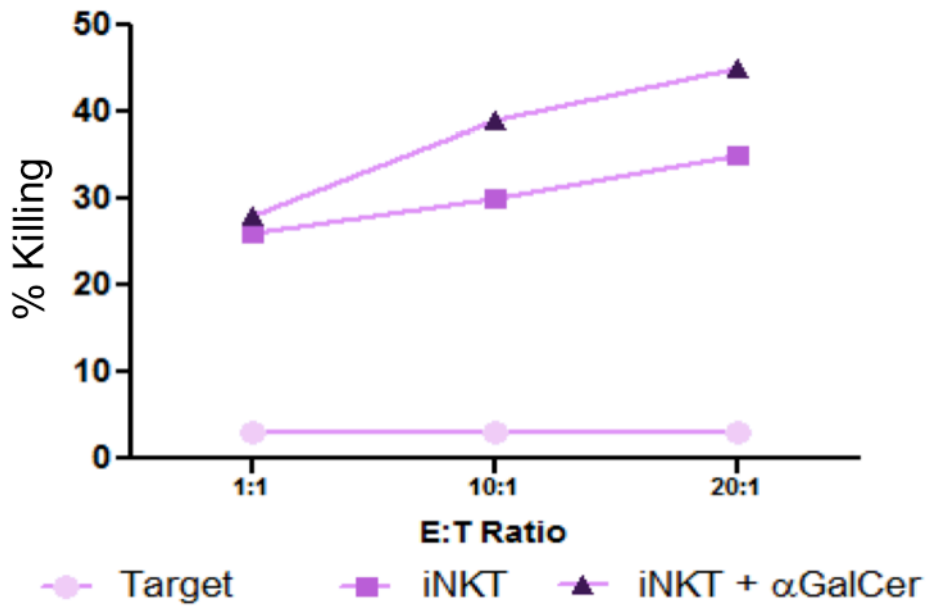
Figure 4.14: Killing of K562 target cells by iNKT cell lines. Line graph displaying lysis of CFSE labelled K562 cells by iNKT at 3 effector target ratios, 1:1, 10:1 and 20:1. CFSE labelled K562 target cells were incubated with iNKT cells at different ratios for 4 hours. The induction of apoptosis was identified using the apoptotic stain 7-AAD.
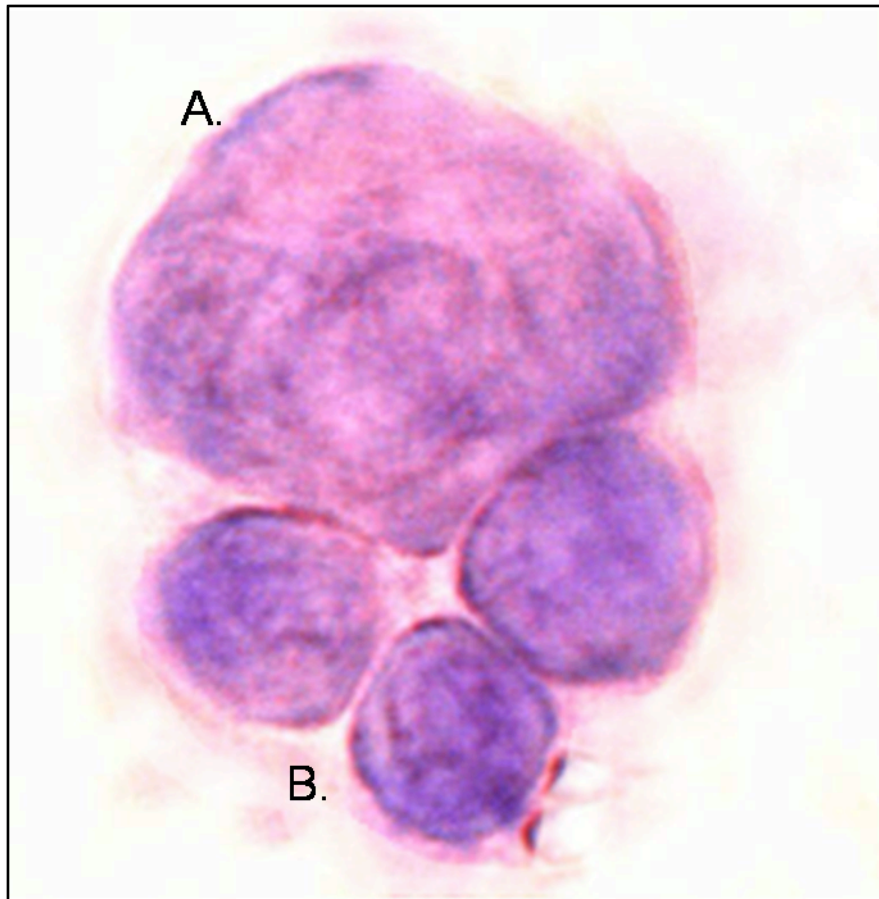
Figure 4.15: iNKT tumour cell cytotoxicity. Histology picture showing a H&E stained (A) K562 tumour cell in contact with (B) 3 iNKT cells. The identity of the cells is achieved by comparison with H&E stained K562 and iNKT cells cultured alone. K562 targets cells were incubated for 4 hours with iNKT cells then smeared on a microscope slide, dried for 24 hours and stained using hematoxylin and eosin. The slides were dried for 24 hours and a covered with a cover slip.

4.4 Discussion

Several investigators have described the expansion of iNKT cells using αGalCer stimulation in the presence of irradiated feeder cells and IL-2 (Tahir *et al* 2001, Exley *et al,* 2003). We used this expansion method (Method 1) starting with magnetic bead enriched iNKT cells cultured with irradiated PBMC from 2 separate donors, 100 ng/ml of αGalCer and 50 U/ml of IL-2. Method 1 resulted in very weak expansion with low purities of iNKT cells. The highest yield achieved after 3 rounds (6 weeks) of stimulation was 2×10^6 cells of which iNKT purity was 1.5%. This is not concurrent with Tahir *et al* (2001) who obtained pure populations of iNKT cells with good yields.

In Method 2, the T cell mitogen PHA was substituted for the specific iNKT agonist αGalCer as used in Method 1. Stimulated iNKT cells were cultured the presence of irradiated feeder cells and IL-2 (Exley *et al* 1997). Method 2 resulted in higher yields of cells and better iNKT purities. The highest yield achieved by Method 2 was 5×10^6 cells, with a purity of 25% iNKT cells from 5 lines plated (Figure 4.2B). Again this method was reported in the literature as a procedure for the expansion of highly pure iNKT cell lines (Exley *et al* 1997).

After personal correspondence with Prof Mark Exley, Harvard Medical School, Method 3 using plate bound anti-CD3 mAb clone OKT-3 was used to expand iNKT populations. Method 3 resulted in good yields, with up to 5×10^7 cells with purities ranging from 30% up to 97% (Figure 4.5). Method 3 was used as the optimal technique for iNKT expansion, however the failure rate of lines as with Methods 1 and 2 remained high with only 1 line from every 3 expanding. The reason for this

"failure" of cells to expand could be attributed to low responding individuals. Croudace *et al* (2008) reported that healthy PBMC donors could be divided into those who responded to αGalCer stimulation and those who responded poorly or not at all, These authors observed that these good and weak responder phenotypes remained stable over time and upon restimulation, only the individuals who responded initially showed a secondary response to αGalCer stimulation (Croudace *et al* 2008).

As outlined above, Methods 1 and 2 have been successfully used to expand human iNKT cells *in vitro* (Tahir *et* al 2001, Exley *et* al 1997, Exley *et al* 2003). Several reasons could explain the failure of Method 1 and 2 to expand iNKT cells; (1) It is possible that the iNKT cells were overstimulated. In Methods 1 and 2, cells were re-stimulated every 14 days, whereas the authors of the previous studies stimulated their cells every 3-6 weeks. Method 3 only used a single stimulation and expanding cells where maintained with IL-2. iNKT cells upon stimulation internalise their TCR to avoid overstimulation (Crowe *et al* 2002), therefore it is possible that iNKT cells are not detected by surface mAb staining. (2) iNKT cell purities or yields were not assessed after magnetic bead separation, to maximise the number of iNKT cells used. It is possible that low yields and purities were obtained using this procedure and this could explain the low rates of expansion and the proliferation of non-iNKT CD3+ cells. Importantly, the authors of the above-mentioned papers sorted their cells using a high speed flow cytometric cell sorter. (3) Non-optimal cell densities: purified iNKT cells where cultured with irradiated PBMC, the presence of which made it difficult to assess the levels of iNKT cells microscopically, cell cultures may have been overcrowded or split before full proliferation was induced. Method

3, because of the lack of feeder cells, allowed easier maintenance and visualisation of cell densitiy of 1×10^6 iNKT cells/well. (4) The reagents may not have been optimal: after lengthy discussions with several collaborators, the question of reagents was raised (FBS, PHA, αGalCer, OKT-3 anti CD3 mAb) and differences between sources could explain why the Methods outlined worked for the original authors but failed in this body of work.

During identification of iNKT cells it was noticed that the numbers of iNKT cells within each line differed depending on the mAb used for their detection by flow cytometry. A sample of each iNKT cell line was stained using either; Vα24 and Vβ11 mAbs, CD3 and 6B11 mAb or αGalCer loaded CD1d PE tetramers with CD3 mAb. In one iNKT cell line, αGalCer loaded CD1d PE tetramers and CD3 mAb stained 97% of cells, while 65% of the same population stained with 6B11 and CD3 mAb, and the mAb pairs Vα24 and Vβ11 stained 43%. Possibly the staining of the iNKT TCR with multiple mAbs versus a single mAb or CD1d tetramer could explain the difference in cell number between methods, where one mAb could partially block the second directed against the same receptor. Also noted in the iNKT line dot plots was a perception of streaking populations, initially flow cytometric compensation settings where adjusted but the numerous "streaking" populations did not change. Godfrey *et al* previously reported that iNKT cell internalize their TCR upon TCR stimulation to prevent over activation and the iNKT cells then re-introduce the receptor to the surface as they become rested (Crowe *et al,* 2003). This could explain the differential expression of the TCR by the iNKT cell lines as they were stimulated using plate bound OKT-3 anti CD3 mAb and IL-2 during culture.

Expanded iNKT cells were characterised by flow cytometry to determine the iNKT surface molecule phenotypes. Of 20 iNKT cell lines generated we phenotypically assessed 7 lines, each which had a purity in excess of 30% 6B11 positivity. Previous studies have described subsets of CD4+, CD4-CD8- in mice and humans and CD8+ iNKT cells in humans but not mice (Bendelac *et al*, 2007, Matsuda *et al* 2008). Analysis of our iNKT lines showed mixed populations with ~50% cells expressing the CD4+ phenotype, 43% expressing the DN phenotype and 7% expressing CD8.

Different subsets of iNKT cells which express different surface marker repertoires have previously been described (Bendelac *et al* 2007, Exley *et al* 1997, Exley *et al* 1998). The iNKT cell lines generated in the present work consisted of mixed subsets. The generation of iNKT clones or homogenous iNKT cell lines was not attempted, due to time/equipment limitations. Furthermore we aimed to minimize the protocol for the generation of iNKT cell lines so that the method could be used in future iNKT cell based immunotherapy. Thus minimization of time and *in vitro* procedures would be beneficial. The surface phenotype of iNKT cells can determine their function. Exley *et al* (1997) reported that cytotoxicity by iNKT cells is CD1d restricted, but later Nicol *et al* showed that cytotoxicity by iNKT cells can occur independently of CD1d. These authors suggested that the expression of CD94 by the iNKT cell lines may facilitate TCR-independent cytotoxicity (Nicol *et al,* 2000). We assessed our lines for 2 inhibitory/stimulatory molecules, CD94 and CD161 expression by flow cytometry. CD94 was expressed at low frequencies

(<10%) whilst CD161 was expressed by ~50% of the iNKT cells examined (Figure 4.7).

iNKT cells are well described as potent cytokine producers, with the ability to simultaneously secrete IFN-γ and IL-4 in response to αGalCer stimulation (Exley *et al,* 1997, Chen and Paul 1997). We examined our iNKT cells' ability to produce cytokine by co-culturing at 1:1 ratio with CD1d transfected C1R cells in the absence or presence of 100 ng/ml αGalCer. Optimal IL-4 secretion was observed 24 hours after stimulation and optimal IFN-γ secretion was observed after 72 hours as described previously and in personal correspondence with Prof Mark Exley. In earlier cytokine experiments with PBMC and purified CD3+ populations, IL-4 was not readily detectable so IL-13 was substituted in its place. In hindsight the time point of 72 hours was probably too long for the optimal detection of IL-4 in our assay supernatants. Addition of CD1d mAb partially blocked both IL-4 and IFN-γ secretion, verifying the role of CD1d in the activation of the iNKT cell lines (Figure 4.8). The secretion of both cytokines could be due to the presence of a mixed iNKT cell subset population in our lines, previous studies have described different subsets preferentially secreting either both IFN-γ and IL-4 (CD4+ iNKT cells) or IFN-γ alone (DN iNKT cells) (Gumperz *et* al 2002, Lee *et* al 2002, Chen *et al* 2007).

iNKT cells have been reported to lyse tumour cells in vitro (Exley *et al*, 1997, Nicol *et al,* 2000). We tested the cytotoxic abilities of our iNKT cell lines using CFSE labelled tumour cells as targets, we can report that our iNKT cell lines can efficiently lyse the CD1d transfected C1R cell line and the NK sensitive K562 tumour cell line in the absence of αGalCer. The percentage specific lysis of both

targets was increased with the addition of αGalCer. iNKT cell lines efficiently lysed targets at effector target ratios of 1:1, 10:1 and 20:1, with 10:1 chosen as the optimum ratio due to cell number restraints. Activation of the iNKT cells in the absence of αGalCer in the cytokine and cytotoxicity assays may be influenced by their previous expansion and culture with IL-2. To reduce the possibility of activation by IL-2 cells where rested for 24 hour before use in assays. The lysis of the CD1d negative NK sensitive K562 tumour cell line by the iNKT cell lines shows the CD1d independent cytotoxic capabilities of iNKT cells, which may be mediated by surface receptors such as the NKG2 family of stimulatory/inhibitory molecules. Microscopic examination of the lysis cultures between K562 tumour cells and iNKT cell shows cell to cell contact, indicating this may be necessary for CD1d independent killing. Unexpectedly, the addition of αGalCer resulted in an increase in K562 killing. Cross presentation of the glycolipid by other CD1d expressing iNKT cells or other contaminating CD1d-positive cells, could be a explanation for this contribution of αGalCer to CD1d-independent cytotoxicity. The thorough examination of the killing mechanism was limited by the number of iNKT cells available.

This chapter of work has optimized a previously described method for the isolation and expansion of human iNKT cells from peripheral blood. Furthermore it examined the subsets within the cell lines established. The functional ability of the iNKT cell lines to produce cytokine and lyse tumour target cells was also investigated. We aimed to optimize and establish a method of expanding functional iNKT cells which provide a essential tool in subsequent chapters.

4.5 Conclusions

- Purified iNKT cells expanded in response to the plate bound anti-CD3 mAb OKT-3 and IL-2 in the absence of irradiated feeder cells.

- iNKT cell lines expressed phenotypes of mixed subsets (CD4+, CD4-CD8-, CD8+)

- iNKT cell lines secreted IFN-γ and IL-4 in the presence of CD1d and αGalCer

- iNKT cell lines efficiently lysed tumour cell targets *in vitro* in the absence of αGalCer, however the % specific lysis was increased in the presence of αGalCer

- The iNKT cells expanded from human peripheral blood were both phenotypically and functionally concurrent with the literature (potent cytokine secreting cells with the ability to lyse target cells) and provided a valuable asset in the following body of work

5: Results: Reciprocal interactions between Human iNKT cells and Dendritic cells

Chapter 5: Results

5.1: Introduction

Dendritic cells (DCs) are a population of cells which act as sentinels for the immune system, surveying the local environment, capturing and processing Ag for presentation to T cells. Thus DCs provide a link between the innate and the adaptive immune systems (Banchereau *et al* 2000). DCs exist in two main states with numerous intermediates; the Ag capturing and processing immature DC (iDC) and the Ag presenting and co-stimulatory mature DC (mDC). DCs are classed as professional antigen presenting cells (APCs) due to their ability to sample, capture and present a large antigenic load and their ability to provide both TCR stimulation (signal 1) and co-stimulation (signal 2) and the release of cytokines that polarize T cell responses (Signal 3) (Blanko *et al* 2008, Banchereau *et al* 2000). The provision of signal 1, 2 and 3 results in full activation of naïve T cells, and thus elicits a full and effective adaptive immune response (Banchereau & Steinman1998, Figor *et al* 2004).

Full maturation of the iDC is required for the provision of signal 2. Maturation is a process whereby the iDC encounters a maturation stimulus such as various pathogen products, inflammatory cytokines or TLR agonists. In this body of work the TLR agonists; LPS and poly I:C where used as maturation stimuli. LPS binds to cell surface TLR 4, while poly I:C binds to TLR3 found within intracellular vesicles. Upon binding a cascade of intracellular signals is initiated leading to the transcription of maturation associated genes. This leads to the upregulation of cell surface expression of MHC and co-stimulatory molecules such as CD40, CD80 and CD86. Other receptors induced on mature DC include CCR7, a chemokine involved in cell migration, CD40 which binds its ligand CD40L on T cells and augments the

co-stimulatory signal. CD54 is an adhesion molecule which binds LFA-1 on T cells and increases the avidity of the interaction between the DC and the T cell allowing ultimately T cell activation. CD80 is a co-stimulatory molecule expressed on DCs and binds CD28 expressed on T cells. CD83 is a classical maturation molecule, whose function or ligand is unclear. CD86 is a co-stimulatory surface molecule similar to CD80 and also binds CD28 on T cells. The resulting activation varies depending on which molecule CD28 binds. HLA-DR is human MHC II and is presentation molecule found on DCs and interacts with T helper cells (Banchereau *et al* 2000).

The maturing DC leaves the tissue and migrates to the peripheral lymph nodes. The DC morphology changes and it develops the dendritic like structures to which it owes its name. Some DC reside in the lymph nodes but the subset detailed in this study are peripheral DC. The mature (mDC) can also produce cytokines such as IL-12 and IL-10, which polarize adaptive immune responses. Once the mDC reaches the lymph node it encounters naïve T cells and presents the antigenic peptide bound to MHC molecules to T cells. The responding T cell receives co-stimulation signals via the CD80/CD86-CD28 interaction. This activates the naïve T cell to proliferate and respond in a specific manner. In the absence of co-stimulation the T cell becomes anergic and does not respond to the Ag presented, thus resulting in tolerance (Banchereau *et al* 2000, Figdor *et al* 2004).

Due to the powerful abilities of DCs to elicit and skew a T cell response, they are now widely manipulated for therapeutic use. For successful elicitation of an effective T cell response the DC vaccine must be a fully mature DC providing both

signal 1 and 2. Adjuvants such as LPS can be used to mature the DC in conjunction with the target Ag (e.g. tumour Ag) (Steinman & Banchereau 2007, Figdor *et al* 2004, Dubsky *et al* 2005) Another adjuvant used in DC vaccine studies is tocotrienol-rich fraction (TRF). TRF is a non-toxic natural compound, which has been shown to enhance the effectiveness of DC vaccines in murine models, with inhibition of tumour growth and increased IFN-γ and IL-12 (Hafid *et al* 2010).

The reciprocal interactions between DCs and innate T cells (iNKT cells and γδ T cells) have been previously described in murine models (Brigl *et al* 2003, Fujii *et al* 2004, Munz *et al* 2005, Ishikawa *et al* 2005). Murine iNKT cells have the ability to fully mature myeloid iDCs into antigen presenting cells expressing MHC, CD80/CD86, which produce bioactive cytokines (IL-12 and IL-10) (Munz *et al*, 2005). Cytokines released by iNKT cells, and signals mediated by CD40-CD40L interactions achieved through direct contact between the DC and the iNKT cell are required for full activation of the DC and subsequent linking of innate and adaptive immunity (Fujii *et al*, 2003, Sporri & Reise-Sousa 2005). Vincent *et al* reported the interactions between self reactive human CD1 restricted cells and DCs in the absence of antigen result in DC maturation, they also report that distinct mechanisms of costimulation lead to profound differences in concomitant interleukin 12 p70 production (Vincent *et al* 2002*)*. Yang *et al* reported that human Vα24+ NKT cells exhibited the same cross talk with monocyte derived DCs as their murine counterparts, and that iNKT cells select for a Th1 adaptive response through the production of IL-12 by DCs. The authors also described specific lysis of DCs by this NKT subset, and concluded this is a possible feedback control mechanism (Yang *et al* 2001). Ishikawa *et al* compared the interactions between Vα24 iNKT

cells and either monocyte derived DC and IL-2/GM-CSF cultured PBMC. The authors concluded the interactions resulted in the expansion of iNKT cells and the production of IFN-γ. PBMC cultured in the presence of IL-2/GM-CSF exhibited the better stimulatory effect compared to monocyte derived DCs (Ishikawa *et al* 2005). The study of the interactions between human iNKT cells and monocyte derived DC is not as extensively studied their murine counterparts, which highlights the need for in-depth studies of the reciprocal interactions between the two.

This influence of iNKT cells on DC could possibly be harnessed as a cellular adjuvant for cell based therapies. Another benefit for using iNKT cell mediated maturation of DC is that, iNKT cells have direct anti-tumour effects and can mobilize an innate immune response (NK cells) and an adaptive response via B & T cell activation (Munz *et al* 2005). Nieda *et al* demonstrated that iNKT activated by αGalCer loaded DC induced NK and T cell expansion in cancer patients (Nieda *et al* 2004).

DCs are rare in human peripheral blood, therefore the study of DCs therefore requires the *in vitro* generation of DC from precursors. The method most widely used for the generation of monocyte derived DCs was first published in 1994 by Sallusto & Lanzavecchia.

In this body of work we aimed to thoroughly investigate the reciprocal interactions between human monocyte derived DCs and iNKT cell *in vitro*. We aimed to establish a system to test novel αGalCer analogues potential for therapeutic use. Using the method described by Sallusto and Lanzavecchia we derived DCs from

monocytes and examined their immature phenotype, and determined if the TLR

agonists LPS and poly I:C could induce phenotypic and functional DC maturation

for use as positive controls. We investigated the ability of human iNKT cell and

non-iNKT cells in the absence or presence of αGalCer to mature iDC into fully

functional mDCs with the ability to elicit a T cell response (Figure 5.1).

5.2: Objectives

1. Establish a DC maturation system

2. Investigate iNKT ability to activate immature DC

3. Examine the reciprocal functional interactions and responses of iNKT and
    DC

4. Investigate the T cell maturating capabilities of iNKT activated DC

Whole PBMC

Anti-CD14 Microbead

CD14 Positive Cells: Monoctyes

7 Day culture with GM-CSF and IL-4

Immature Dendritic Cell

No Stimulation: Negative Control   **NKT cells +/- Glycolipid**   **LPS or Poly I:C: Positive Control**

24 hour culture with Stimuli

Mature Dendritic Cell
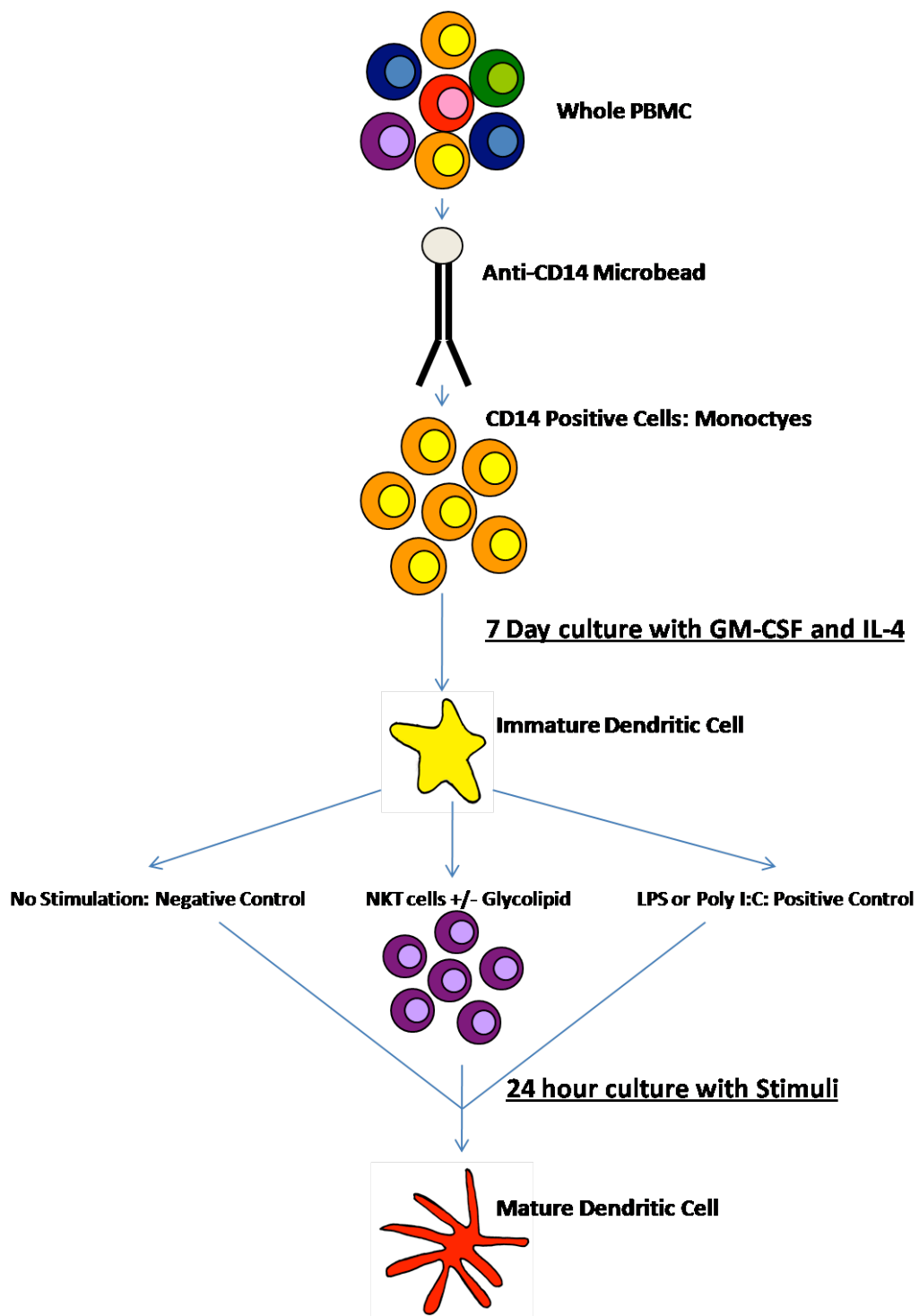
Assay for Maturation & Activation: Cytokine Analysis and Flow Cytometry

Figure 5.1: Strategy for the investigation of interactions between human iNKT and dendritic cells: Monocyte derived immature DC were cultured in the presence or absence of αGalCer. LPS or poly I:C were included as positive control for DC maturation whilst non iNKT cells or culture medium serve as negative control. After stimulation DCs were examined for surface marker expression, cytokine production and their ability to activate naïve T cells.

5.3.1: Effect of LPS and Poly I:C on dendritic cells.

Monocyte-derived dendritic cells (DC) were cultured from human peripheral blood as described in chapter 2. The starting number of PBMC and the purities of isolated monocytes were assessed over 8 experiments. The average starting number of PBMC was 4.96×10^8 and after magnetic bead separation the average number of CD14+ monocytes retrieved was 1.67×10^7. Monocyte purities were examined by flow cytometry and are shown table 5.1. The generation of immature DC from monocytes resulted in the downregulaton of CD14 so expression of CD14 was analysed in order to assess DC maturation (Figure 5.2).

The effects of LPS and poly I:C on iDC maturation was investigated by examining changes in cell surface marker repertoires by flow cytometry (Figure 5.3) and by quantification of secreted cytokines by ELISA (Figure 5.4). iDC were incubated for 24 hours in medium alone (control) or in the presence of LPS or poly I:C. The levels of expression of seven surface molecules were investigated for each set of DC, namely CCR7, CD40, CD54, CD80, CD83, CD86 and HLA-DR.

CCR7 was undetectable on iDC having similar fluorescence intensities to iDC stained with isotype control antibodies. Stimulation with LPS or Poly I:C resulted in mean inductions of 1.7 and 2 fold (p =0.03) increases in fluorescence intensities of CCR7 surface expression respectively. CD40 was also expressed on iDC at low levels and the mean surface expression was increased 7 fold (MFI) with LPS stimulation (p =0.003) and 5.5 fold with poly I:C stimulation (p =0.008). CD54 was expressed at moderate to high levels on iDC, and the mean expression levels were increased 3 and 2 fold with LPS (p=0.008) and poly I:C (p=0.015) respectively.

CD80 was expressed at low levels on iDC (compared to isotype control staining intensities), and this was not increased by LPS or Poly I:C stimulation within 24 hours. CD83 was expressed at very low levels on iDC, with intensities similar to those of the isotype control. LPS stimulation induced a (mean 3.5 fold) increase in expression (p =0.002), whilst poly I:C resulted in smaller (mean 1.7 fold) increase in surface CD83. CD86 was expressed at low levels by iDC, stimulation with LPS and poly I:C resulted in (4.5 and 3.7 fold respectively) increases in surface expression(p= 0.0007 and p =0.02). HLA-DR was expressed at moderate levels on iDC with fold increases in expression of 2 and 1.6 with LPS and poly I:C stimulation respectively (Figure 5.3). These results show that LPS and poly I:C treatment of iDC result in the upregulation of Ag presentation and co-stimulation molecules. This confirms that iDC could be obtained from healthy human peripheral blood and matured using the TLR ligands LPS and poly I:C.

Cytokine secretion by DC incubated with LPS or poly I:C was examined by ELISA. Levels of IL-10 and IL-12 were compared to those released by iDC incubated in medium alone (control). Cytokine secretion was quantified from 5 experiments and expressed as fold increases over the levels released by iDC cultured in medium alone (IL-12 range 41-88 pg/ml, IL-10 range 54-108 pg/ml). Stimulation of iDC with LPS resulted in a mean 5 fold increase in IL-12 (p =0.041) (Figure 5.4A) and 8 fold increase in IL-10 secretion (p =0.007) (Figure 5.4C). Poly I:C stimulation resulted in 2.5 fold increase in IL-12 secretion (p =0.001) (Figure 5.4B) and ~1.5 fold increase in IL-10 (p =0.04) (Figure 5.4D). These results show that iDC can be isolated from healthy human peripheral blood and that the TLR agonists LPS and

poly I:C can induce DC maturation and cytokine production. For maturation purposes LPS was better than poly I:C and thus selected for use in later experiments.

Table 5.1: Yields and purities of CD14 magnetic bead enrichment of monocytes from PBMC

	PBMC **Starting Number X10⁶**	Isolated **CD14+ cell number X10⁶**	Purity **% of Monocyte gate**
1	400	9.1	87.1
2	685	9.7	98.6
3	438	10.3	91.6
4	450	17.8	96.9
5	500	12	98
6	675	18.1	91.3
7	500	16.8	94.2
8	325	39.7	81.6
Mean	496.6	16.7	94.2
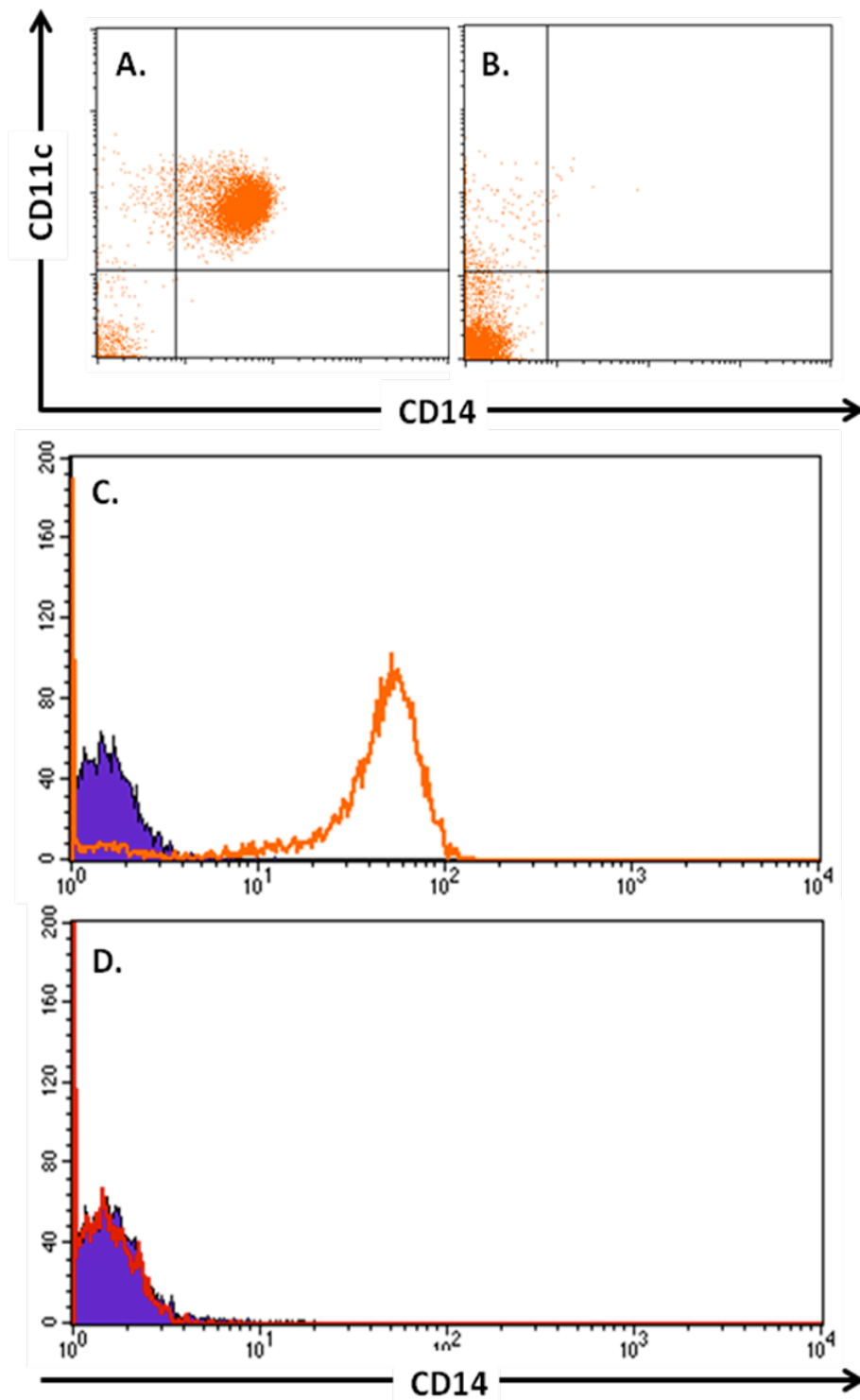
Figure 5.2. Downregulation of CD14 during the generation of iDC from monocytes. A & B, Flow cytometry dot plots showing the expression of (A) CD14 and CD11c by monocytes sorted by magnetic-bead separation and (B) the CD14 negative fraction. C & D, Flow cytometry histograms showing that CD14+ monocytes cultured with GM-CSF and IL-4 downregulate CD14 during the transition from (C) monocytes to (D) immature DC. Purple histsograms respresents isotype control staining. Results are representative of 8 donors.

Figure 5.3. LPS and Poly I:C induce DC maturation. Flow cytometric histograms showing the intensities of CCR7, CD40, CD54, CD80, CD83, CD86 & HLA-DR expression by iDC cultured for 24 hours in medium alone (Purple), iDC cultured with LPS (Green) or iDC cultured with Poly I:C (Blue). Bar charts showing average mean fluorescence intensity (MFI) of surface molecule expression observed in 5 independent experiments.
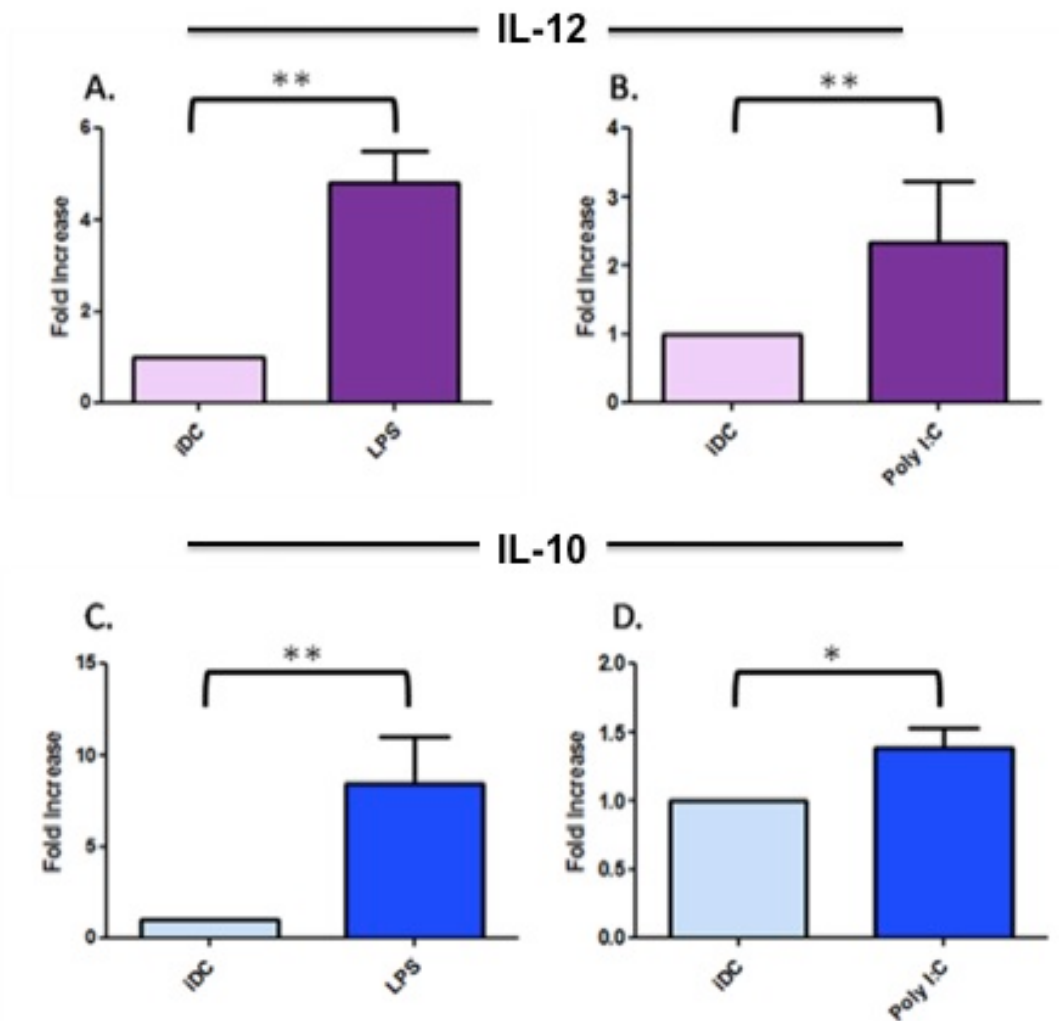
Figure 5.4: LPS and Poly I:C induce cytokine production by DC. Supernatants from iDC cultured with LPS or poly I:C for 24 hours were examined for levels of secreted IL-10 and IL-12 by ELISA. Bar charts show the fold increase in cytokine levels over those from unstimulated DC which released 41-88 pg/ml IL-12 and 54-108 pg/ml IL-10 respectively. Results show levels of IL-12 (Purple) in response to LPS (A) and Poly I:C (B) and IL-10 (Blue) in response to LPS (C) and poly I:C (D) Results are mean of 5 independent experiments.

5.3.2: Effect of co-culturing iNKT cells with dendritic cells.

Monocyte derived dendritic cells (DC) were incubated in medium alone (control) or co cultured with equal numbers of iNKT cells expanded from human peripheral blood (using Method 3, described in chapter 4) with or without the addition of 100 ng/ml of αGalCer. Expression of seven surface molecules was investigated for each set of DC.

The MFI of CCR7 expression on DC was increased 1.7 fold with iNKT cell co-culture compared to DC cultured in media alone. CCR7 expression was further increased (final 2.5 fold) with the addition 100 ng/ml of αGalCer to the co-culture. CD40 surface expression was increased 1.5 fold with iNKT cell co-culture and 2.5 fold with addition of αGalCer to the co-culture. CD54 expression was increased 3 and 5 fold with co-culture of iNKT cells (p= 0.07) in the absence or presence of αGalCer (p =0.003) respectively. CD80 was expressed at low levels on iDC (similar to the isotype control), this was not increased by either co-culture after 24 hours. iNKT cell co-culture induced a 2 fold increase in CD83 expression, whilst co-culture with iNKT cells and 100 ng/ml of αGalCer resulted in 4 fold increase in surface CD83 on DCs (p =0.01). Co-culture with iNKT cells resulted in a 2.5 fold increase in CD86 expression compared to iDC alone (p =0.03), whilst co-culture in the presence of αGalCer resulted in increase of 5 fold (p =0.01). HLA-DR expression was increased 1.25 fold and 1.5 fold on DC co-cultured with iNKT cells and iNKT cells in presence of αGalCer respectively (Figure 5.5).

Cytokine secretion by DC incubated with medium alone or co-cultured with iNKT cells in the presence or absence of 100 ng/ml of αGalCer was examined by ELISA.

Culturing DC with αGalCer resulted in a 7 fold increase in IL-12 secretion compared to iDC cultured in medium alone (range 41-88 pg/ml). Levels of IL-10 released by DC cultured in medium alone (range 54-108 pg/ml) were not altered in response to the same stimulus of culturing with 100 ng/ml of αGalCer (Figure 5.6). Co-culture of DC with iNKT cells resulted in an average 12 fold increase in IL-12 (p =0.0001), a 1.5 fold increase in IL-10 and 2.5 fold increase in IFN-γ release compared to the levels released by iDC alone (Figure 5.7). It should be noted that The cellular origin of the IFN-γ was not tested but is likely to be the iNKT cells. The co-culture of DC loaded with 100ng/ml of αGalCer with iNKT cells resulted in further increases of IL-12 (p =000.1) and IFN-γ (p =0.0079) (23 and 6.2 fold respectively) but no increase of IL-10 were recorded for 3 individual experiments (Figure 5.7). These results show that iNKT cells can induce DC maturation and cytokine production and that the maturation and cytokine production was significantly increased with the addition of 100 ng/ml of αGalCer.
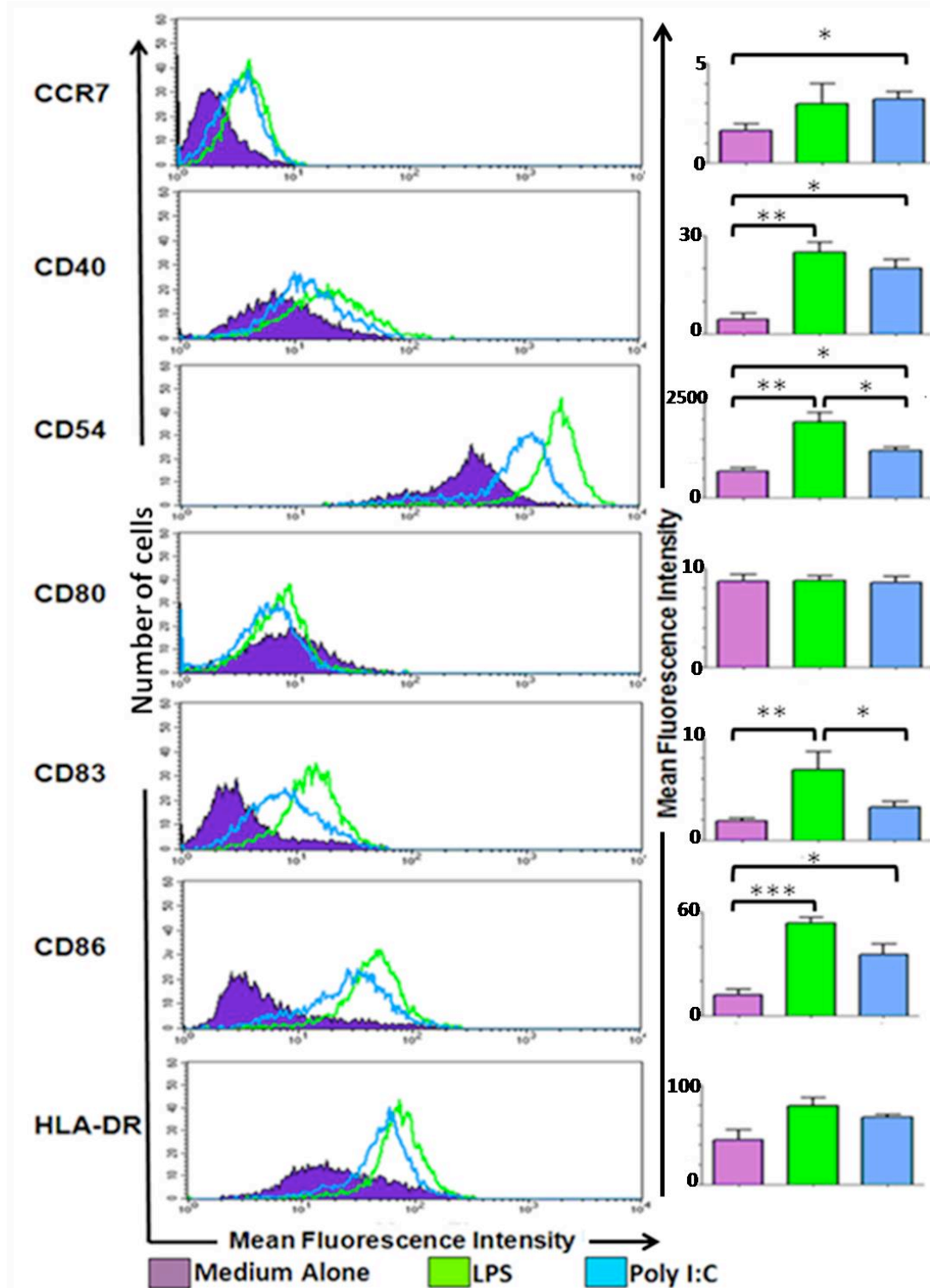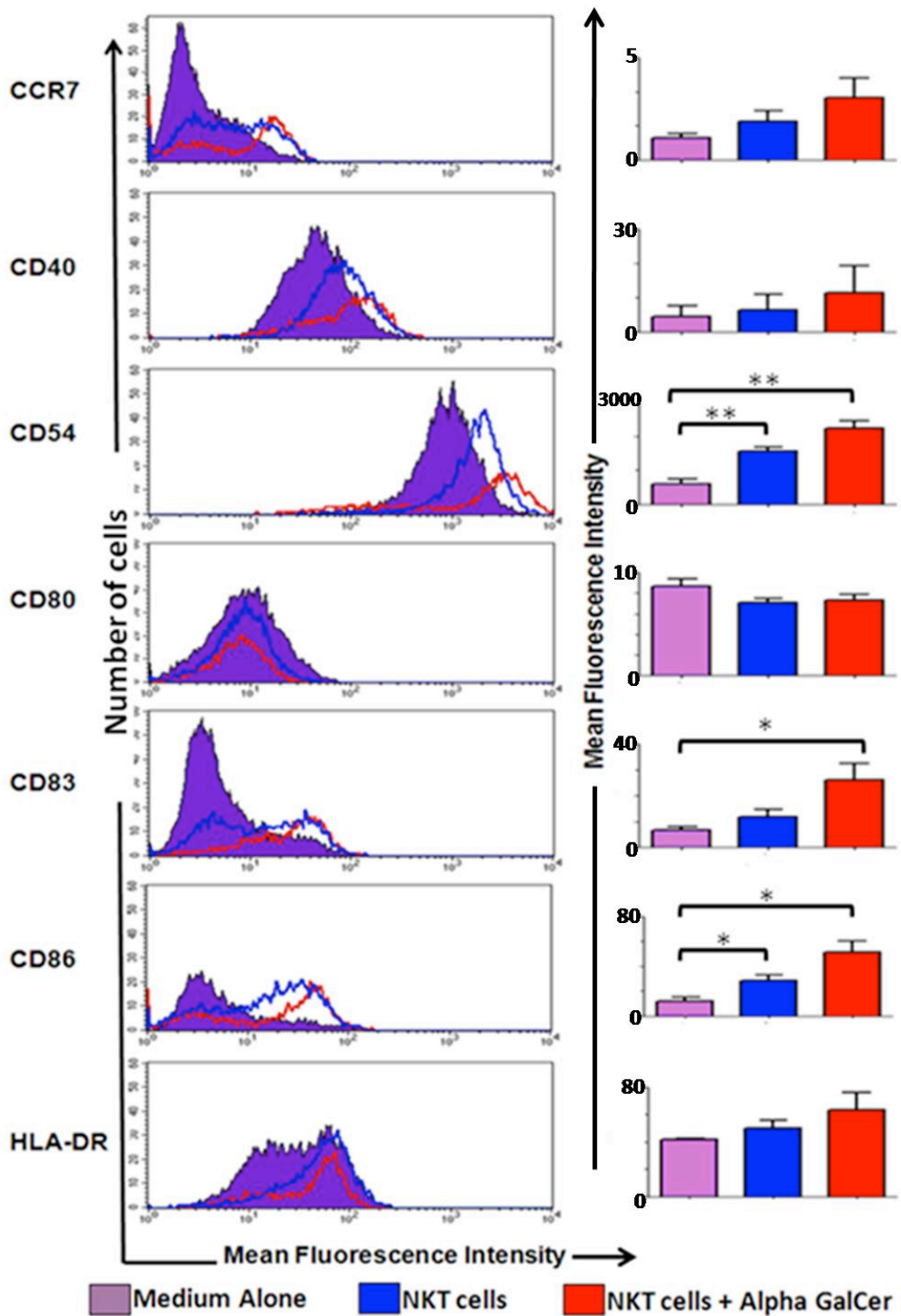
Figure 5.5. iNKT cells induce DC maturation. A, Flow cytometric histograms showing the intensities of CCR7, CD40, CD54, CD80, CD83, CD86 & HLA-DR expression by DC cultured in medium alone (Purple), DC co-cultured with iNKT cells (Blue), and DC loaded with 100 ng/ml of αGalCer and co-cultured with iNKT (Red). B, Bar charts showing average mean fluorescence intensity (MFI) observed in 5 independent experiments.
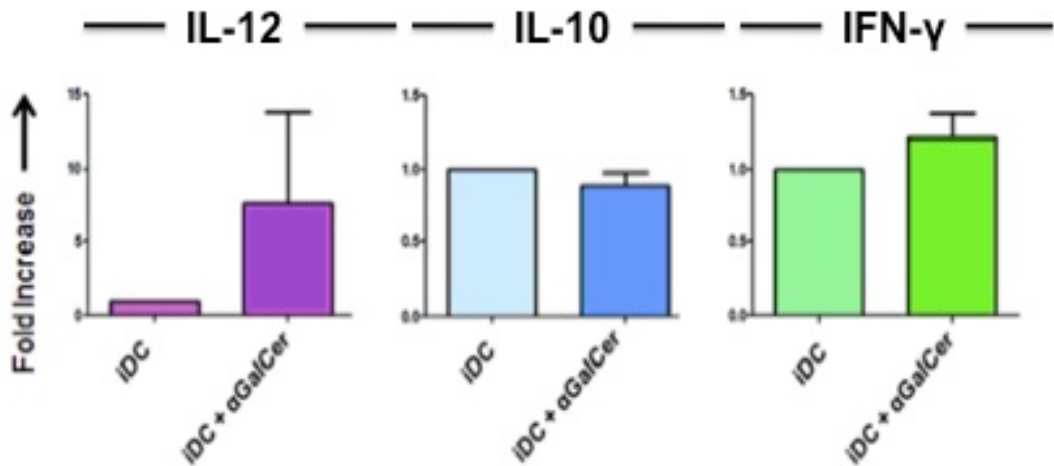
***Figure 5.6: Effects of αGalCer on cytokine production by monocyte derived DC.
Bar graphs show fold increases in levels of IL-12 (purple), IL-10 (blue) and IFN-γ
(green) released by iDC cultured in the presence of 100 ng/ml of αGalCer
compared with those released by iDC alone. Results are a mean of 5 separate
experiments.***
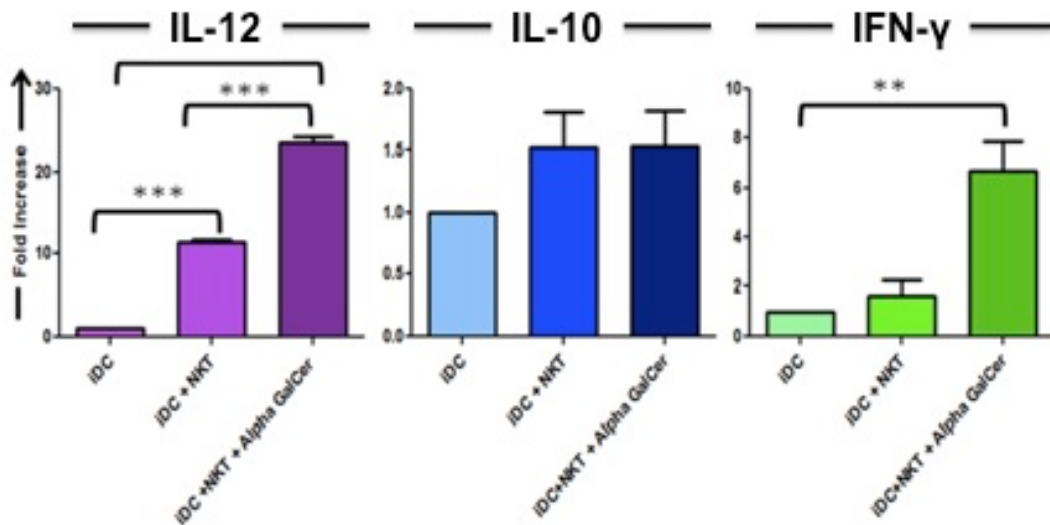
***Figure 5.7: Effects of iNKT cells cytokine production by DC. Bar graphs show
fold increases in levels of IL-12 (purple), IL-10 (blue) and IFN-γ (green) released
by iDC cultured in the presence of iNKT cells in the absence or presence of 100
ng/ml of αGalCer compared with those released by iDC alone. Results are a mean
of 5 separate experiments.***

5.3.3: Effect of co-culturing non-iNKT on dendritic cells

To investigate the specificity of the observed iNKT induction of iDC maturation, non purified iNKT cells consisting of total PBMC stimulated with PHA and cultured in the presence of IL-2, were co-cultured as previously described with DC in medium alone or in the absence or presence of 100 ng/ml of αGalCer. As previously observed, iDC incubated in the presence of αGalCer and expanded iNKT cells upregulated CCR7, CD40, CD54, CD83, CD86 and HLA-DR (Figure 5.8). However when PHA/IL-2 expanded PBMC were substituted for iNKT cells, no upregulation of these markers was observed. This data indicates that iNKT cells are required for the induction of DC maturation and that total non-iNKT cells do not have the same ability.
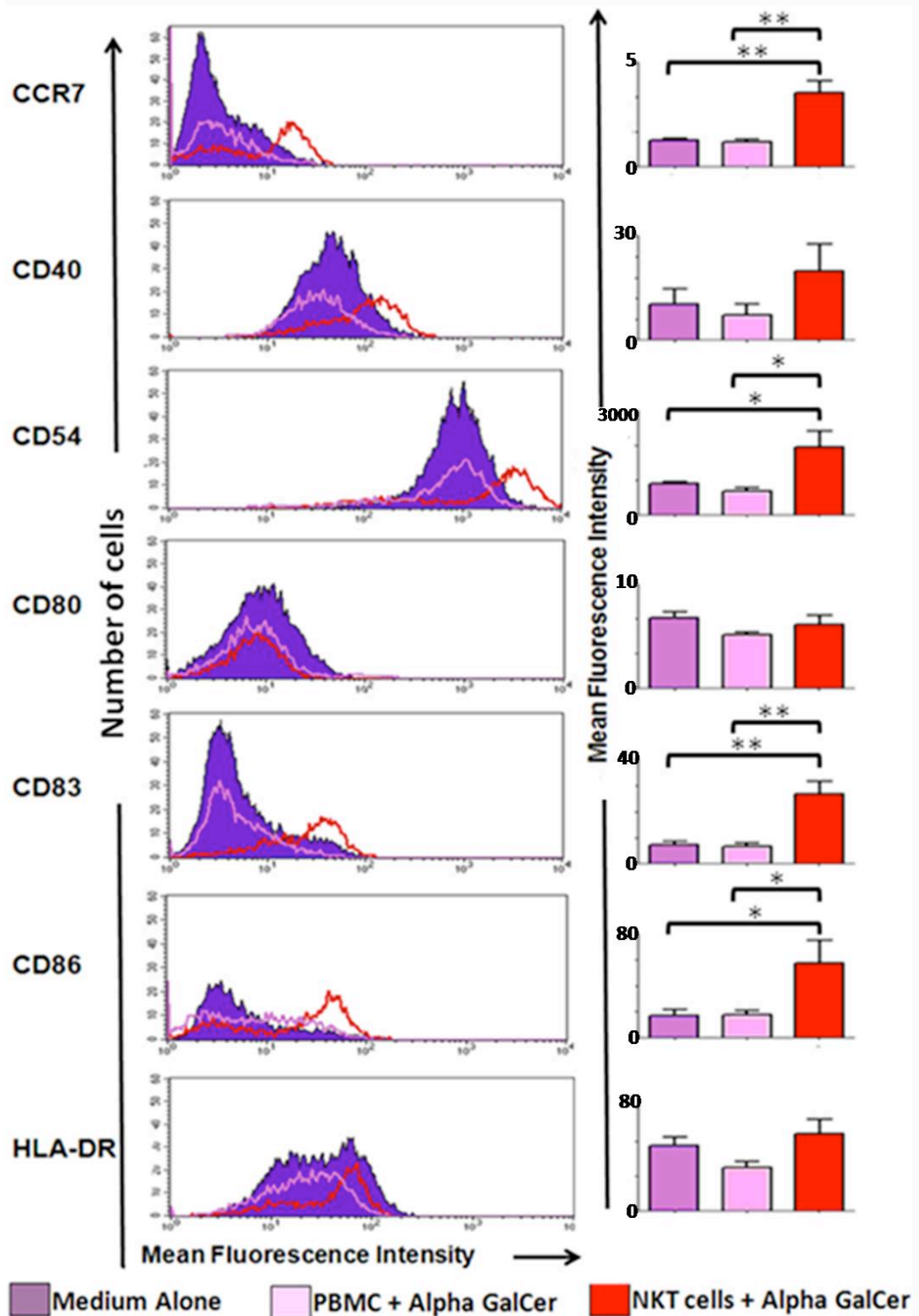
Figure 5.8: iNKT cells are required for αGalCer induced DC maturation. iDC were cultured in medium alone (purple histograms) with PBMC expanded with PHA and IL-2 in the presence of αGalCer (pink histograms) or iNKT in the presence of αGalCer (Red). Flow cytometry histogram showing the expression of CCR7, CD40, CD54, CD80, CD83, CD86 or HLA-DR. Bar charts show average MFI. Results are a mean of 5 separate experiments

5.3.4: Effects of dendritic cells matured with iNKT cells on allogeneic T cell proliferation proliferation and cytokine production

The capacity of DC matured with iNKT cells in presence or absence of 100 ng/ml of αGalCer to activate naïve allogeneic T cells was investigated in T cell proliferation and cytokine release assays. DC were cultured in medium alone, in the presence of the TLR agonist LPS, or with equal numbers of iNKT cells or stimulated PBMC in the presence or absence of the iNKT stimulant αGalCer for 24 hours. The DCs were then diluted and co-cultured with purified peripheral naïve T cells at 1:10 ratios for 72 hours. The naïve T cells were stained with CFSE prior to the addition of the DC in order to distinguish them from the DC and iNKT cells. The CFSE proliferation assay was used to investigate T cell proliferation after co-culture with DCs under the various conditions. The parent population has highest intensity of CFSE and this is reduced with each round of daughter cells, resulting in populations of decreasing CFSE intensities.

In the experiment shown in Figure 5.9, CFSE labelled T cells incubated in medium alone did not divide with a single parent population detected on day 3 (Figure 5.9A). CFSE labelled T cells incubated with DCs cultured in medium alone proliferated with 3 daughter populations detected (Figure 5.9B). CFSE labelled T cells incubated with DCs cultured in presence of LPS resulted in > 4 daughter populations (Figure 5.10C). CFSE labelled T cells incubated with DCs co-cultured with iNKT cells resulted in 4 daughter populations (Figure 5.9D). CFSE labelled T cells incubated with DCs loaded with 100 ng/ml αGalCer co-cultured with iNKT cells also resulted

in >4 daughter populations (Figure 5.9E). The results were similar for 3 independent experiments.

The supernatants of 3 day co-cultures of T cells and DCs were assessed for the T cell cytokines IFN-γ and IL-4 by ELISA (Figure 5.10). T cells cultured with DC that were matured with LPS, iNKT cells or iNKT cells in the presence of 100 ng/ml αGalCer produced higher levels of IFN-γ (2000-2320 pg/ml) in comparison to T cells incubated with immature DC (Figure 5.10A). However the quantities of IL-4 (58-65 pg/ml) released by T cells cultured with iDC were similar to those released by T cells cultured with DC that had been matured with LPS, iNKT cells and iNKT cells in the presence of 100 ng/ml αGalCer (Figure 5.10B). These results show that iNKT cells matured DC can induce T cell proliferation and Th1 biased cytokine production and αGalCer compliments this effect.
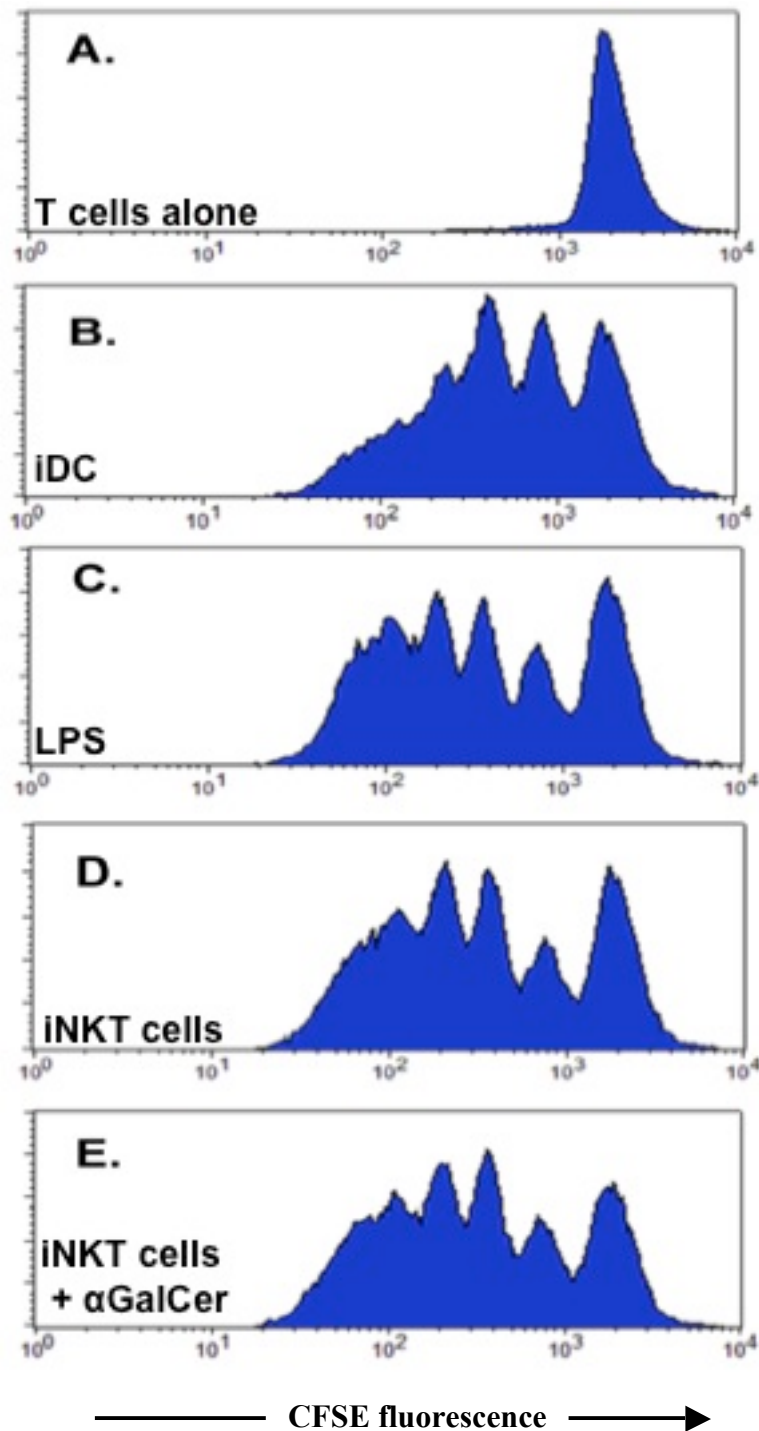
Figure 5.9: DC matured by co-culture with iNKT cells induce proliferation of allogeneic T cells. Allogeneic T cells were labelled with CFSE and incubated with DC cultured under various conditions A-E. Representative flow cytometric histograms show CFSE positive T cells, cultured alone (A) or with DC previously cultured with medium alone (B), LPS (C), iNKT cells (D) or iNKT cells in the presence of 100 ng/ml αGalCer (E). Results are representative of 3 separate experiments.
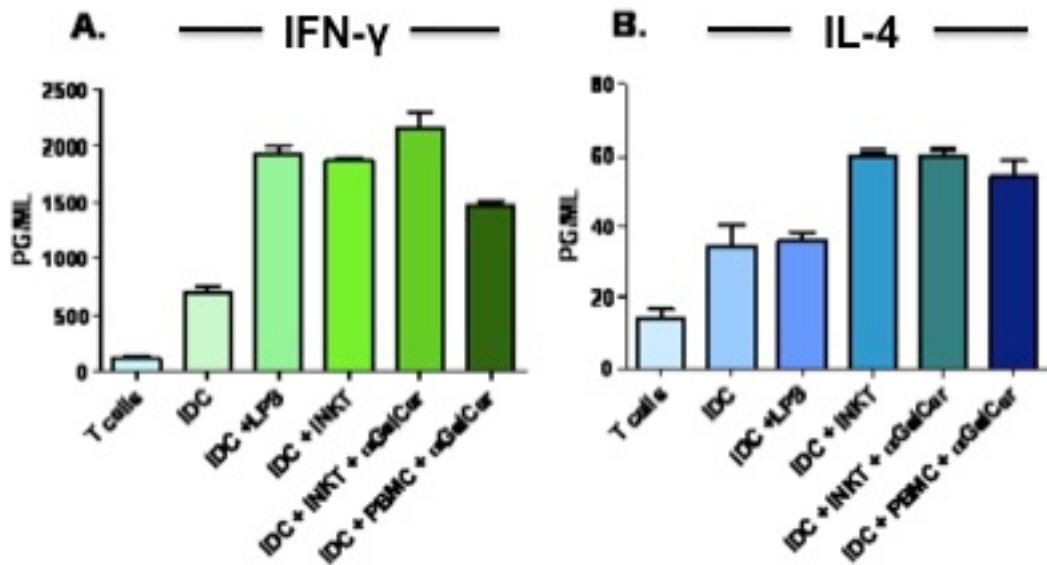
Figure 5.10: DC matured using iNKT cells stimulate IFN-γ and IL-4 production by naïve allogeneic T cells. iDC were cultured in medium alone, with LPS, iNKT cells, iNKT cells in the presence of 100 ng/ml αGalCer or expanded PBMC in the presence of 100 ng/ml αGalCer. After 24 hours the cells were washed, diluted and co-cultured at 10:1 ratios with naïve allogeneic T cells. After 3 days levels of secreted IFN-γ and IL-4 were measured by ELISA. Bar charts show the levels of (A) IFN-γ (2000-2320 pg/ml) and (B) IL-4 (58-65 pg/ml) secreted by allogeneic T cells incubated in the presence of DCs stimulated as aforementioned. Results are means of 3 separate experiments.

5.4: Discussion

In the investigation into the reciprocal interactions between iNKT and DCs we optimized a technique for the generation of DCs from monocyte precursors described previously by Sallusto and Lanzavecchia (1994). On average the yields of CD14+ cells isolated from a mean of 496 x10^6 PBMC by magnetic bead separation was 16.7 x10^6 CD14 positive cells. The purities ranged from 81.6% to 98.6%. CD14+ monocytes were cultured with GM-CSF and IL-4 for 6 days. On day 6 CD14 was downregulated indicating the transition from monocytes to immature DC as described by Sallusto and Lanzavecchia (1994). The DCs where classed as immature due to their low expression of the DC surface molecules; CD40, CD54, CD80, CD83 and CD86 (Banchereau *et al* 2000). We used 2 TLR agonists, LPS and Poly I:C to mature our DCs and investigate the phenotype and function of a mature DC (Rescigno *et al* 1998, De Jong *et al* 2002). As described in studies by Rescigno *et al* (1998) and De Jong *et al* (2002)*,* LPS and Poly I:C induced the upregulation of the surface molecules, CD40, CD54, CD83, CD86, HLA-DR and the chemokine receptor CCR7. Interestingly the expression of surface molecule CD80 was not upregulated in response to LPS or poly I:C after a 24 hour stimulation. Maerten *et al* (2003) reported that the expression of various surface markers occurs at different time points after stimulation, and thus later time points could see the up regulation of CD80. A time curve study was beyond the scope of this project due to time and budget restrictions. Both LPS and poly I:C stimulation resulted in the secretion of IL-10 and IL-12 by DCs indicating a functionally mature DC.

Chapter 5: Results

The main aim of this body of work was to investigate the interactions between iNKT cells and monocyte derived DCs in order to establish a system of DC maturation by iNKT cells activated by novel antigens (Chapter 6). The co-culturing of iNKT cells and iDC resulted in the maturation of the iDC into APCs expressing the maturation molecules CD40, CD54, CD83 and CD86. The addition of αGalCer resulted in an increase in upregulation of these maturation molecules.

We also examined the effects of αGalCer on cytokine production by DC and found that this glycolipid on its own induced the production of IL-12 but not IL-10. The cytokine produced can not be atttributed solely to the glycolipid, since it is possible the αGalCer was contaminated with endotoxin. However Yue *et al* recently showed that CD1d ligation on DC with multiple CD1d mAbs resulted in the secretion of IL-12 (Yue *et al* 2010). Next we co-cultured iDC in the presence or absence of αGalCer with iNKT cells, and examined the surface phenotype of the DCs and the cytokine production by the co-cultures. iNKT cells in the absence of αGalCer induced the upregulation of DC surface markers associated with maturation and this upregulation was significantly increased in the presence of αGalCer. It is a point to note that the iNKT cell lines were cultured in the presence of IL-2 and thus were previously activated. The maturation in the absence of glycolipid could be due to CD40-CD40L interaction as described by Fujii and colleagues (2003) or the presentation of unknown endogenous glycolipid in the system. However the interactions between DC and iNKT cells in the absence of αGalCer resulted in significantly less IL-12 and IFN-γ than in its presence. This round of experiments was repeated but the PBMC were substituted for the highly pure iNKT cell line.

PBMC in the absence or presence of αGalCer failed to mature the immature monocytes derived DCs.

The interactions between iNKT cells and DC resulted in a Th1 biased cytokine profile with increased IL-12 and IFN-γ but no IL-10 produced. This is concurrent with previous studies in both mice and humans (Yang *et al* 2000, Fujii *et al* 2003). Other groups have reported increased or decreased secretion of IL-12 by DCs in response to interactions with iNKT cells, depending on the experimental conditions and whether they were carried out *in vivo* or *ex vivo*. (Naumov *et al* 2001, Vincent *et al* 2002). This indicates that iNKT cells have differential abilities to drive a response in different ways i.e Th1 or Th2.

Next we investigated if the interaction between the iNKT cells and DCs was sufficient to elicit T cell responses. Fujii *et al* reported that elicitation of a T cell response via DC and iNKT cell interactions requires TCR/CD1/Ag complexing, CD40-CD40L interactions (Fujii *et al* 2003). To examine the elicitation of human T cell responses we co-cultured iNKT matured DC with CFSE labelled allogeneic CD3+ T cells and examined T cell proliferation and their cytokine profiles after 3 and 5 days. CD3+ allogeneic T cells were stained with CFSE and cultured alone as a control. The parent population did not divide after 3 or 5 days. Allogeneic CFSE stained T cells co-cultured with iDC proliferated after 3 and 5 days. Culturing allogeneic T cells with LPS matured DC resulted in greater T cell proliferation, exhibiting 4 divisions after 3 days. Co-cultures of iNKT cells and iDC in the absence of αGalCer resulted in comparable proliferation of allogeneic T cells to those of LPS matured DC. This could be due the slightly activated iNKT cell and the

milieu of pro-inflammatory cytokines detected in the supernatant of the cultures in the absence of αGalCer, this mode of activation is described previously by Blanko *et al* (2008). The addition of αGalCer resulted in increased T cell proliferation but this increase was not significant. The supernatants of the cultures were examined for the presence of Th1 and Th2 cytokines to determine the type of response elicited, IFN-γ was produced in large quantities by allogeneic T cells incubated with DCs matured in the presence of LPS, iNKT cells and iNKT cells and αGalCer. IL-4 was also produced but at low levels in cultures of DCs matured in the presence of LPS, iNKT cells and iNKT cells and αGalCer. (58-65 pg/ml).

The results described in this chapter show that co-culturing of iNKT cells and iDC resulted in the maturation of the iDC into APCs that produced abundant IL-12 but little or no IL-10. The addition of αGalCer resulted in an increase in upregulation of maturation molecules and IL-12 secretion. These iNKT cell matured iDC drove a pro-inflammatory response with abundant IFN-γ and IL-12, which induced proliferation of allogeneic circulating T cells and skewed them to produce high levels of IFN-γ but low IL-4. This body of work mirrors murine work by *Munz et al,* which described the resultant Th1 response from the culture of iNKT cells with iDCs (Figure 5.13) *(Munz et al* 2005). The understanding of the interactions between iNKT cells and DC and the resulting T cell response is essential for the design of immunotherapies. We have established a platform for the analysis of human iNKT cell/DC interactions which can be effectively used to test novel glycolipid antigens with the ultimate aim of tailoring T cell responses for immunotherapy. The application of this system for the testing of synthetic glycolipids is the subject of Chapter 6.

5.5 Conclusions

- Immature DCs can be generated *in vitro* from monocytes isolated from human peripheral blood

- LPS and poly I:C stimulation induce DC maturation and production of DC derived cytokine

- iNKT cells can induce DC maturation and DC derived cytokine secretion

- DCs matured in the presence of iNKT cells and αGalCer can induce proliferation and cytokine secretion by allogeneic T cells

- The use of iNKT cells as a DC adjuvant results in a Th1 biased immune response

6: Results: Investigations into the biological activity of novel αGalCer analogue glycolipids

6.2: Introduction

In 1997 Kawano *et al* and Cui *et al* reported that Vα14 iNKT cells activated using αGalCer, a glycolipid derived from marine sponge, cleared B16 melanoma in mice (Kawano *et al* 1997, Cui *et al* 1997). Smyth *et al* subsequently reported that IFN-γ from iNKT cells and from NK cells was essential for the anti-metastatic effect of αGalCer (Smyth *et al* 2002). In 1999 Kawano *et al* reported that human Vα24 iNKT cells could kill tumour cells *in vitro* and were reduced in number in human cancers (prostate, melanoma and liver). These studies highlighted the possible potential of iNKT cells for cancer immunotherapy. Since 2002 numerous clinical trials have attempted to harness the anti-tumour ability of iNKT cells in human cancer patients. Several approaches have been applied in phase I and II trials including, the injection of the iNKT stimulating ligand αGalCer into patients with solid tumours (Giaccone *et al* 2002), infusion of *in vitro* expanded iNKT cell into patients with recurrent non small cell lung cancer (Motohashi *et al* 2006) or infusions of dendritic cells pulsed with αGalCer into patients with non small cell lung cancer, myeloma, or head and neck cancer (Nieda *et al* 2004, Ishikawa *et al* 2005, Chang *et al* 2005, Uchida *et al* 2008, Motohashi *et al* 2009) (Figure 1.12) The studies were well received with low toxicity but overall they proved disappointing with regard to tumour regression/clearance.

The failure of iNKT cells to induce tumour regression in clinical trial directed studies towards development of synthetic analogue ligands, which could potentially increase the anti-tumour effect of human iNKT cells. In 2002, Miyamoto *et al* synthesised an analogue of αGalCer, named OCH (Figure 1.13). OCH differs from αGalCer in that it has a truncated sphingosine chain. OCH was found to skew a Th2

like response with predominant IL-4 production. OCH activation of iNKT cell resulted in specific protection against EAE, whilst αGalCer failed to induce protection. The development a Th1 biased analogue followed in 2003 with the synthesis of α−C-GalCer by Schmieg *et al* (Figure 1.13). α−C-GalCer replaces the *O* glycoside bond found in αGalCer' with a *C* glycoside bond. The authors reported 1000-fold increase in anti malarial activity and 100-fold anti metatastic activity compared to αGalCer in murine models. Responses using α−C-GalCer resulted in a Th1 biased response with increased IFN-γ and IL-12 and decreased IL-4 compared to αGalCer. The development of αGalCer analogues with structural changes to increase/skew activity is a very attractive concept for immunotherapy.

Colleagues from University College Dublin synthesised several analogues with small structural changes predicted to increase either stability or affinity with respect to TCR-CD1d-lipid contact. α-S-GalCer was synthesised by the group of Dr. Xiangming Zhu *et al* and replaced the glycosidic oxygen atom link with a sulfur atom link (Figure 6.1). Thioglycosides are very attractive substitutes for *O*-glycosides as it is well known that they are much less susceptible to enzymatic cleavage as well as chemical degradation, moreover, their anomeric sulphur may interact with CD1d through hydrogen bonding. Thus authors proposed the structural changes would increase the stability of the linkage and thus increase the presentation time of the glycolipid (Dere *et al* 2008). This lipid was kindly provided by Dr Zhu for testing in our biological assays along with a truncated version α-S-GalCer-14, which differs in the length of the acyl chains. Four αGalCer analogues (W1-W2) were synthesised by the group of Professor Paul Murphy at the National University of Ireland Galway (Figure 6.2). The analogues W1-W4 had trucated sphingosine
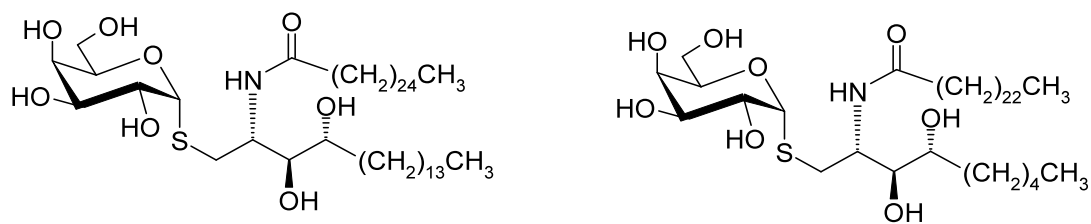
Figure 6.1: Structures of the synthetic analogue α-S-GalCer glycolipid and the truncated α-S-GalCer Structures provided by Zhu et al.
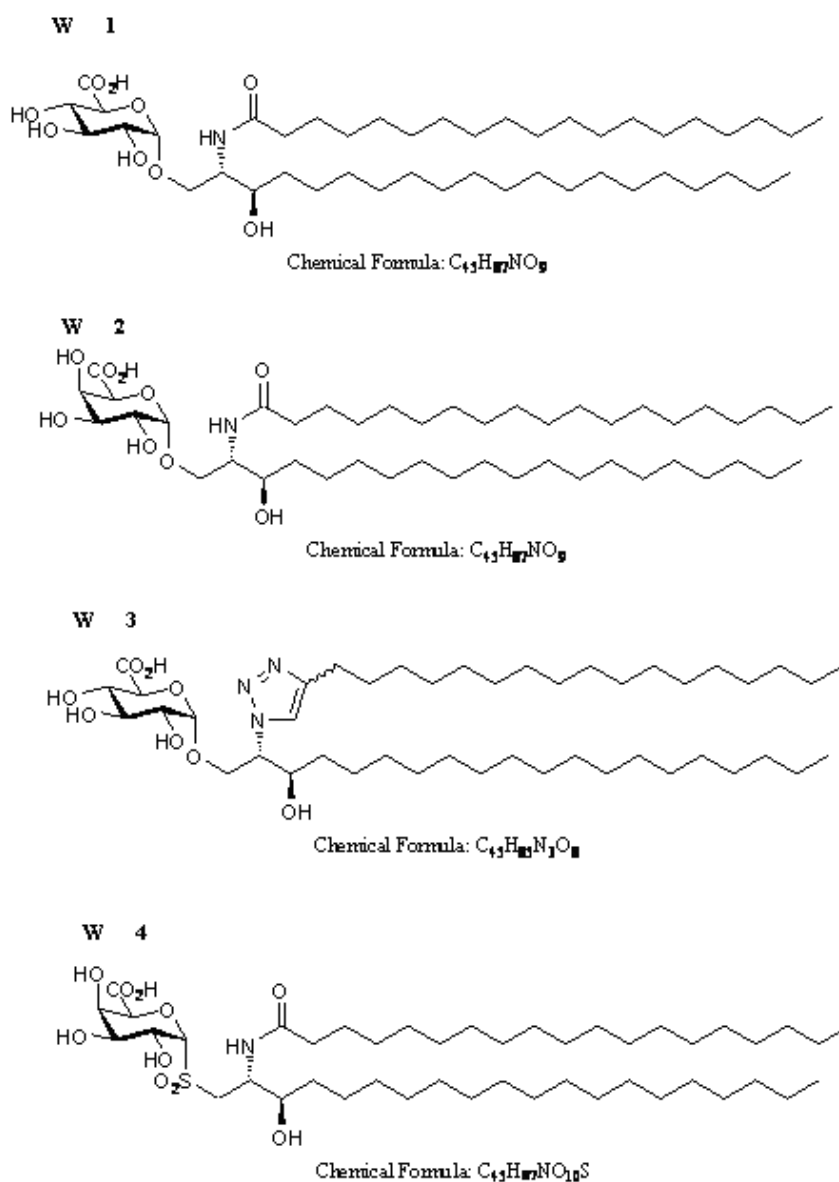
Figure 6.2. Structures of the synthetic αGalCer analogues glycolipids W1-W4.

chains. W1-W4 contained several structural changes to the carbohydrate head and in analogue W4 the *O*-glycoside was replaced with *O$_2$S*-glycoside. The authors proposed these analogues would have biological activity.

In this body of work we aimed to investigate the biological activity of these six novel αGalCer analogues. We aimed to test the functional responses (cytokine production and cytotoxicity) of our human iNKT cell lines expanded from peripheral blood to the novel analogue glycolipids. The analogues were also used in the DC-iNKT system established in chapter 5 to test both the reciprocal interactions between iDC and iNKT and the subsequent T cell responses to the novel analogues. The results obtained with the novel analogues are compared to αGalCer throughout (Figure 6.3).

6.2: Objectives

1. To test the iNKT stimulatory ability of novel αGalCer analogues

2. To investigate ability of the glycolipid analogue activated iNKT to activate immature DC

3. To examine the reciprocal functional interactions and responses by the glycolipid analogue activated iNKT and DC

4. To investigate the T cell maturating and activating capabilities of the glycolipid analogue activated iNKT activated DC
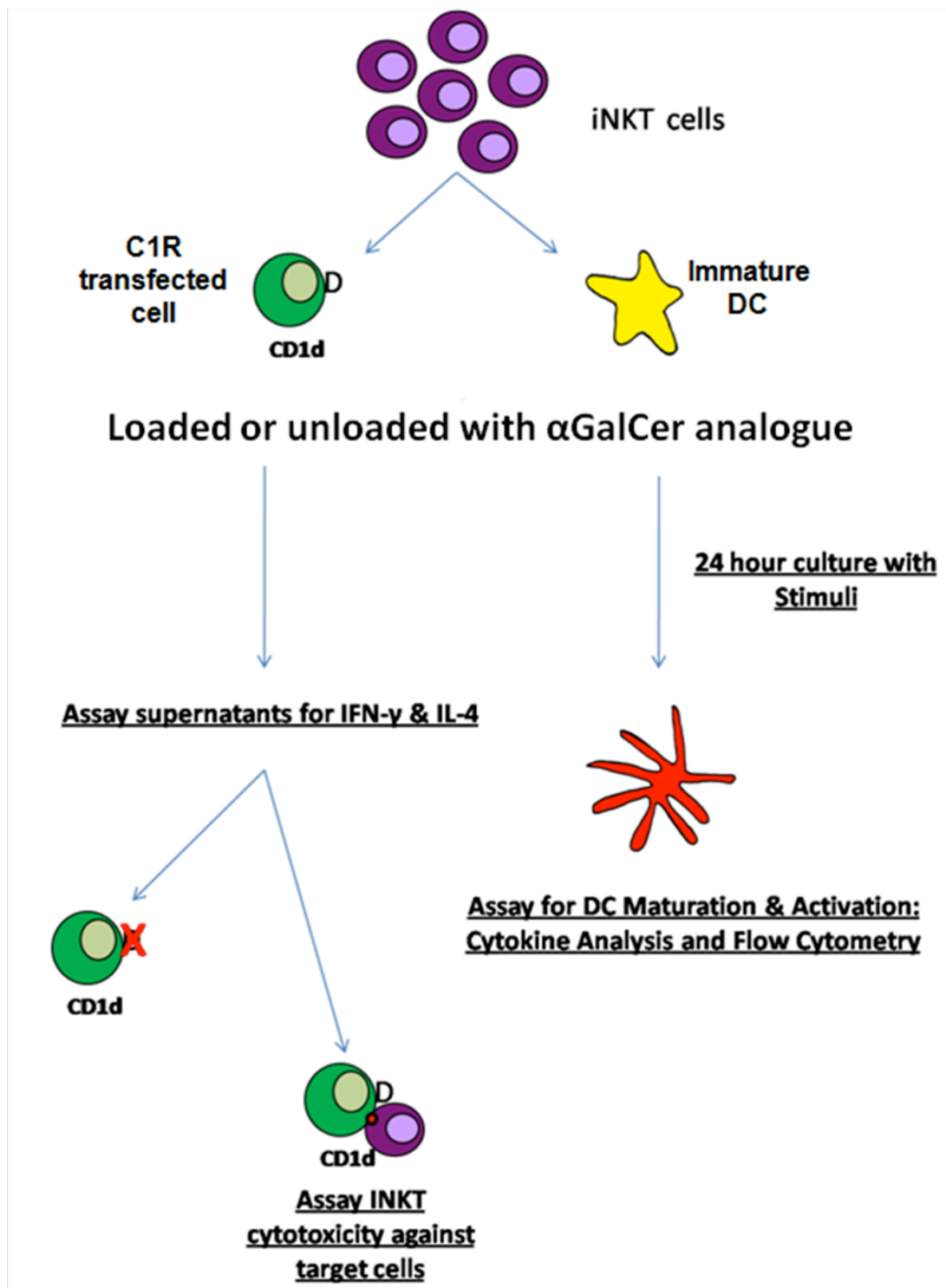
Figure 6.3: Strategy for investigation of biological activity of synthetic αGalCer analogue glycolipids. Analogues were tested for iNKT cell stimulatory capabilities using the C1R reactivity, cytotoxicity and DC activation assays optimized and described in previous results chapters. The analogues were compared to the current gold standard of iNKT cell activation, αGalCer in all assays.

Results: Chapter 6

6.3.1: Investigation of iNKT cell cytokine responses to novel synthetic αGalCer analogues

Currently αGalCer remains the "gold standard" ligand for the activation of iNKT cells. αGalCer stimulates potent cytotoxicity and the early release of both IFN-γ and IL-4 by iNKT cells. As discussed previously clinical trial therapies using αGalCer have resulted, in at best modest successes, and new analogues provide the best chance of improved iNKT activators. In response to this, the iNKT stimulating properties of 6 novel αGalCer analogues, differing in small structural changes were investigated. iNKT cell reactivity to six novel αGalCer analogues was assessed using functional assays. iNKT cells expanded from human peripheral blood using Method 3 (described in chapter 3) were co-cultured with CD1d transfected C1R cells loaded or unloaded with αGalCer or single analogues for 24 or 72 hours (100 ng/ml). The mock transfected C1R cells were used as a negative control and T cell mitogen PHA was used as positive control. Reactivity to the lipid ligands was measured by quantifying secreted IFN-γ and IL-4 in the culture supernatants by ELISA.

Six analogues were tested (W1, W2, W3, W4, S-α-GalCer & S-α-GalCer14). Of the six analogues tested for iNKT cell cytokine secretion, only one analogue showed biological activity which justified further investigation with none of the remaining analogues stimulating IFN-γ or IL-4 release by iNKT cells over and above the amounts released by unloaded CD1d transfected C1R cells (IFN-γ (311-480 pg/ml) and IL-4 (152.3-164.2 pg/ml) (Figure 6.4 and 6.5).

The reactive analogue termed α-S-GalCer, differs from αGalCer in the bond which links the carbohydrate head to the fatty acid chain, glycosidic S link replaces glycosidic O link in αGalCer. Stimulation of iNKT cells with α-S-GalCer-pulsed CD1d+ C1R cells resulted in a mean 7 fold increase in levels of IFN-γ over the levels released in response to mock transfected C1R cells (p =0.001). This compared to a 5 fold increase with αGalCer stimulation (Fig 6.5A). α-S-GalCer stimulation of iNKT cells resulted in a mean 9.5 fold increase in levels of IL-4 compared to the levels released in response to mock transfected C1R cells (p =0.01). This compared to 11 fold increase with αGalCer stimulation (Fig 6.5B). There was no significant difference between the cytokine produced by α-S-GalCer stimulated iNKT cells and the that produced by αGalCer stimulated iNKT cells. To ensure the specificity of α-S-GalCer, CD1d was blocked using a blocking mAb. The blocking of CD1d on the C1R transfected C1R cells resulted in a 4.2 fold reduction in IFN-γ compared to α-S-GalCer stimulation and 1.8 fold reduction in IL-4 secretion (Fig 6.6 A & B). These studies show that of the 6 analogues test only 1, α-S-GalCer stimulated cytokine production by iNKT cell lines in a CD1d dependent manner.
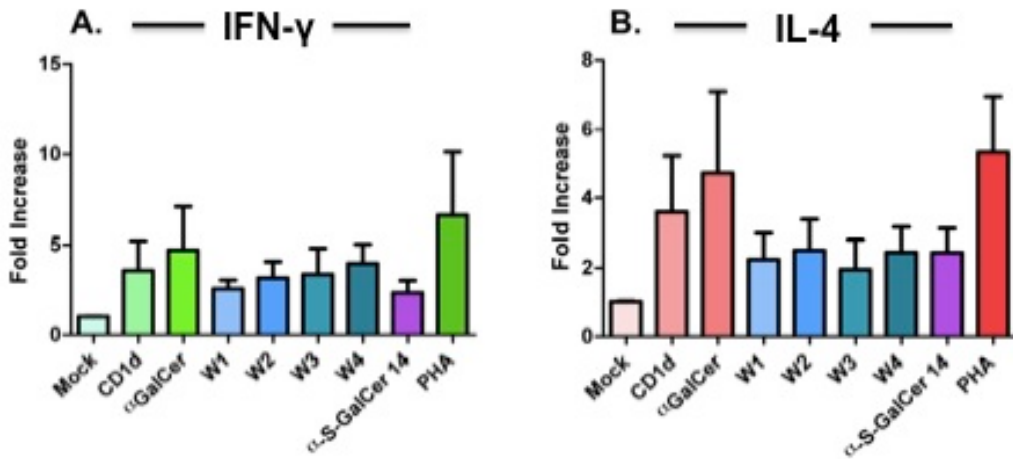
Figure 6.4: Analogues W1-W4 and α-S-GalCer 14 failed to induce cytokine secretion by iNKT cells. iNKT cells were co-cultured with CD1d transfected C1R cells unloaded or loaded with either αGalCer or αGalCer analogues. Supernatants were examined for IFN- γ after 72 hours (A) and IL-4 after 24 hours (B). Bar graphs display the mean fold increase over in cytokine levels compared to those released in response to mock transfected C1R cells (IFN-γ 100.1-124.3 pg/ml & IL-4 40-62.7 pg/ml). Results are means from 5 independent experiments.
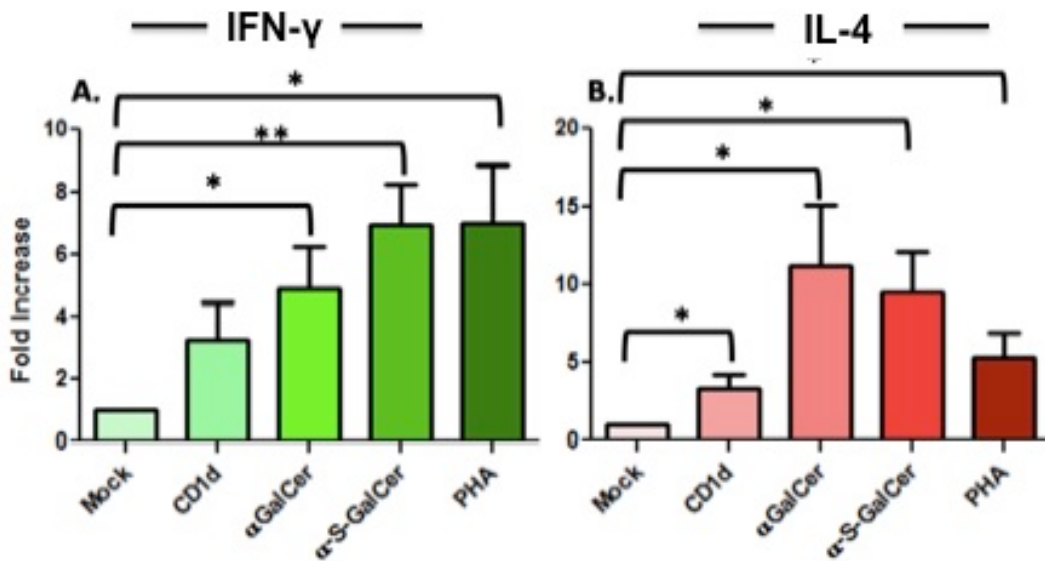
Figure 6.5: S-α-GalCer induces IFN-γ and IL-4 secretion by iNKT cells. iNKT cells were co-cultured with CD1d transfected C1R cells unloaded or loaded with either 100 ng/ml of αGalCer or α–S-GalCer were examined for IFN- γ after 72 hours (A) and IL-4 after 24 hours (B) by ELISA. Bar graphs display the mean fold increase in cytokine secretion compared to those released in response to mock-transfected C1R cells (IFN-γ 100.1-124.3 pg/ml & IL-4 40-62.7 pg/ml). Results are means of 5 independent experiments. *P<0.01; **P<001.
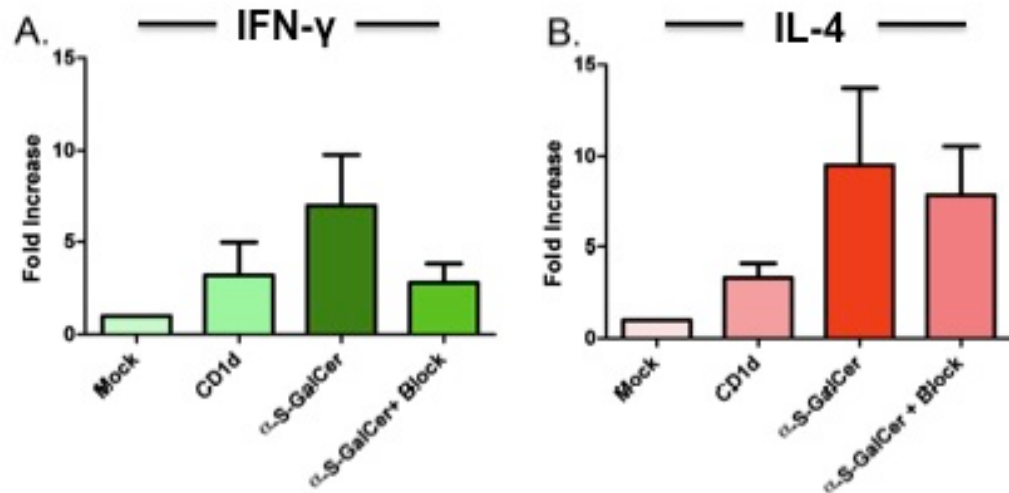
Figure 6.6 Monoclonal antibody blocking of CD1d reduces α-S-GalCer induced cytokine secretion by iNKT cells. iNKT cells were co-cultured with CD1d transfected C1R cells unloaded or loaded with 100 ng/ml of α–S-GalCer in the absence or presence of CD1d mAb were examined for IFN- γ after 72 hour (A) or IL-4 after 24 hours (B) ELISA. Bar graphs display the mean fold increase in cytokine levels over those released by mock transfected C1R cells (IFN-γ 100.1-124.3 pg/ml & IL-4 40-62.7 pg/ml). Results are from 5 independent experiments.

6.3.2: Investigation into α−S-GalCer induced cytotoxicity of CD1d transfected C1R cell lines by iNKT cells

The ability of α-S-GalCer to prime CD1d+ targets for cytotoxicity by iNKT cells cytotoxic capabilities were assessed using the CFSE incorporation cytotoxicity assay. C1R cells stably transfected with CD1d were labelled with CFSE and were incubated with iNKT cells in the presence or absence of αGalCer or α-S-GalCer for 4 hours at effector target ratio of 10 to 1. After incubation cells were co stained with 7-AAD, a marker of recent apoptosis. Double positive cells staining for CFSE and 7-AAD represented apoptotic lysed target cells. C1R CD1d target cells incubated in the absence of effector cells contained a mean of 3% apoptotic cells (Figure 6.7A, 6.8). Addition of iNKT cells resulted in mean lysis of 7% of target C1R CD1d cells (p =0.03) (Figure 6.7B, 6.8). The further addition of αGalCer resulted in a mean increase in lysis to 13% of the target cells (p =0.002) (Figure 6.7C, 6.8). Addition of α-S-GalCer resulted in mean lysis of 18% of CD1d transfected target cells (p <0.0001) (Figure 6.7D, 6.8). These studies show that of the α-S-GalCer stimulated iNKT cell can lyse CD1d transfected target cells.
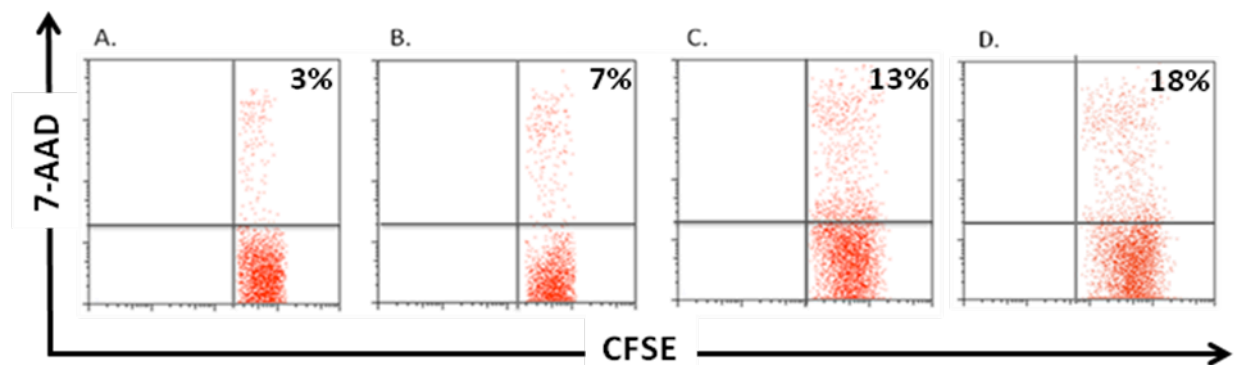
Figure 6.7: α-S-GalCer primes CD1d transfected C1R tumour cells for lysis by iNKT cells. Representative flow cytometric dot plots showing iNKT cytotoxicity of CFSE labelled CD1d transfected target cells incubated with, iNKT cells alone (B), iNKT cells with αGalCer (C) or iNKT cells with s-αGalCer (D). Target cells alone (A) act as negative control. Lysed cells stained positive with the apoptotic stain 7-AAD so cells in the upper right quadrants represent lysed cells. Results are representitive of 3 independent experiments.
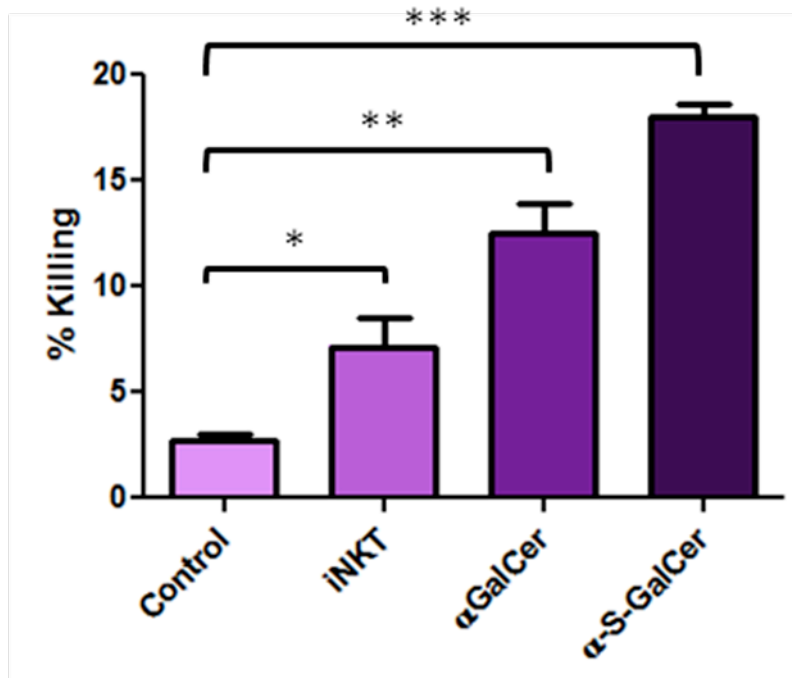
Figure 6.8: α-S-GalCer primes CD1d transfected C1R tumour cells for lysis by iNKT cells. iNKT cells were co-cultured at 10:1 ratio with CFSE labelled CD1d transfected C1R target cells in the absence or presence of 100 ng/ml of αGalCer or α-S-GalCer. Lysed cells stained positive with the apoptotic stain 7-AAD. Bar chart showing the mean of 3 separate cytotoxicity assays, target cells alone act as negative control. *P=0.03; **P=0.002; ***P=0.0001.

6.3.3: Investigation into α–S-GalCer induced iNKT cell cytotoxicity of K562 cell lines

To further assess the biological activity and iNKT cell stimulatory properties of s-α-GalCer; iNKT cell cytotoxicity was examined against the NK cell sensitive K562 cell line, in the presence of s-α-GalCer. iNKT cells were co-cultured with either K562 at 10:1 effector to target ratio in the presence or absence of either 100 ng/ml of αGalCer or α-s-GalCer. iNKT cells stimulated with α-S-GalCer lysed a mean of 30% of target cells (p =0.0002) (Fig 6.9D & 6.10). This was an increase over αGalCer stimulated iNKT cells which lysed a mean of 23% (p =0.003) (Fig 6.9C & 6.10) and over iNKT without additional exogenous ligand which lysed 19% (p

<0.0001) (Fig 6.9B & 6.10). These studies show that of the α-S-GalCer stimulated

iNKT cell can lyse CD1d negative target cells.
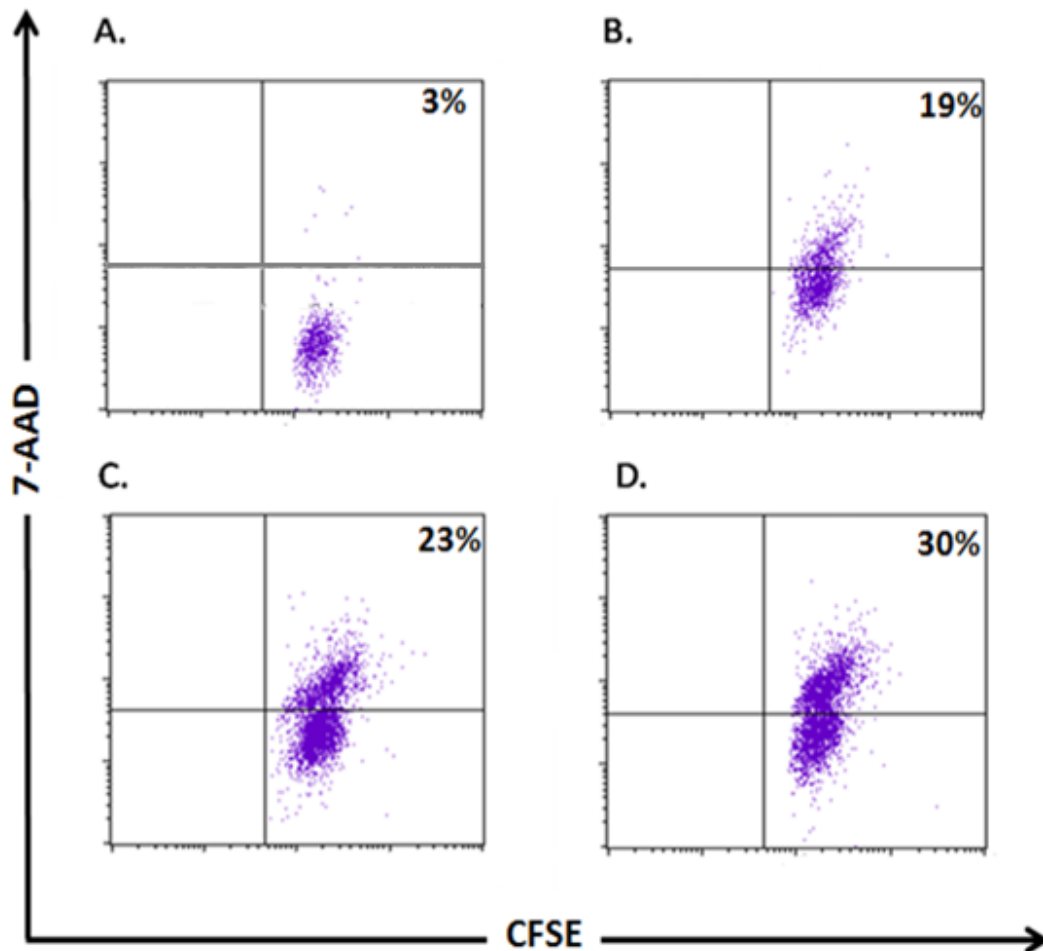
Figure 6.9: S-αGalCer induces K562 tumour cell lysis by iNKT cells. Representative flow cytometric dot plots showing iNKT cytotoxicity of CFSE labelled K562 target cells incubated with, iNKT cells alone (B), iNKT cells with αGalCer (C) or iNKT cells with s-αGalCer (D). Target cells alone (A) act as negative control. Lysed cells stained positive with the apoptotic stain 7-AAD in the upper right quadrants represent lysed cells. Results are representative of 3 independent experiments.
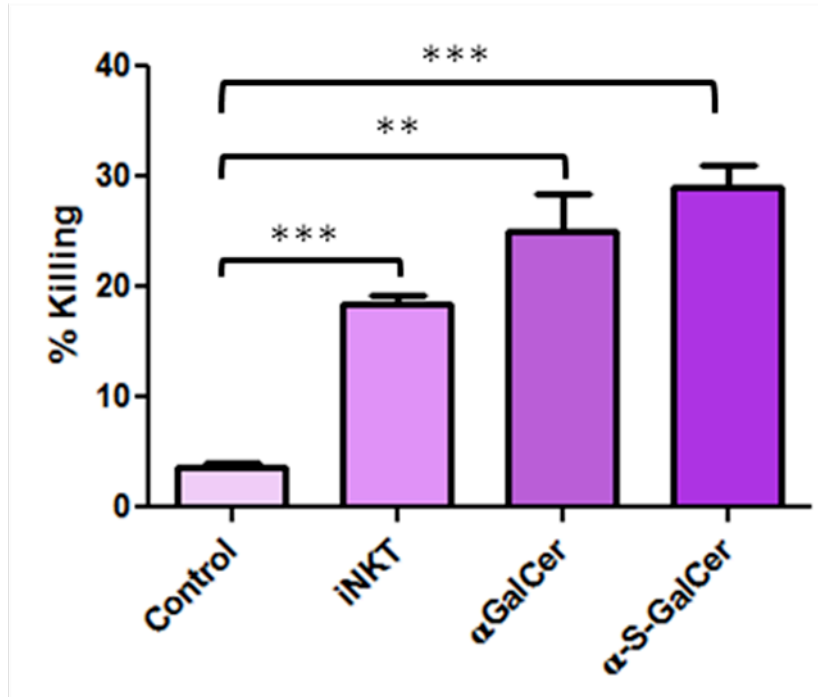
*Figure 6.10: S-αGalCer induces K562 tumour cell lysis by iNKT cells. iNKT cells were co-cultured at 10:1 ratio with CFSE labelled K562 target cells in the absence or presence of αGalCer or α-S-GalCer. Lysed cells stained positive with the apoptotic stain 7-AAD. Bar chart showing the mean percentage of killed target cells from 3 separate cytotoxicity assays. Target cells alone act as negative control. **P=0.003 ***P=0.0002.*

6.3.4: Effect of s-α GalCer stimulated iNKT on monocyte derived dendritic cells.

Monocyte derived dendritic cells (DC) were incubated in medium alone (control) or co cultured with equal numbers of iNKT cells expanded from human peripheral blood (using Method 3, described in chapter 4) with or without the addition of 100 ng/ml of αGalCer or s-α-GalCer for 24 hours. Expression of seven surface molecules was investigated for each set of DC as outlined in chapter 5.

Co-culture of iDC with iNKT cells in the absence of glycolipid antigen resulted in a 1.7 fold upregulation of CCR7 expression by DC. CCR7 expression was increased 2.5 fold with the addition 100 ng/ml of αGalCer to the co-culture (p =0.008) and 2.35 fold with 100 ng/ml α-S-GalCer (p =0.008) compared to iDC cultured in medium alone. The surface expression of the co-stimulatory molecule CD40 was increased 1.9 fold upon co-culture with iNKT cells with 100 ng/ml of αGalCer (p =0.009) and 1.6 fold with the addition of α-S-GalCer (p =0.02). Surface expression of the adhesion molecule CD54 was increased 2.1 fold with co culture with iNKT cells and 100 ng/ml of αGalCer and 1.6 fold with addition of α-S-GalCer. CD80 was expressed at low levels on iDC with MFI comparable to that of iDC stained with isotype control mAbs. This was not increased by co-culture with iNKTs in the presence or absence of either αGalCer or α-S-GalCer. Surface expression of the classical DC maturation marker CD83 was increased 3.4 fold upon co-culture with iNKT cell of 100 ng/ml of αGalCer (p =0.002) and 2.7 fold with addition of α-S-GalCer to the co-culture (p =0.004). Surface expression of CD86 was increased 1.5 2.5 fold upon co-culture with iNKT cell of 100 ng/ml of αGalCer (p =0.01) and 2.15 fold with addition of α-S-GalCer (p =0.03) to the co-culture. Expression of HLA-DR

was increase 1.25 fold upon co-culture with iNKT cell of 100 ng/ml of αGalCer and 1.2 fold with addition of α-S-GalCer to the co-culture (Figure 6.11). The effects of iNKT in the absence of glycolipid on DC maturation is detailed in chapter 5. There was no significant difference between the maturation induced by α-S-GalCer stimulated iNKT cells and the maturation induced by αGalCer stimulated iNKT cells.

Cytokine secretion from co-culture supernatants of DCs incubated with iNKT cells in the presence or absence of 100 ng/ml of αGalCer or α-S-GalCer were examined by ELISA. Levels of IL-10 and IL-12 secreted by DC were compared to those released by iDC incubated in medium alone (control). Cytokine secretion was quantified in 3 experiments by ELISA. Co-culture of DC with iNKT cells resulted in an average 2 fold increase in IL-12 (p =0.04) and 1.26 fold increase in IL-10 over that released by the iDC alone (54-66 pg/ml IL-12, 79-84 pg/ml IL-10) (Figure 6.12). The co-culture of DC loaded with 100 ng/ml of αGalCer with iNKT cells resulted in 16 fold increase of IL-12 (p =0.002) (Figure 6.12) and 2.26 fold increase in IL-10 production (p =0.0007). DC loaded with 100 ng/ml of α-S-GalCer and co-cultured with iNKT cells resulted in 10 fold increase of IL-12 release (p =0.002) (Figure 6.12) and 1.7 fold increase in IL-10 production (p =0.0001). These results indicate that iNKT cells stimulated with αGalCer and α-S-GalCer have similar adjuvant effect on cytokine production by monocyte derived DC.
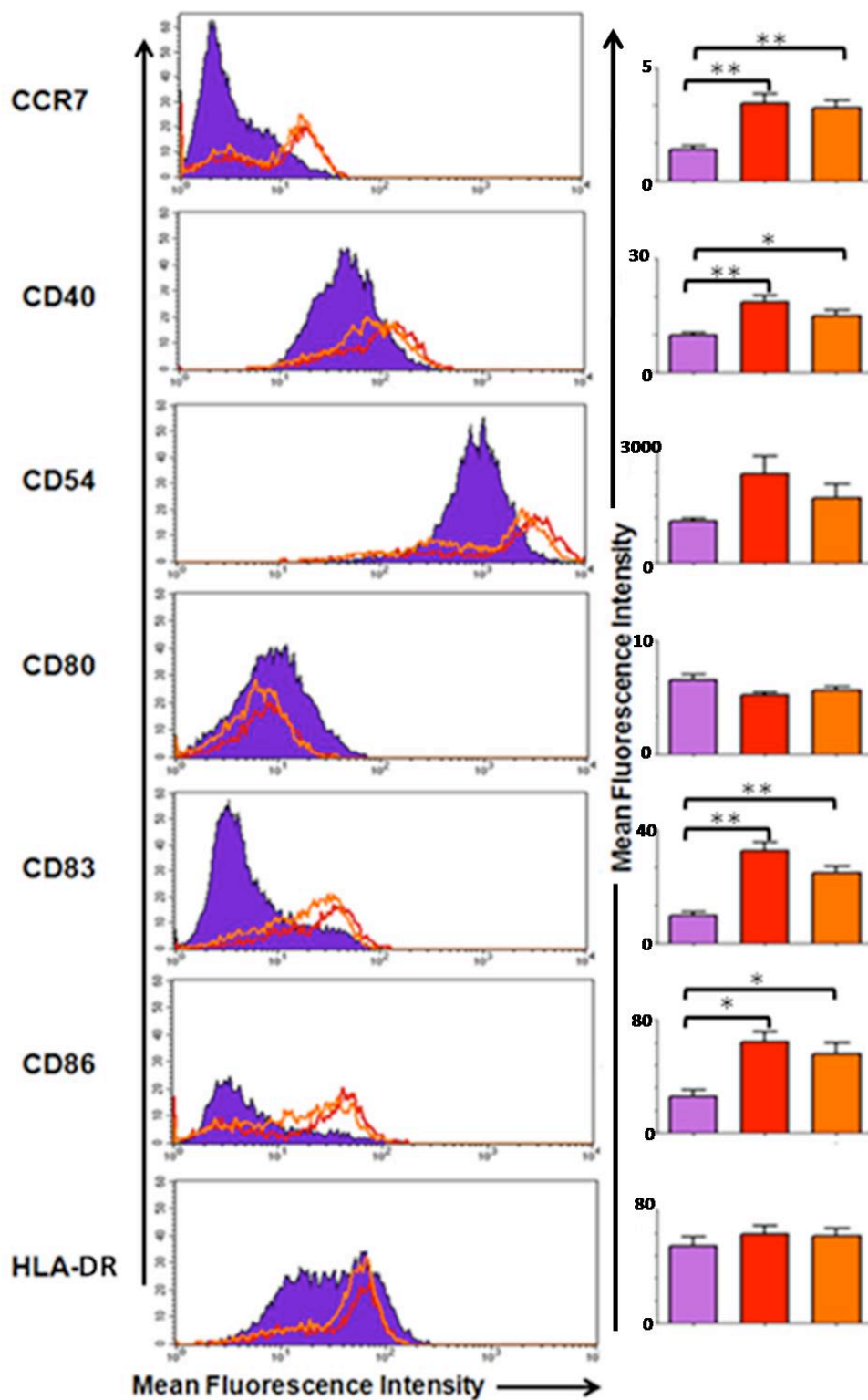
Figure 6.11. α-S-GalCer stimulated iNKT induce DC maturation comparable to αGalCer. A, Flow cytometric histograms showing intensities of CCR7, CD86, CD83, CD40, CD54, HLA-DR & CD80 expression by iDC cultured alone (Purple), cultured with iNKT in the presence of αGalCer (Red) or cultured with iNKT in the presence of α-S-GalCer (Orange). Bar charts showing the average

mean fluorescence intensities (MFI) observed in 3 independent experiments. iDC (Purple), iNKT + αGalCer (Red) and iNKT + α-S-GalCer (Orange). *P<0.03; **P<0.009.
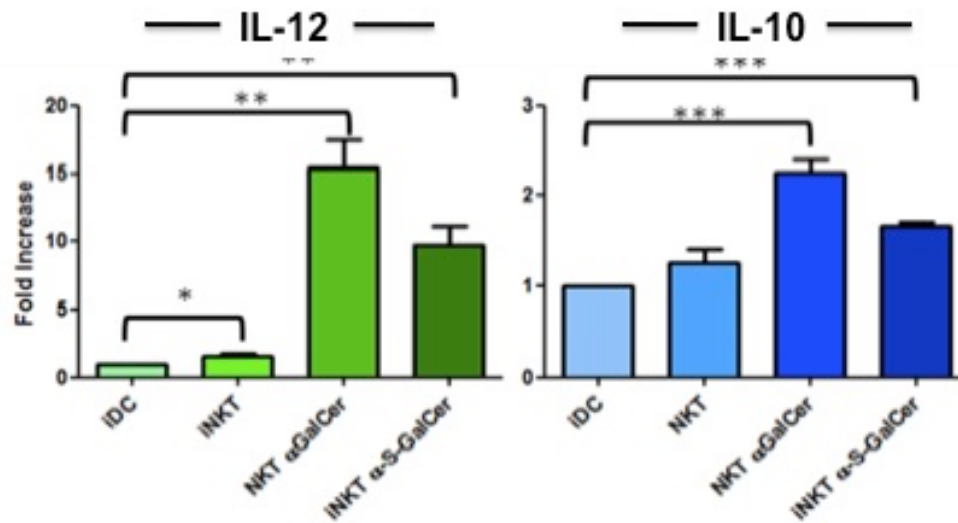
Figure 6.12.: α-S-GalCer stimulated iNKT cells induce cytokine secretion by DC that is comparable the levels released by DC cultured with to αGalCer stimulated iNKT cells. iDC were cultured in medium alone, co-cultured with iNKT cells in the absence or presence of 100 ng/ml αGalCer or α-S-GalCer and cytokine production measured by ELISA. Bar graphs show the fold increase in the secretion of IL-12 (green) and IL-10 (blue) compared to iDC cultured in medium alone (54-66 pg/ml IL-12, 79-84 pg/ml IL-10). Results are the means of 3 separate experiments. *P<0.04; **P=<0.002; ***P<0.0007

6.3.5: DC matured with iNKT cells in the presence of α-S-GalCer can stimulate the proliferation and activation of allogenic T cells

The capacity of DC matured with iNKT cells in presence or absence of either 100 ng/ml of αGalCer or 100 ng/ml of α-S-GalCer to activate naïve allogenic T cells was investigated in T cell proliferation and cytokine release assays as previously described in chapter 5. DCs were cultured in medium alone, in the presence of the LPS or with equal numbers of iNKT cells or PHA stimulated PBMC (non-iNKT cell control cells) in the presence or absence of αGalCer or its analogue α-S-GalCer for 24 hours. The DCs were then co-cultured with CFSE labelled purified peripheral

naïve T cells at 1:10 ratio for 72 hours. CFSE proliferation assay was used to investigate T cell division after co-culture with DCs cultured matured under the various conditions. The parent population of T cells had highest intensity of CFSE stain and this was reduced with each round of daughter cells, resulting in populations of decreasing CFSE intensities.

In the experiment shown in figure 6.11, CFSE labelled T cells incubated in medium alone did not divide with a single parent population present after 72 hours culture (Figure 6.13A). CFSE labelled T cells incubated with DCs cultured in medium alone proliferated with 3 daughter populations detected (Figure 6.13B). CFSE labelled T cells incubated with DCs cultured in presence of LPS resulted in 5 daughter populations (Figure 6.13C). CFSE labelled T cells incubated with DCs co-cultured with iNKT cells resulted in 5 daughter populations (Figure 6.13D). CFSE labelled T cells incubated with DCs loaded with 100 ng/ml αGalCer and co-cultured with iNKT cells resulted in 5 daughter populations (Figure 6.13E). or DC loaded with 100 ng/ml α-S-GalCer and co-cultured with iNKT cells resulted in 5 daughter populations (Fig 6.13F). Similar results were obtained in 2 other separate experiments.

The supernatants of T cell and DC 3 three day cultures where assessed for levels of the T cell cytokines IFN-γ and IL-4 by ELISA (Figure 6.14). T cells cultured in the presence of DC that were matured with LPS, iNKT cells or iNKT cells in the presence of 100 ng/ml αGalCer or α-S-GalCer produced high levels of IFN-γ (1918-2154 pg/ml) in comparison to T cells incubated with immature DC, but produced small quantities of IL-4 (36-57pg/ml) (Figure 6.14). Addition of αGalCer and or α-

S-GalCer to the DC-iNKT cell co-cultures did not lead to increased secretion of
IFN-γ or IL-4 by the allogeneic T cells.
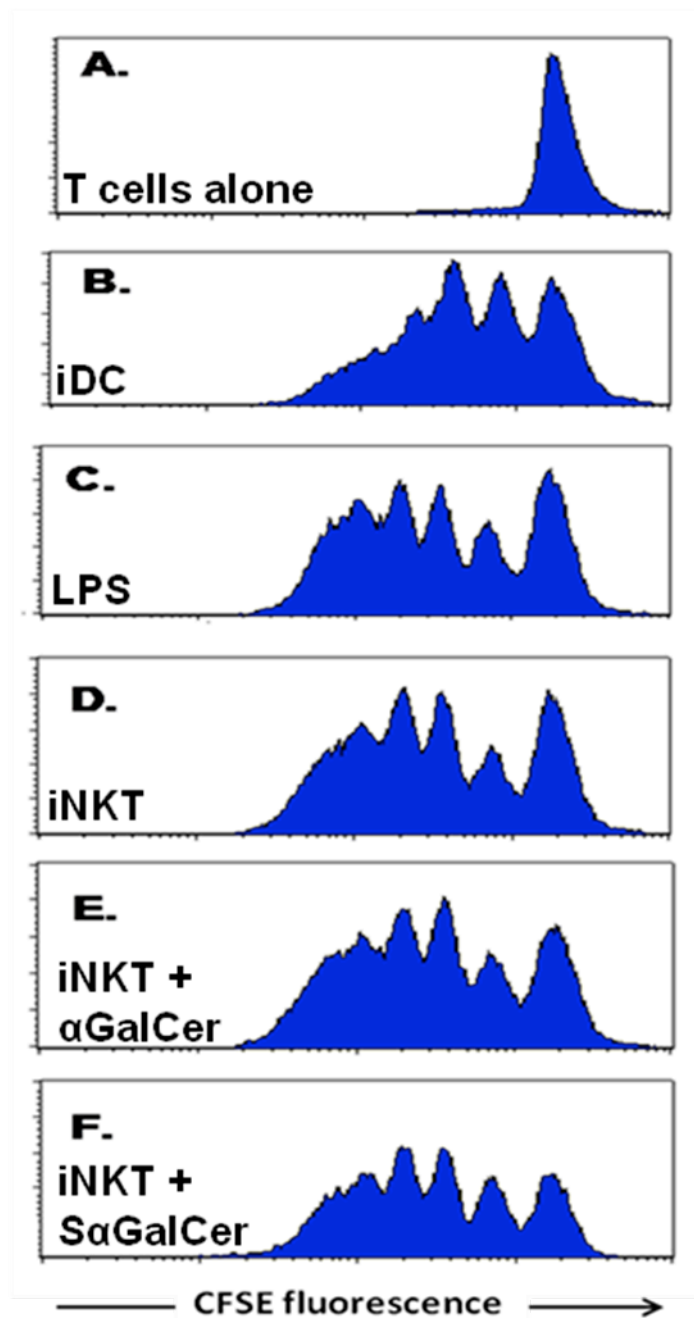
***Figure 6.13: DC matured with αGalCer and α-S-GalCer stimulated iNKT cells
induce proliferation of allogeneic T cells. Representative flow cytometric
histograms showing CFSE positive T cells cultured in medium alone (A) or with
DC previously cultured with medium alone (B), LPS (C), iNKT cells (D), iNKT
cells in the presence of 100 ng/ml αGalCer (E) or iNKT cells in the presence of
100 ng/ml α-S-GalCer (F). Results are representative of 3 separate experiments***
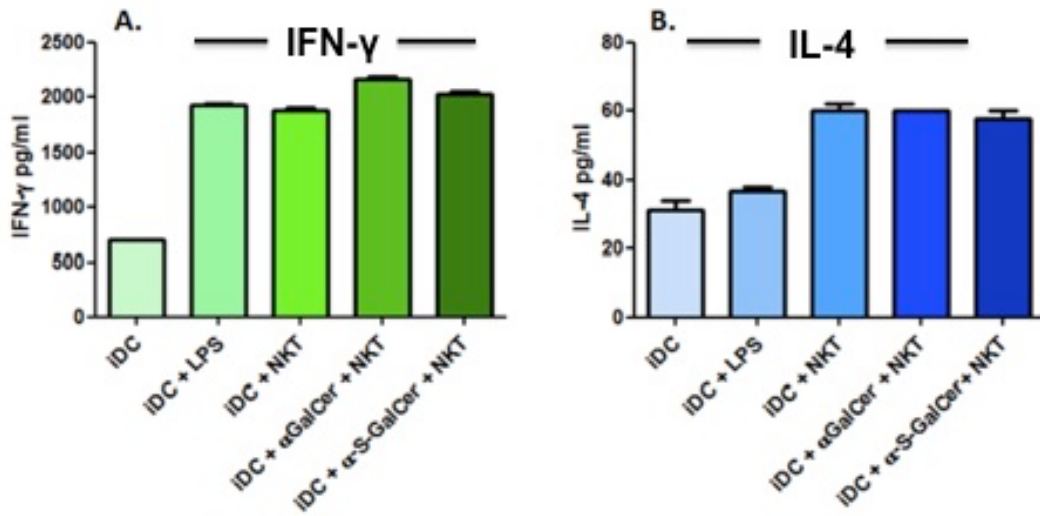
Figure 6.14: DC matured with iNKT cells in the absence and presence of αGalCer and α-S-GalCer stimulated iNKT cells induce IFN-γ and IL-4 release by allogeneic T cells. Bar graphs show secretion of IFN-γ (A) and IL-4 (B) by allogeneic T cells incubated for 72 hours in the presence of DCs that were previously cultured for 72 hours in medium alone, with LPS, with iNKT cells in the presence or absence of 100 ng/ml αGalCer or α-S-GalCer. Results are means of 3 separate experiments.

6.4: Discussion

iNKT cells are a rare subset of innate T cells which express an invariant TCR and NK cell like surface molecules. iNKT cells have potent cytotoxic capabilities and can rapidly release cytokines that activate, polarise and regulate adaptive immune responses. iNKT cell cytokines include GM-CSF, IL-2, IL-5, IL-6, IL-8, IL-10, IL-17 and TNF-α, although their simultaneous production of IFN-γ and IL-4 is most noteworthy (Exley *et al* 1997, Gumperz *et al* 2002, Kronenberg 2005) (Chapter 4). iNKT cells are also widely reported to activate other immune cells and skew the subsequent immune responses (Nakagawa *et al* 1998, Carnuad *et al* 1999, Fujii *et al* 2004, Munz *et al* 2005).

In 1997 Cui *et al*, and Kawano *et al* reported the identification of a glycolipid isolated from the marine sponge *Agelas mauritianus* whilst screening for anti cancer agents, called αGalCer. αGalCer activated iNKT cells and cleared B16 melanoma in murine models (Cui *et al* 1998, Kawano *et al* 1997). Subsequent studies highlighted the potent anti-tumour ability of murine iNKT cells activated with αGalCer (Smyth *et al* 2002, Crowe *et al* 2002).

iNKT cells are also implicated in immunity against tumours in humans, with reduction in iNKT numbers in several human cancers (Kawano *et al* 1999, Tahir *et al* 2001, Kenna *et al* 2003). Human iNKT cells are reported to kill iNKT cells *in vitro* and activate potent anti-tumour innate and adaptive immune responses (Brossay *et al* 1998, Kawano *et al* 1999, Metelista *et al* 2001, Fujii *et al* 2004). However clinical trials based on iNKT immunotherapy in various cancers have proved disappointing compared to the results observed in murine models. iNKT cell based immunotherapies have frequently been based on the activation of peripheral or infused iNKT cells with αGalCer pulsed DC (Section 1.10, Figure 1.12).

As mentioned in section 6.2, several investigators have designed and synthesised modified αGalCer analogues in improving and skew iNKT cell function. Thus, while αGalCer stimulates the simultaneous production of Th1 and Th2 cytokines (Exley *et al* 1997, Prussin & Foster 1997), analogue glycolipids have been synthesised that polarise cytokine production by iNKT cells towards Th1 or Th2

Results: Chapter 6

(Franck *et al* 2003, Miyamoto *et al* 2001, Schmieg *et al 2003)*. The analogues are based on small structural changes to αGalCer, aimed to improve affintiy, stability and contact between CD1d, glycolipid and the TCR.

In this body of work we tested the iNKT stimulatory capabilities of 6 novel αGalCer analogues, W1-W4, α-S-GalCer and α-S-GalCer14 (Figure 6.1, Figure 6.2). CD1d transfected C1R cells were pulsed with the glycolipids and then used as stimulators of iNKT cell lines generated using Method 3 and described in chapter 4. Of the analogues tested only α-S-GalCer stimulated iNKT cells to induce cytokine production at levels comparable to αGalCer (Exley *et al* 1997, Gumperz *et al* 2002). α-S-GalCer stimulation resulted in greater IFN-γ but less IL-4 production compared to αGalCer (Figure 6.4, Figure 6.5). This augmented IFN-γ/IL-4 ratio suggests a Th1 bias which could be beneficial in therapy for viral infection and cancer. Mab blocking of CD1d on the CD1d transfected C1R cells, used as stimulators, caused inhibition of cytokine secretion by iNKT cells in response to α-S-GalCer. This provides compelling evidence that α-S-GalCer is loaded onto CD1d. We also found that iNKT cells killed CD1d expressing and CD1 negative tumour cell lines. α-S-GalCer activated iNKT cells lysed more tumour cells than αGalCer stimulated iNKT cells, again suggesting that α-S-GalCer may have superior anti-tumour properties (Figure 6.7-6.10).

In chapter 5 we described the ability of iNKT cells in the presence of αGalCer to mature iDC and stimulate them to produce IL-12 *in vitro.* Here, we investigated the ability of α-S-GalCer to mature iDC (Figure 6.11). The maturation of monocyte derived DC by iNKT cells stimulated with α-S-GalCer was comparable to the DC

maturation of iDC by αGalCer stimulated iNKT cells, when upregulation of cell surface molecules involved in cell adhesion, antigen presentation and costimulation were examined. Furthermore, the ratio of IL-12 to IL-10 released by the DC was similar for αGalCer and α-S-GalCer activated iNKT cells (Figure 6.12). Indeed α-S-GalCer resulted in comparable allogeneic T cell proliferation and cytokine production to that observed with αGalCer (Figure 6.13, Figure 6.12). These data suggests that α-S-GalCer has a similar adjuvant effect to that of αGalCer and could be a candidate for immunotherapy.

Previous studies have reported the lack of stimulatory effect by similar α-S-GalCer analogues (Blauvent *et al* 2008). The methods used to derive these α-S-GalCer analogues are reported as different to those used for the synthesis of the α-S-GalCer used in the present study (Dere *et* al 2008). The study by Blauvent and co-workers investigated the stimulatory properties of αGalCer and α-S-GalCer in mice, *in vivo* and *in vitro*. The failure of α-S-GalCer to induce cytokine production by mouse splenocytes [is that correct?] was reported as lack of a stimulatory effect. In collaboration with Professor Padraic Fallon at Trinity College Dublin, we tested our α-S-GalCer analogue in wild type and CD1d knockout mice. Concurrent with the study by Blauvent and co-workers, no stimulatory effect was observed with α-S-GalCer in mice, while injection of mice with αGalCer resulted in increased levels of IFN-γ and IL-4 in serum and stimulated recall responses of splenocytes. We propose that α-S-GalCer is only stimulatory in humans and therefore merits further investigation *in vitro* and in primates as an anti-tumour agent.

6.5 Conclusions

- α-S-GalCer induces cytokine production by iNKT cells, with a Th1 biased ratio

- α-S-GalCer induces the lysis of CD1d positive and CD1d negative cells by iNKT cells

- iNKT cells stimulated with α-S-GalCer can induce DCs maturation and cytokine secretion

- DCs matured in the presence of iNKT cells and α-S-GalCer can induce proliferation and cytokine secretion by allogeneic T cells

- α-S-GalCer to stimulation of iNKT cells as a DC adjuvant results in a Th1 biased immune response

7: General Discussion

7. Discussion

Although current treatment modalities for malignant tumours, such as surgery, radiation and chemotherapy have been improved markedly in the past three decades, the prognosis for these neoplasms remains poor (Soling & Rainov 2001). Alternate treatments such as immunotherapy, are gaining more momentum, with some immunotherapies in phase II/III clinical trials. Immunotherapy is the use of the bodies natural defence system, the immune system, in the treatment of a variety of human diseases. Cellular immunotherapy has focused on the concept of eliciting a specific T cell response. The cells of the innate immune system such as DCs and innate T cells have been widely used in many immunotherapy trials against cancer to date (Banchereau 2007, Bennouna 2008, Fujii 2009). Cancer immunotherapy has been well tolerated and specific but it's efficacy remains variable.

Invariant Natural Killer T cells are a subset of innate T cells and unlike conventional T cells they are restricted by the MHC like molecule CD1d and respond to glycolipids (Bendelac 2007). As the name suggests iNKT cells express an invariant TCR (Vα24 Jα18) and NK like molecules on their surface. iNKT cells are present at very low frequencies in human peripheral blood (0.01%-1%). However humans have T cells restricted by the CD1a, CD1b and CD1c as well as non iNKT cells restricted by CD1d. Due to the lack of the group 1 CD1 molecules (CD1a, CD1b and CD1c) in mice, T cells restricted by CD1 are poorly defined in the absence of microbial infections (Vincent *et al* 2003). In our studies we aimed to investigate the CD1 reactivity in human peripheral blood. We established an assay using CD1 transfected C1R cells co-cultured with PBMC from healthy donors, we examined the culture

supernatants by ELISA for the classical Th1 and Th2 cytokines, IFN-γ and IL-4 respectively after 72 hours. We failed to detect IL-4 in the supernatants of the PHA stimulated positive controls. We substituted IL-4 with another Th2 cytokine IL-13. We tested for IFN-γ and IL-13 in subsequent experiments. No significant reactivity was observed in the PBMC cultures so purified CD3+ T cells from peripheral blood were substituted for PBMC. Reactivity in the form of IFN-γ and IL-13 production was observed for CD1d but not for the group one CD1 molecules (CD1a, CD1b and CD1c). Previous studies in the literature describing CD1 group 1 reactive T cells from peripheral blood have described the presence of microbial lipids (Kronenberg 2005, Wingender & Kronenberg 2006, Ulrichs *et al* 2008). We can hypothesize that CD1a, CD1b and CD1c reactive T cells are only found in the presence of a microbial infection. With the identification of CD1d reactivity, we repeated the C1R assay with the addition of the CD1d binding glycolipids αGalCer and iGb3. The addition of the glycolipids resulted in increases in both Th1 and Th2 cytokines in culture supernatants. This indicated that the major group of CD1 restricted T cells in human peripheral blood are iNKT cells.

iNKT cells due to their low frequency in human peripheral blood, are difficult cells to study *in vitro*. Studies described in the literature used expanded iNKT cell lines and clones (Exley *et al* 1997, Tahir *et al* 2001, Exley *et al* 2003). In order to study the biology of iNKT and DC interactions we aimed to establish iNKT cell lines from human PBMC. We tested 3 methods described previously. Expansion of iNKT proved difficult, and several problems were identified. (1) The failure to isolate a pure population of iNKT cells from peripheral blood resulted in the expansion of non iNKT cells. Other groups use high speed fluorescence assisted cell sorting (FACS)

(Exley *et al* 2003), this equipment was not available during this section of work. (2) The variability amongst donors resulted in some lines failing to expand and others expanding at different rates, this variability was also observed by Croudace *et al*, who noted that iNKT cells from some donors fail to respond to stimulation. (3) The quality of reagents can affect iNKT cell expansion, (personal communications with Prof Mark Exley and Prof Steve Porcelli). A method described by Exley *et al* which utilizes a plate bound anti-CD3 mAb OKT-3 as a stimulus proved to be the best of the methods tested for the expansion of iNKT cells. This method resulted in highly pure iNKT cell lines with good numerical yields after 4-6 weeks of culture. We phenotyped the expanded iNKT cells to compare to those described in the literature. iNKT cells are predominately described as either CD4+ or double negative (DN) for CD4 and CD8 (Dellabona *et al* 1994, Exley *et al* 1997, Gumperz *et al* 2002). The iNKT cell lines established in this body of work had mixed populations of CD4+ iNKT cells and DN cells. Functionally iNKT cells are described as potent cytokine producers, with the unique ability to produce IFN-γ and IL-4 simultaneously (Chen and Paul 1997, Exley *et al* 1997). We examined the ability of the expanded iNKT cells to produce cytokine upon stimulation with αGalCer, and consistant with the literature, the cells produced both IFN-γ and IL-4 simultaneously. This data shows that our adapated method of purifying iNKT cells from human peripheral blood, results in functional iNKT cells as described previously by Exley *et al* (1997). iNKT cells as the name indicates are also effective cytotoxic cells. iNKT cell cytotoxicity has been described in both CD1d dependant and CD1d independent manner (Exley *et al* 1997, Nicol *et al* 2000). We tested the ability of our iNKT cell lines to lyse CD1d transfected C1R target cells *in vitro,* iNKT cells in the absence αGalCer could lyse significant numbers of target cells, the number of cells lysed was increased with

the addition of αGalCer. We also tested the ability of our cells to lyse the NK cell sensitive cell line K562 which is CD1d negative. iNKT cells lysed significant levels of the K562 target cells, the addition of αGalCer resulted in an increase in the lysis levels. Interestingly as mentioned previously K562 cells are CD1d negative, in 2000 Nicol *et al* reported iNKT lyses of the CD1 negative U937 cell line. The authors outlined the invariant Va24+T-cell lines used in the studies by Exley *et al* which indicated the necessity of CD1d for cytotoxic activity, co-expressed CD94, whereas their iNKT cells did not. They further implicated that the different results can be resolved if CD94/NKG2 inhibits CD1d-TCR-independent cytotoxic activity against otherwise sensitive CD1d-negative targets. This hypothesis can explain the lysis of our K562 cells as our cell lines had variable levels of CD94 expression. The increase in lysis with the addition of αGalCer suggests that either the iNKT cell lines or the K562 cell line expresses very low levels of CD1d, due to cell number restraints, the expression of CD1d was not tested on either line. These results show that functional iNKT cells can be readily expanded from human peripheral blood *in vitro* but the success of expansion can be attributed to the donor "response", reagent grade and the stimulation time and frequency.

iNKT cells are attractive candidates for cancer immunotherapy due to their potent adjuvant ability (Fujii *et al* 2009). In murine models, activation of iNKT cells using αGalCer resulted in the clearance and prevention of tumours (Cui *et al* 1997, Kawano *et al* 1998, Smyth *et al* 2000). iNKT cell based immunotherapies have under gone several phase I and phase II clinical trials, and whilst specific and well tolerated, results have been mediocre (Giaccone *et al* 2002, Nieda *et al* 2004, Motohashi *et al* 2006, Motohashi *et al* 2009). The basis of the most recent set of

clinical trials is the infusion of monocyte derived DC primed with a glycolipid, with or without an infusion of *in vitro* expanded iNKT cells. In order for immunotherapy to be successful, the DC must be fully matured and provide the correct signals to T cell populations. This has proven one of the biggest challenges in DC based immunotherapy (Banchereau and Steinman 1998, Figdor *et al* 2004). The interaction between the iNKT cell and the DC has been well established in murine models but only a few studies have examined these interactions in humans (Vincent *et al* 2002, Munz *et al* 2005, Yang *et al* 2005). We aimed to address this and our studies have in detail studied the reciprocal interactions between iNKT expanded from peripheral blood and monocyte derived DC. Our data shows that iNKT cells can induce maturation of DCs, the maturation was stronger with the addition of αGalCer. Maturation of the DC was determined by the upregulation of the maturation marker CD83, the co-stimulatory molecules CD40 and CD86, the adhesion molecule CD54, the antigen presentation molecule HLA-DR and the chemokine receptor CCR7 after 24 hours co-culture. The maturation induced was comparable to maturation induced by the TLR agonists LPS and poly I:C, which have been previously described (Rescigno *et al* 1998, De Jong *et al* 2002). This data shows that iNKT can pheotypically mature human DCs, this has been reported previously, but our studies have more extensive in the investigation of the effects of iNKT cells on the expression of, co-stimulation molecules (CD40, CD80, CD86), adhesion molecules (CD54), antigen presentation molecules (HLA-DR) and chemokine receptors (CCR7) than the previous studies (Munz *et al* 2005, Yang *et al* 2005) We investigated the functional properties of iNKT cell matured DC. iNKT cell matured DC produced significant levels of IL-12 p70 and also produced levels of IL-10. The interaction in turn resulted in significant levels of IFN-γ produced by the iNKT cells.

The production of cytokine revealed a Th1 bias (IL-12/IFN-γ) this has been described previously by Vincent *et al*. Next we investigated if this bias resulted in a Th1 response by allogeneic naïve peripheral T cells. iNKT cell matured DC induced the proliferation of allogenic peripheral T cells again with comparable results to the proliferation induced by LPS. The proliferated T cells secreted large amounts of IFN-γ and small amounts of the Th2 cytokine IL-4. These results also show that iNKT have a strong adjuvant effect, resulting in a Th1 bias immune response and correlates with the clinical trial data which showed increases in IFN-γ producing cells after iNKT/DC immunotherapy. This body of work is also the most comprehensive study of the interactions between iNKT and monocyte derived DC and the subsequent T cell response.

The natural ligand for iNKT cells remains to be elucidated but αGalCer a glycolipid isolated from marine sponge is currently the best known stimulator of iNKT cell in both mice and humans. With the disappointing clinical results observed with iNKT immunotherapy, many research teams aimed to produce a synthetic ligand with the hope of improving iNKT activation. In 2002 Miyamoto *et al* synthesised OCH, a αGalCer analogue, with truncated fatty acid chains. Stimulation of iNKT cell with OCH resulted in a Th2 bias, and the prevention of encephalomyelitis in mice. In other murine models OCH provided protection against other Th1 driven autoimmune disease such as collagen induced arthritis (Chiba *et al* 2004), insulitis and diabetes (Mizuno 2004 *et al*). In 2003 a Th1 biased αGalCer analogue was reported by Schmieg and colleagues. α-C-GalCer had a *C*-glycoside as opposed to the *O*-glycoside found in αGalCer. Stimulation of iNKT cells with α-C-GalCer resulted in a Th1 biased ratio of cytokine and 1000 fold increase in anti-malarial activity and

100 fold increase in anti-tumour activity compared to αGalCer (Schmieg *et al* 2003). The same group are currently investigating the use of α-C-GalCer as an adjuvant in a attenuated live influenza vaccine (Kopecky-Bromberg *et al* 2009). With the potential of analogues for immunotherapy reported, we tested the biological activity of several novel αGalCer analogues. Of the 6 tested only one, α-S-GalCer, stimulated iNKT cell lines. The ability of α-S-GalCer to induce cytokine production by human iNKT cells was tested in the CD1d transfected C1R assay described previously. α-S-GalCer induced the production of both IFN-γ and IL-4 by iNKT cell lines, however exhibited a greater Th1:Th2 ratio compared to αGalCer. α-S-GalCer induced greater iNKT cell lyses of both CD1d transfected C1R targets and CD1d negative K562 cells compared to αGalCer. In keeping with the iNKT based immunotherapy investigation, we investigated the ability of α-S-GalCer stimulated iNKT cells to mature monocyte derived DC and their ability to subsequently elicit a T cell response. α-S-GalCer stimulated iNKT cells induced DC maturation and the production of high levels of IL-12 but low levels of IL-10. The α-S-GalCer stimulated iNKT cell matured DC also proliferated allogeneic T cells and stimulated the same to produce high levels of IFN-γ and low levels of IL-4. Previously Blauvelt *et al* investigated the biological activity of their own α-S-GalCer. In contrast to our findings, the authors reported the α-S-GalCer had no biological activity. This study was in conducted in mice and examined proliferation of iNKT cells and production of cytokine, both *in vivo* and *in vitro*. The activity was not examined in a human system. In collaboration with Prof P Fallon (TCD) we repeated these murine studies and found that our α-S-GalCer also failed to induce iNKT activation in mice. The authors proposed that α-S-GalCer did not bind the CD1 molecule. To note, α-S-GalCer was first synthesised and described by Xhu *et al*, the method to synthesize

this α-S-GalCer varies from the method used by Blauvelt and colleagues (Dere *et al* 2008, Blauvent *et al* 2008). The data from the human studies using α-S-GalCer shows it stimulates iNKT cells *in vitro* with a Th1 bias. The conflicting murine data suggest that further investigations into both the stimulatory and structural properties of α-S-GalCer are necessary. However the success of tailoring iNKT responses via novel analogues provides strong evidence that iNKT cell therapy could be ultilized as specific immunotherapy adjuvants in the fight against human disease in the future.

Chapter 8: References

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8.1: Primary References

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