UNIVERSITY OF LIVERPOOL

NKT Cells in Rheumatoid Arthritis

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by

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Abstract

Rheumatoid arthritis (RA) is a common autoimmune disease, affecting around 1% of the population worldwide. The inflammatory processes which affect the joints and other tissues in the course of the disease lead to a significant decrease of quality of life and increased mortality. Although RA has been known for centuries, the pathomechanism of the disease development remains far from clear. Numerical or/and functional deficiencies in a regulatory, CD1d-restricted T cell population, known as invariant NKT cells (*i*NKT cells) have been indicated as a potential cause of immunodysregulation observed in human autoimmunity, including RA. Recently, a non-invariant subset of NKT cells has been described in murine B-cell lymphoma and autoimmune hepatitis. Surprisingly however, this non-invariant population seems to execute opposite roles to their invariant counterparts, aggravating the pathology observed in both of these models.

Based on these recent findings a hypothesis was formed that the non-*i*NKT cell population in RA may also execute a functionally different role and be actively involved in exacerbating the disease in patients. Therefore the aim was to appraise the involvement of both NKT cell subsets in the disease by assessing their prevelance and functional outcome of their activation. Firstly the frequency of *i*NKT cell subset was measured in the patient population by anti-Vα24 staining. The results indicated that the tendency for reduced *i*NKT cell numbers was not statistically significant, suggesting that a decreased prevalence is not an obligatory requirement for the development of RA. The disease can clearly develop even when the frequency of these cells is in the same range as that in the control population.

Next the αGalCer-driven proliferative response of $V\alpha24^+$ cells was measured in mononuclear cell cultures at multiple time points during a two weeks period. These experiments detected a striking overall proliferation defect in both the peripheral blood and the synovial fluid of RA patients. As the diminished response was uniformly seen in the entire patient cohort it could be argued that this defect is more closely linked to the development of RA than the relative paucity of these cells in itself.

The abundance of the total CD1d-restricted cell population was measured using αGalCer-loaded CD1d tetramers. The simultaneous enumeration of $V\alpha24^+$ cells allowed the calculation of the prevalence of non-*i*NKT cells in patients and controls. This work detected a relatively high proportion of the non-invariant CD1d-restricted cells being present in the synovial fluid, but not in the blood, of the patients.

αGalCer-driven cytokine secretion was measured using an 11-plex multi bead assay. The results indicated that the levels of six cytokines (IL-1β, IL-2, IL-4, IL-6, TNF-α and IL-10) were significantly altered in RA. Exposure of NKT cells from the synovial fluid to αGalCer resulted in a pattern of increased IL-10 and diminished TNF-α levels. Lower IL-4 secretion observed in

previous studies in the peripheral blood of patients was also reproduced but showed less clear cut association with the disease.

The work presented provided findings which help in clarification of the involvement of NKT cell subsets in RA. The initial hypothesis was proven as the increase in non-*i*NKT cells at the inflammation sites suggests a distinct role for this population in the immunopathogenesis of the disease. By accumulating in affected joints, these cells may alter the cytokine milieu, which in turn could influence the course of the inflammation.

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Abbreviation List

AB	assay buffer
ACR	the American College of Rheumatology
αGalCer	α-glucosylceramide
AHR	airway hyperresponsiveness
AIA	antigen-induced arthritis
AbIA	antibody-induced arthritis
AICD	activation-induced cell death
ANOVA	analysis of variance
AP	adapter protein
APC	antigen presenting cell
ARA	the American Rheumatism Association
ATP	adenosine triphosphate
β 2 m	β 2 microglobulin
B7RP-1	B7-related protein-1
BD	Behçet's disease
bp	base pair
CCL	CC chemokine ligand
CCP	citrullinated proteins
CCR	CC Chemokine Receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CDR	complementarity-determining regions
CFA	complete Freund's adjuvant
CFSE	carboxyflurescein diacetate, succinimidyl ester (CFDA SE)
CLIP	class II-associated invariant chain peptide
CIA	collagen-induced arthritis
CM	complete medium
CNS	central nervous system
ConA	concanavalin A
CPJ	cartilage–pannus junction
CRP	C-reactive protein

CS	contact sensitivity
CtIA	*Chlamydia trachomatis*-induced arthritis
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
CV	cardiovascular
CXCR	CXC chemokine receptors
DC	dendritic cell
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DP	double positive
ds-DNA	double stranded DNA
DUPGD	uridin diphospgoglucose dehydrogenase
EAE	experimental encephalomyelitis
EBV	Ebstein-Barr virus
EDTA	ethylenediamineetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
ER	endocytic reticulum
ERp	endoplasmic reticulum protein
ESR	erythrocyte sedimentation rate
FACS	fluorescence-activated cell sorter
FasL	Fas ligand
FcγR	Fc gamma receptors
FCS	fetal calf serum
FDC	follicular dendritic cells
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
FS	forward scatter

GC	germinal centre
GD3	disialoganglioside
Gg3Cer	gangliotriaosylceramide
GITR	glucocorticoid-induced TNF receptor
GRP94	glucose-regulated protein 94
H	healthy
h-HPRT	human hypoxanthine-phophoribosyl-transferase
HLA	human leukocyte antigen
HTLV-1	human T-lymphotropic virus type 1
Hex	hexaminidase
HSA	heat stable antigen
Hsp	heat shock protein
hTERT	human telomerase reverse transcriptase
IAV	influenza A virus
ICAM	inter-cellular adhesion molecule
ICOS	inducible T-cell co-stimulator
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
iGb3	isoglobotrihexosylceramide
Ii	invariant chain
*i*NKT	invariant Natural Killer T cell
IL	interleukin
IP-10	IFN-gamma-inducible protein-10
ITAM	immunoreceptor tyrosine-based activation motif
*i*TCR	invariant T cell receptor
KAR	killer activation receptor
KIR	killer cell Ig-like receptors
KIR	killer immunoglobin-like receptor
LAM	lipoarabinomannan
LAMP	lysosome-associated membrane protein

LADA	latent autoimmune diabetes in adults
Lck	leukocyte-specific protein tyrosine kinase
LFA	lymphocyte function-associated antigen 1
LMP	membrane protein
LPG	lipophosphoglycan
LTP	lipid transfer protein
MIIC	MHC class II-enriched compartment
MAIT	mucosal-associated invariant T cells
MAPK	mitogen-activated protein kinase
mBSA	methylated bovine serum albumin
MC	mononuclear cell
MCP	metacarpophalangeal joints
MDDC	monocytes-derived dendritic cell
MDSC	myeloid-derived suppressor cell
MECL	multicatalytic endopeptidase complex-like
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
ml	millilitre
MLB	multilamellar body
mM	millimolar
MMPs	matrix metaloproteases
MPT	metatarsophalangeal joints
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MTP	microsomal triglyceride transfer protein
MVB	multivesicular body
NFκB	nuclear factor κB
NHS	National Health Service
NK	natural killer cell
NKT	natural killer T cell
nm	nanometre

NOD	non-obese diabetic mouse
NPC1	Niemann-Pick type C1 protein
PAMP	pathogen-associated molecular pattern
PB	peripheral blood
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PE	phycoerythrin
PI	phosphatidylinositol
PIP	proximal interphalangeal joints
PLZF	promyelocytic leukaemia zinc finger, Zbtb16
PPDF	pentamethyldihydrobenzofuran
PTPN22	protein tyrosine phosphatase, non-receptor type 22
RA	rheumatoid arthritis
RAG	recombination-activating genes
RAPB	RA peripheral blood
RASF	RA synovial fluid
RDB	reagent dilution buffer
RER	rough endoplasmic reticulum
RF	rheumatoid factor
RPM	revolutions per minute
RTE	recent thymic emigrant
RT-PCR	real-time PCR
SAP	sphingolipid activator protein
SAR	structure-activity-relationship
SCID	severe combined immunodeficiency
SE	shared epitope
Sec61p	endoplasmic reticulum (ER) membrane protein translocator Sec61

SF	synovial fluid
SFMC	synovial fluid mononuclear cell
SLAM	signalling lymphocytic activation molecule
SLE	systemic lupus erythematosus
SM	standard mix
SNP	single nucleotide polymorphism
SS	Sjögren's syndrome
SSc	systemic sclerosis
SSC	side scatter
T1D	type 1 diabetes
TAP	transported associated with antigen processing
TCR	T cell receptor
TGF	transforming growth factor
TGN	trans-Golgi network
ThrCer	threitolceramide
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAIL	TNF related apoptosis inducing ligand
TREC	T-cell receptor excision complexes
T_{reg}	regulatory T cell
µl	microlitre
µg	microgram
VCAM-1	vascular cell adhesion molecule-1
WT	wild type
ZAP-70	zeta-associated protein of 70kDa

Chapter 1: Introduction

1.1.1 Overview

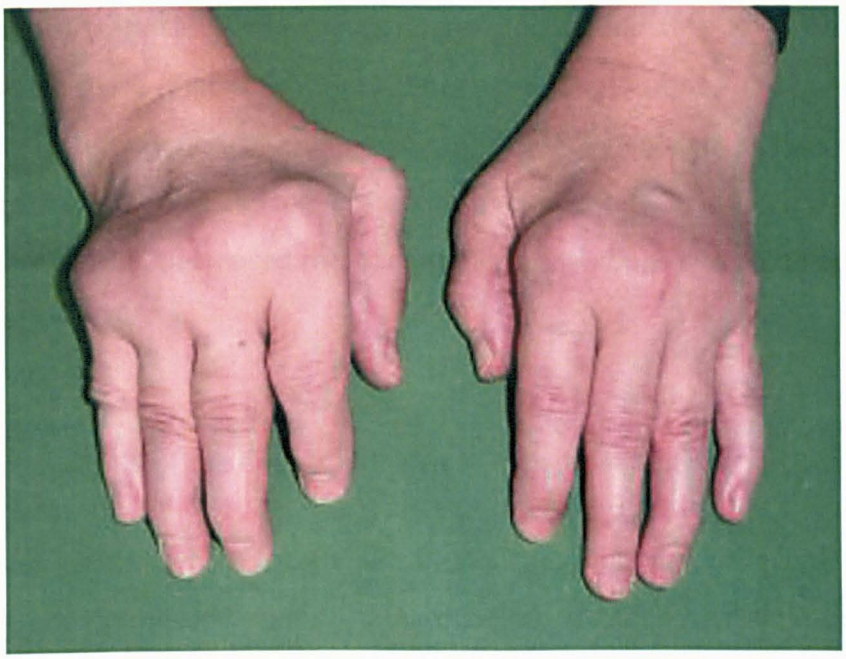
Rheumatoid arthritis (RA) is a common autoimmune disease, affecting around 1% of people worldwide. The inflammatory process can begin at any age, although it typically starts in the fifth or sixth decade of life. The disease development is not dependent on race, but it affects women more often than men (the female to male ratio for RA is 3 : 1). There is also increased occurrence of RA in some families, indicating a genetic link.

1.1.2. Clinical picture of RA

The main site of disease development is the joint, where a chronic inflammation process results in the degradation of cartilage and bone erosions. A classical feature of the disease is that the joints are involved symmetrically, which helps in distinguishing RA from some other diseases with joint involvement.

All the surrounding tissues, namely tendons, ligaments and muscles can also be inflamed. This in consequence provides the base for remodelling, which includes obliteration of joint space, subluxations and deformity (Figure 1.1 A and B). Clinically, the joints are swollen and tender, often containing substantial amounts of accumulating synovial fluid causing severe pain. Other tissues may also be affected by the ongoing inflammatory process. Infiltration can form subcutaneous nodules in the soft tissues, affect the pericardium or pleura, adding a range of systemic manifestations to the clinical picture of the disease.

A



B

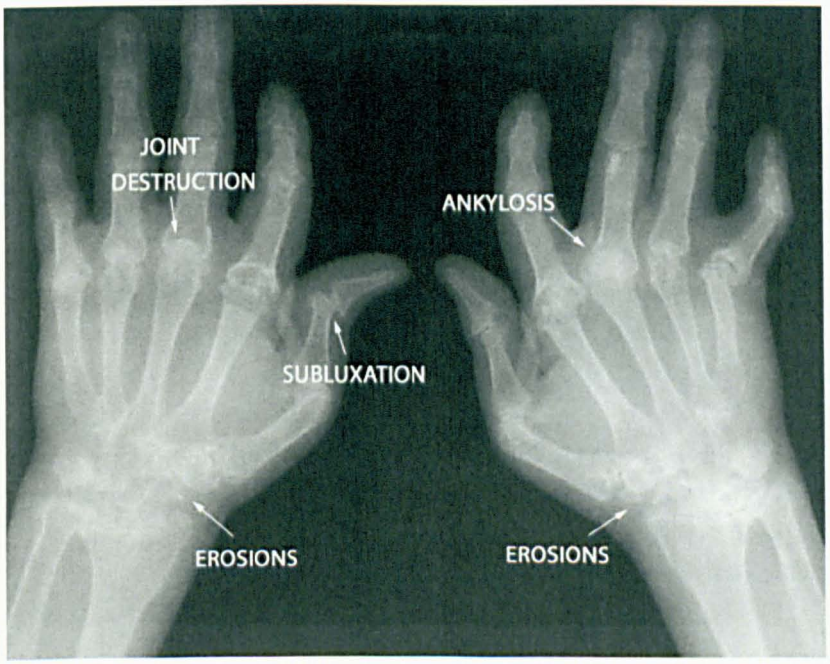


Figure 1.1 Clinical appearance of RA. A) hands of a patient with advanced RA, from www.sciencenewsden.com B) typical X ray picture at the later stage of RA, showing multiple signs of joint destruction, subluxations, ankylosis and erosions; from www.rheumatologia.pl; modified.

Cardiovascular (CV) risk is also increased in RA sufferers (reviewed in Gabriel 2008), which is reflected in common CV episodes in this group. There is an increase in mortality due to a higher risk of heart attacks. Moreover, it is also recognized that RA patients frequently experience lung disease (pleural effusions, interstitial lung disease) and anaemia, which is also intensified by the use of anti–inflammatory drugs. The medications that are now widely prescribed are also responsible for a spectrum of side effects, such as gastrointestinal ulcerations, weight gain, decreased resistance against infections and osteoporosis, liver and kidney damage, etc.

1.1.3. Diagnostic criteria of the American College of Rheumatology

The diagnosis of RA is based on the classification of the American College of Rheumatology (ACR, formerly the American Rheumatism Association, ARA). The symptoms which patients present with in clinic are assessed against 1987 revised criteria for RA (Arnett *et al.* 1988), as showed in Table 1.1.

Criterion	Definition
1. Morning stiffness	Morning stiffness of the joints, at least 1 hour before maximal improvement
2. Arthritis of 3 or more joints	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone)
3. Arthritis of hand joints	At least 1 area swollen in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of symmetrical joint areas
5. Rheumatoid nodules	Subcutaneous nodules over bony prominences or extensor surfaces, or in juxtaarticular regions
6. Serum rheumatoid factor	Abnormal amounts of serum rheumatoid factor
7. Radiographic changes	Radiographic changes typical of RA on hand and wrist X - rays, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

Table 1.1 ACR criteria for RA (from Arnett *et al.* 1988). A patient can be diagnosed with RA if he/she satisfies at least 4 of these 7 criteria. In addition, criteria 1-4 must have been present for at least 6 weeks.

1.1.4. Genetic predisposition

There are several risk factors that are linked to disease onset and flare–ups. The genetic susceptibility mentioned before is associated with the presence of some specific HLA-DR alleles (especially HLA-DR1, HLA–DR4 and HLA–DR10). It is thought that the presence of these in the genotype is associated with expression of a so called "shared epitope" (SE) which triggers an activation of self–reactive cells (Winchester 2006). In agreement with this observation, a large multicenter study on single nucleotide polymorphisms (SNPs) found accumulation of those in the HLA-DR1 alleles in the genome of RA patients (Wellcome Trust 2007). The other "sensitive" locus which was found in an SNP study is located on chromosome 1. One of the genes located there encodes a protein tyrosine phosphatase, non-receptor type 22 (PTPN22), which was shown to be associated with increased risk of RA.

1.1.5. Environmental factors

In addition, other factors are considered as having an effect on the onset and course of the disease. These include stress and lifestyle changes, hormonal disturbances, diet, smoking, infections and other environmental determinants (Solomon *et al.* 1975; Oliver and Silman 2009).

1.1.6. Histological appearance of a healthy joint

As mentioned above, the primary manifestation of RA is joint destruction. However, in order to illustrate histological changes that take place in RA it is essential to briefly outline the physiology of joints.

In healthy individuals a synovial joint is formed between two or more bones, each of them covered by a thick layer of cartilage and encapsulated by a fibrous membrane to create an enclosed joint space, filled with a small amount of lubricant, synovial fluid, which is needed for smooth movement and to reduce friction between the surfaces. This so called "capsule" comprises a fibrous sheet and a double layered synovium: an outer subintimal layer and the interior lining of synoviocytes (intima). The former provides the supply of nutrients for the joint by means of a capillary plexus; it also contains nerve endings and lymphatic vessels. The intimal layer is formed by cells of mixed origin; these cells are derived from a monocytic linage (type A synoviocytes), or are fibrocyte–like cells (type B synoviocytes). These two cell types form a discontinuous layer of synovium, embedded in an amorphous matrix.

Type A synoviocytes are relatively rare in normal joints; they are considered to represent a population of "resident macrophages", located more superficially towards the lumen (joint space). Despite their relatively low abundance in the synovial intima, due to their migratory abilities these cells can be more frequently seen in the synovial fluid. The function of these cells is related to the uptake of particles from the synovial fluid, which is reflected in the morphology of these cells which are rich in Golgi system and have a prominent network of vesicles (lysosomes, vacuoles). In addition, these cells express the CD16 receptor, which is required for opsonin-facilitated phagocytosis, and MHC class II molecules, which enable them to serve as antigen presenting cells in the joints (reviewed in Iwanaga *et al.* 2000).

The other cell population, type B synoviocytes, are fibroblast–like cells, which can stretch throughout the intimal layer, with dendritic villi protruding towards the lumen. These cells are much more abundant; the processes of many type B cells form a cellular plexus on the surface of the intima. Type B synoviocytes contain well-developed rough endoplasmatic reticulum (RER) and bundles of actin microfilaments. These structures play a role in

secretory function. These fibroblast-like cells produce matrix constituents (such as hyaluronan, collagen and fibronectin) and also secrete the synovial fluid. In addition, they also participate in the transfer of nutrients from the sub-intimal capillaries towards the lumen.

Synovial fluid is a physiologically aseptic, viscous lubricant required for a smooth joint operation. It consists of an ultrafiltrate of plasma. However, some of its constituents are secreted by type B synoviocytes as mentioned above. Normal synovial fluid also contains a small number of leukocytes (less than 200 cells per μl), mostly monocytes and macrophages (comprising around 2/3 of all the cells). Other cell populations, including neutrophils and lymphocytes can also be found, but less frequently. As the cartilage is devoid of blood vessels, the synovial fluid also provides the only supply of nutrients and oxygen to chondrocytes within the articular cartilage.

The cartilage is a smooth matrix of various collagens (predominantly type II) and proteoglycan dispersed in water, the latter constituting over a half of the cartilage mass. Other components include hyaluronan and fibronectin. The matrix of the cartilage is secreted by chondrocytes.

1.1.7. Histology of a joint in RA

One of the prominent features in the development of chronic inflammation in RA is an uncontrolled proliferation of synoviocytes. This hyperplasia increases the number of layers of cells (from the initial 1–3 to 10–20) and results in the thickening of the synovial membrane and the formation of villous structures protruding into the joint space. This is shown schematically in Figure 1.2. Simultaneously, the process of angiogenesis starts, which is essential in the development of the disease as it provides local blood supply for proliferating cells. This pathological synovium shares some characteristic with neoplastic cells, i.e. ability of uncontrolled growth and invasive properties. The enlarging tissue expands and acquires invasive capabilities, forming the so called "pannus".

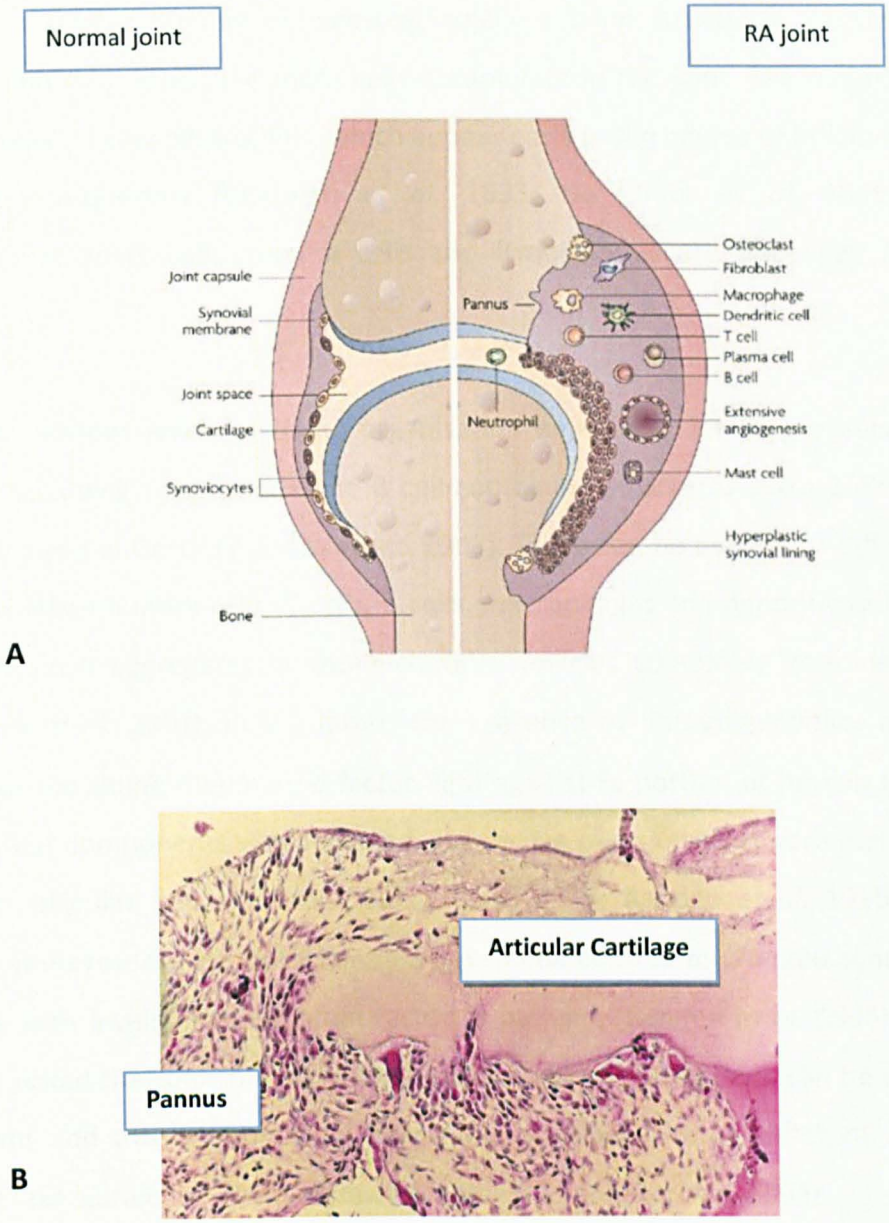


Figure 1.2 Inflammatory process in RA joint A) advanced RA (from Strand *et al.* 2007; modified); B) Histology of pannus invading the cartilage at cartilage-pannus junction in the joint affected by RA; from a front cover of Annals of Rheumatic Diseases, March 1999, 58/3, modified.

At places where the pannus is in contact with the cartilage, at the, so called "cartilage–pannus junction" (CPJ), pathogenically altered synoviocytes start tissue degradation. The histological section shown in Figure 1.2 B illustrates the process of invasion. The enzymes

observed at the interface are matrix metalloproteinases (MMPs), which apart from a cartilage, are also capable of digesting adjacent bone structures. As the inflammatory process escalates, more and more cells accumulate in the joint. The majority of those are granulocytes, mostly neutrophils, which appear early in the course of inflammation and bear destructive properties (Chatham *et al.* 1993; De Clerck *et al.* 1995). In addition, macrophages, mast cells, plasma cells and lymphocytes are also seen *in situ* in large numbers.

Moreover, various levels of tissue organisation have been for long observed in different patients, including lymph node-like B cell-containing structures (Sato 1986; Randen *et al.* 1995, reviewed in Goronzy and Weyand 2005). These can be present as diffuse infiltrates of individual inflammatory cells (T cells, B cells, macrophages and dendritic cells), B and T cells can form small aggregates or more complex follicles containing germinal centres (GCs) (Takemura *et al.* 2001). In the latter, the presence of immunoglobulins and monoclonal antibodies (including rheumatoid factor, IgM against Fc portion of human IgG) and various complement components was detected, suggestive of an ongoing germinal centre reaction involving follicular dendritic cells (FDCs) (Sato 1986; Randen *et al.* 1995). Furthermore, clinically the presence of these lymphoid germinal centres in affected synovium seems to correlate with levels of rheumatoid factor in patients (Randen *et al.* 1995). Importantly, it was also noted that only one type of these histological formations can be found in a given RA patient and this is also stable over time, suggesting three different subtypes of the disease (Takemura *et al.* 2001, reviewed in Weyand and Goronzy 2003).

The abundance of leukocytes (over 2,000 cells per µl) changes the appearance of the synovial fluid which becomes cloudy. The accumulating cells release inflammatory mediators, participating in a vicious circle of inflammatory processes. Moreover, the permeability of capillaries greatly increases, resulting in enlarged volumes of synovial fluid produced. This causes a visible swelling of affected joints and stretches the capsule resulting in pain. Other symptoms of the inflammatory state, such as an increased temperature of the surrounding tissues and redness are also present, especially during flare–ups.

1.1.8. Immunopathology of RA

Various immunological disturbances have been suggested to play a role in the development of chronic inflammation seen in RA. These include changes in the synovium, emergence of autoreactive T cells, antibody production by plasma cells and others. However, despite years of research the key mechanism which initiates the disease is still unknown.

One of the first explanations of RA pathogenesis identified B cells as a major causative factor in disease development. This was based on findings of autoantibodies (such as rheumatoid factor; RF or antibodies recognizing citrullinated proteins; anti–CCP reviewed in Mewar and Wilson 2006) in the patients. These arthritogenic antibodies were presumed to be produced as a consequence of molecular mimicry after a microbial infection (van Eden *et al.* 1985). When reacting with autoantigens in the joints, they form immune complexes that activate the complement cascade and initiate an inflammatory response (reviewed in Benoist and Mathis 2000). However, as rheumatoid factor and other autoantibodies can also be detected in around 10% of healthy people, while a significant proportion of RA patients do not have them, the concept that an abnormal antibody response causes RA remains unsubstantiated.

One of the widely proposed explanations suggests that a pathological activation of $CD4^+$ T cells is central for the development of RA. These cells represent the predominant mononuclear cell population in joints affected by RA and evidence from a number of animal models of the disease also strongly support their role (Ranges *et al.* 1985; Wooley *et al.* 1985; Ku *et al.* 1990; Kadowaki *et al.* 1994). The summarized concept is known as the "trimolecular complex hypothesis", which assumes the combined existence of three factors in RA patients (VanderBorght *et al.* 2001). First of all, the expression of a favourable MHC class II molecule is required. This molecule must be characterized by a groove suitable to accommodate an endogenous/exogenous ligand, which is the second factor needed. Finally, the abundance of a complementary TCR repertoire (VanderBorght *et al.* 2001) would be required. Overall this concept proposes a central role of T cells in initiating the autoimmune process in the joints. After activation, probably outside the joint space, the activated cells would be recruited to the affected joints where expansion of specific clones would start.

These cells secrete cytokines promoting changes in synoviocytes, which as a consequence proliferate and acquire invasive properties. Moreover, other cells with destructive potential, neutrophils and osteoclasts, are recruited by these cytokines and secrete additional chemokines (reviewed in McInnes and Schett 2007). According to this concept, the antigen presenting cells of the monocytic linage (monocytes, macrophages, type A synoviocytes) are thought to be involved at the stage where tissue destruction is already noticeable. These APCs are presumed to present a spectrum of autoantigens released from joint tissues (e.g. type II collagen or proteoglycans) which is reflected in the increase in variety of T cell clones in synovial fluid seen with the progression of the disease (reviewed in VanderBorght *et al.* 2001).

A recent extension of the trimolecular complex theory is a role for Th17 cells. The emergence of these cells may be fundamental to RA development. IL–17, the effector cytokine produced by these cells, is capable of activating a spectrum of cells involved at the site of autoimmune damage in the disease. For example, neutrophils and monocytes are targeted; they become activated, proliferate and release cytokines. Importantly, synoviocytes also respond to IL-17 stimulation. These cells also undergo activation, proliferate and secrete mediators boosting the inflammatory state. In addition, as discussed above, the activation of synoviocytes triggers the release of various matrix metalloproteinases (MMPs) which results in cartilage destruction and erosion of bone tissue (reviewed in McInnes and Schett 2007).

Although the role of conventional $CD4^+$ T cells in disease progression is undeniable, as supported by class II HLA association with the disease and complementary SNPs data described in paragraph 1.1.4, the assumption of a primary role of this cell population has been questioned. Critically, the severe combined immunodeficiency (SCID) mouse chimera model supports the role of synoviocytes as initiators of RA. In these animals xenogeneic transplantation of synovial tissue from RA patients results in joint inflammation with features of chronicity (Sack *et al.* 1994; Sack *et al.* 1996; Sack *et al.* 1999).

Observations that recombination activating gene knock-out ($RAG^{-/-}$) mice, lacking mature T and B lymphocytes, develop collagen–induced arthritis with similar incidence as control

animals (Plows *et al.* 1999) also question the importance of activated T cells in disease development. Histologically synovial hyperplasia, cartilage and bone destruction and fibrin deposition can be observed in spite of the lack of inflammatory infiltrate, suggesting the importance of innate immune responses elicited by an injection of collagen with complete Freund's adjuvant (CFA). However, there was a delay in disease development and a reduction in severity of joint changes seen in these $RAG^{-/-}$ mice, illustrating the role of adaptive immunity in disease initiation/progression (Plows *et al.* 1999). Moreover, the efficacy of T cell-targeting CTLA-4-Ig treatment, bringing benefits even to RA patients who fail to respond to other biological drugs (Ostor 2008; Taylor 2009), certainly provides the evidence of T cell involvement in the disease.

Finally, a hypothesis of a dysfunction of cells with an immunoregulatory role has also been outlined. The populations proposed to be affected in RA patients are $CD4^+CD25^+$ T_{regs} and NKT cells. The former have received much attention as it was suggested that this cell population is unable to control expansion of autoreactive T cells in various animal models of autoimmunity (reviewed in Anderson and Isaacs 2008). In agreement with this, the antigen–induced arthritis model (AIA) – where arthritis is induced by a single intraarticular injection of mBSA - demonstrated the protective role of adaptively transferred $CD4^+CD25^+$ cells (Frey *et al.* 2005). However, regulatory T cells seen in the synovial fluid of RA patients have an increased suppressive activity (van Amelsfort *et al.* 2004). The authors of this study conclude that the increased activation status of autoreactive T cells makes them less susceptible to suppression by T_{regs} in those patients.

NKT cell involvement in the initialization and development of RA was proposed when reduced frequencies of these cells were identified in other autoimmune diseases, such as type 1 diabetes (T1D) (Wilson *et al.* 1998), systemic lupus erythematosus (SLE) (Yang *et al.* 2007) or multiple sclerosis (MS) (Araki *et al.* 2003). Few research groups investigated the frequencies and function of these cells in RA (Maeda *et al.* 1999; Kojo *et al.* 2001; van der Vliet *et al.* 2001; Linsen *et al.* 2005); these results will be described in detail later. However, due to the lack of a uniform definition of NKT cell populations and the limited number of those investigations the role of these cells still needs to be clarified which is the reason why the present study has been initiated.

1.2. Antigen presentation pathways

1.2.1. Presentation of peptide antigens

1.2.1.1. MHC class I pathway

MHC class I–mediated antigen presentation emerged in jawed vertebrates (Danchin *et al.* 2004) to provide organisms with protection against intracellular pathogens. This system exists in almost all nucleated cells and is responsible for screening the cytosol for exogenously synthesised (e.g. viral) antigens. However, endogenous self–derived peptide epitopes are also presented, providing the means for detecting malignant transformation, or other non-infectious events.

Human Major Histocompatibility Complex genes are located on chromosome 6, within a large complex, containing multiple polymorphic alleles, grouped into class I, class II and MHC-related genes (reviewed in Goldsby *et al.* 2003). The main constituent of the MHC class I molecule is a heavy chain encoded by one of these alleles. These heavy chains comprise threes α domains; two distal domains (α_1 and α_2) form a pocket–like peptide binding site. This binding site (or "cleft") has its opening between two antiparallel α helices overlaying a platform formed by β–strands and is able to accommodate 8–10 amino acid-long peptide antigens or occasionally longer (as reviewed in Rammensee 1995). A domain proximal to the cell membrane, α_3, associates with an additional molecule, β2–microglobulin, to stabilize the conformation of the complex. β2m is necessary for the expression of the class I molecule; these are never expressed at the cell surface of $\beta2m^-$ cell lines and are inevitably degraded in the cytosol (Hughes *et al.* 1997). The class I molecule is anchored in the plasma membrane by a transmembrane segment of the heavy chain.

In addition to the MHC class I molecule itself, various proteins are needed for the purpose of complex formation and peptide loading; these will be outlined next, together with a brief description of antigen processing. As mentioned above, the cell cytosol is under constant scrutiny. In order to be successfully presented at the cell surface, antigens must be first assigned for processing. This is mediated by proteins E1, E2 and E3 which attach polyubiquitin chains to a given protein in a process called ubiquitination (Ciechanover and

Schwartz 1994). Polypeptides marked in this way undergo enzymatic cleavage by a composite protein complex, the proteasome (Figure 1.3). The proteasome forms a funnel or a barrel consisting of four rings, each formed by a set of seven α or β domains. The interior of this structure exhibits a proteolytic activity and cleaves peptides which are fed into the proteasome core. Additional subunits, LMP2, LMP7, LMP10 (also known as MECL1) also participate in the antigen cleavage in mammals. These latter subunits are IFN-γ–induced and their presence affects the cleavage site by enhancing hydrolysis after hydrophobic and basic residues (reviewed in Pamer and Cresswell 1998). In addition, the so called P28 activator, can also participate in the antigen processing. This protein complex forms a ring of α and β subunits which attaches to the ends of proteasome core (Gray *et al.* 1994) and significantly increases the efficiency of proteolysis as well as the diversity of final products (reviewed in Pamer and Cresswell 1998). In addition, a polypeptide complex, PA700, also enhances the turnover of the peptides processed, most likely by unfolding the substrates for cleavage (Chu-Ping *et al.* 1994).

Next, short peptides of the appropriate length are transported into the lumen of the ER in an ATP-dependent process. Peptides are transported through the ER membrane by the "transporter associated with antigen processing" (TAP), a heterodimeric complex that consists of the TAP-1 and TAP-2 subunits, encoded in the MHC gene complex. This transporter binds a given peptide in an ATP-independent process and then trans-locates it into the lumen of the endoplasmatic reticulum using ATP as an energy source (Neefjes *et al.* 1993). The TAP complex is capable of transporting oligopeptides which are longer than ten amino acids, which suggests that additional trimming takes place inside the ER. In fact, it was found that the enzymatic activity in the ER compartment enables the cleavage of long polypeptides, which had not been pre-treated by proteasome (Elliott *et al.* 1995). At this stage peptides can be trans-located back to the cytosol for additional processing and can re-enter the ER via TAP (Roelse *et al.* 1994). TAP-mediated peptide translocation seems to be the main source of epitopes for class I molecules and clinical manifestations can be observed in individuals who are TAP deficient (Gadola *et al.* 2000).

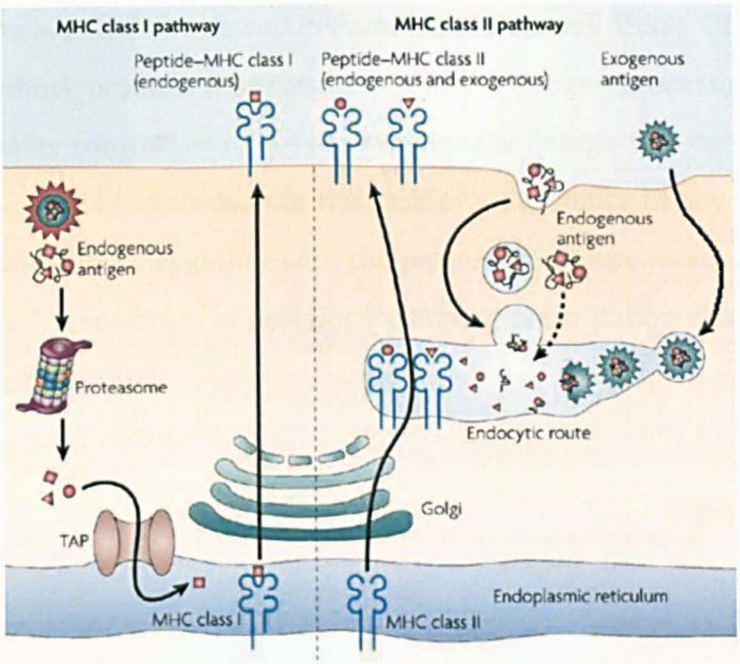


Figure 1.3 Simplified illustration of MHC class I and II antigen processing and presentation pathways, from (Villadangos and Schnorrer 2007). As shown, the MHC class I pathway (visualised on the left) requires antigen processing by proteasome in cytosol and translocation to ER by TAP complex, while class II-mediated pathway (depicted on the right side) is independent from these structures and both antigen processing and loading take place in the vesicles of the endocytic system.

The processes of class I molecule assembly and consecutive antigen loading in the endoplasmatic reticulum is assisted by ER–located chaperones: calnexin, calreticulin and tapasin. These molecules form a part of a "quality control system" for nascent composite proteins and are essential for folding multimeric protein structures. The first chaperone involved in the formation of the MHC class I/peptide complex is calnexin. This membrane–bound chaperone interacts with the heavy chain and retains it in the lumen of the ER for the binding of β2m (Pamer and Cresswell 1998). Calreticulin is the next molecule which joins the complex, replacing calnexin. It has a similar role of stabilizing and retaining nascent class I molecule in the compartment. Tapasin is involved in the process of folding and antigen loading (Sadasivan *et al.* 1996). As the name suggests, it is a protein associated with TAP, inter-joining this transporter with the heavy chain/chaperone complex. The proximity of the folding heavy chain to TAP that tapasin provides enables the acquisition of peptides incoming from the cytosol and also increases the rate of peptide translocation through TAP

and stabilizes the heavy chain (reviewed in Pamer and Cresswell 1998). Other proteins, such as gp96 and heat shock proteins (Hsp) also assist in the antigen processing for MHC class I molecule. The "quality control" enzymes are functionally flexible to a certain extent, which provides continuous class I expression in the case of a deficiency of any of those enzymes (Pamer and Cresswell 1998). Together with the proteasome these molecules are described as a "presentasome", a spectrum of proteins facilitating presentation of antigens, as shown in Figure 1.4 (Srivastava 2002).

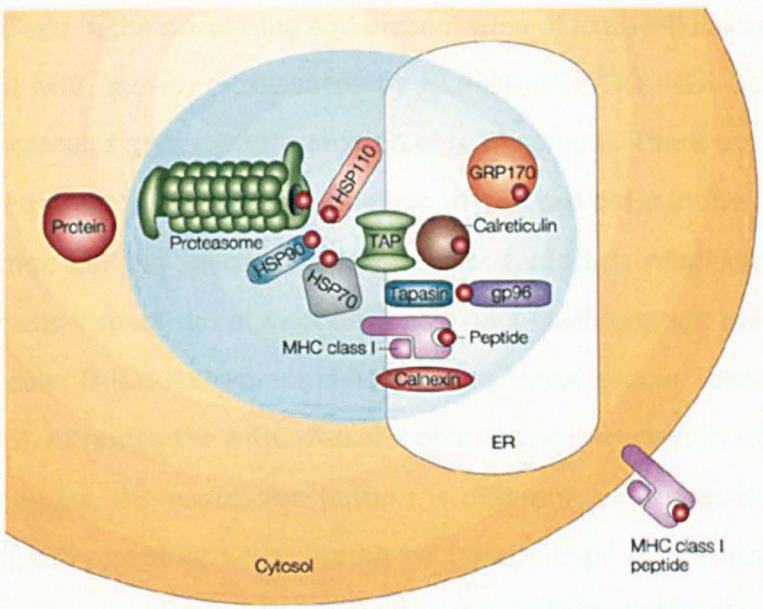


Figure 1.4 Presentasome assisting in antigen processing and formation of MHC class I/antigen complex (from Srivastava 2002). Class I-associated machinery of proteins is complex and consists of chaperons involved in antigen digestion and transport (proteasome and TAP, respectively), class I molecule folding and loading (calnexin, calreticulin, tapasin) and preserving conformation of the intermediates (HSPs, gp96).

If the process of assembly is successful, a complete heavy chain/β2m/peptide complex traffics via the Golgi Apparatus towards the plasma membrane while a misfolded protein undergoes destruction by the proteasome in the cytosol. The transport of these misfolded heavy chains is facilitated by an ER translocation channel, Sec61p (Pilon *et al.* 1997).

1.2.1.2. MHC class II antigen presentation

The MHC class II pathway samples the extracellular environment for the presence of bacteria or parasites. The presented epitopes are protein-derived, as in class I-mediated presentation but instead of being recognized by a specific TCR of cytotoxic lymphocyte population (CTLs), they are recognised by $CD4^+$ (helper) T cells. The differences between class I and II molecules in regard to antigen processing and complex formation are illustrated schematically in Figure 1.3.

Human genes involved in the processing and presentation of extracellular antigens comprise the group II of all MHC genes, represented by HLA-DR, HLA-DQ, HLA-DP and some non-classical class II molecules genes, all situated on chromosome 6. There are several hundred allele variants; in contrast to class I genes, the haplotypes can differ in the number of alleles present. The peptide binding specificity of both class I and II is relatively low, so a given molecule can present a spectrum of peptides and also a given peptide can bind more than one HLA-DR molecule. This is sometimes referred to as "promiscuous" peptide presentation (Rammensee 1995). Although the MHC class II molecules are structurally similar to the MHC class I-peptide complex, the expression pattern is different, with class II molecules being restricted to Antigen Presenting Cells. During bacterial, fungal or parasitic invasion these cells take up antigens, process them and present in the context of MHC class II.

There are two types of APCs: professional APCs – cells expressing MHC class II constitutively (dendritic cells, macrophages, B cells) - and non-professional APCs, on which the expression is inducible under inflammatory stimulation (i.e. fibroblasts, glial cells, keratinocytes) (Goldsby *et al.* 2003; Black *et al.* 2007). Dendritic cells are most potent; they are able to prime naïve T cells due to very high level of constitutively expressed MHC class II molecules (McKeever *et al.* 1992; Liu and MacPherson 1993).

The class II molecule is composed of two heavy chains: α and β. These chains associate together to form a four-domain structure with cytoplasmic anchor segment (reviewed in Cresswell 1994) expressed on a plasma membrane. It is characterized by the

immunoglobulin-fold which is the reason for classifying class II into the immunoglobulin superfamily. While the membrane proximal parts of the α and β chains form the stabilizing α_2 and β_2 domains, the distal α_1 and β_1 domains create the peptide-binding cleft. The binding groove is open to the sides, which enables MHC class II molecules to accommodate longer peptides, up to 13-25 amino acids (Chicz *et al.* 1992).

Both heavy chains are synthesized in the ER simultaneously, with additional chaperones employed, especially ERp72, GRP94 and calnexin. The role of these chaperones is folding large, complicated molecules (i.e. immunoglobulins, TCRs, etc) and taking part in the ER quality control mechanism (Anderson and Cresswell 1994; Meunier *et al.* 2002). Calnexin on the other hand also enables merging of α and β chains (Meunier *et al.* 2002), and assists in the process of attaching the Invariant Chain (Ii) at the time of complex formation (Anderson and Cresswell 1994). This latter molecule is a chaperone with a unique function; it protects the groove from binding random endogenous peptides (Anderson and Miller 1992). Three Invariant Chains associate together in the ER, so the final structure is nonameric $(\alpha\beta Ii)_3$ and the three class II molecule complexes traffic together. Ii also contains a di-leucine targeting signal that directs the nonamers into the endocytic system of the cell (Karlsson 2005).

The latter compartment is a large system of vesicles, vacuoles and tubules within the cytoplasm. Early endosomes are formed by the fusion of phagocytic bodies with ER/Golgi apparatus-derived enzymatic vacuoles. They become gradually more and more acidic forming late endosomes and lysosomes. This process is maintained by the function of an ATP-dependent proton pump in the membrane of these organelles (Mego *et al.* 1972). As the acidity of organelle in the endocytic system is gradually rising, a variety of intracellular microenvironments with the optimal pH for the digestion of a spectrum of proteins, self- and non–self, is created. Some of the vesicles of the endocytic system contain smaller vesicular bodies inside or are of multilamellar structure. These are referred to as multivesicular bodies (MVBs) or multilamellar bodies (MLBs), respectively. The internal membranes of these organelles are thought to be the site of intense release of lipids (Kolter *et al.* 2005). MLBs are usually quite acidic and rich in enzymes. They are also enriched in MHC class II complexes accumulating there before being directed to the plasma membrane. This compartment is therefore also called the MHC class II-enriched compartment (MIIC)

and is recognized as being crucial for MHC class II-mediated antigen presentation (Geuze 1998).

In order to present exogenous antigens within the groove of the MHC class II molecules, these must be internalized into the cell first. The uptake takes place through phagocytosis, macropinocytosis or receptor-mediated endocytosis. The latter is the most specific and involves the B Cell Receptor. Also when antigen is coated by immunoglobulins or complement its uptake can be facilitated by means of specific receptors (Watts 1997). Antigens can be also taken up by pinocytosis or endocytosis, which depend on two different proteins, clathrin and actin, respectively. The former polymerizes into a form of mesh–like structures enabling invagination of the plasma membrane to form pinocytotic pits, while the latter plays a role in the phagocytosis of insoluble particles (Geuze 1998).

When an antigenic molecule is internalized, it enters the endocytic system at the least acidic environment (early endosomes) and then progresses through compartments into the vesicles of lower pH and higher cathepsin content. Both peptidases and low pH act simultaneously in the process of protein degradation releasing smaller peptides, which fit into the groove of MHC class II molecule (Watts 1997). The same enzymes also digest the invariant chain, although the process is not complete and a remaining part of Ii (referred to as the CLIP peptide) still occupies the groove.

Additional molecules, DM and DO, are required in the process of antigen processing and loading. These non-classical class II proteins accumulate in the MIIC compartments (Denzin *et al.* 1997; Karlsson 2005). Their striking similarity to class II molecules is not surprising, as they originate from genes of the MHC complex, but are regarded as non–classical molecules (Alfonso and Karlsson 2000). The HLA-DM gene is thought to be derived from an ancestral protein, giving rise to both classical and non-classical MHC class II molecules and the gene encoding DO is assumed to have evolved from it after a consecutive duplication event (Danchin *et al.* 2004). These molecules never present antigens, but they assist during antigen editing/processing, releasing the CLIP peptide and loading antigens into the groove of class II molecules (Alfonso and Karlsson 2000). DM acts to remove CLIP from the class II molecule, creating the space in its groove for the incoming external antigen. It also protects

the conformation of the presenting molecule, so it is ready to accept an incoming peptide (Alfonso and Karlsson 2000) and, if the peptide is of a low affinity, it releases it back. These events are repeated until the antigen of the highest affinity is bound in the binding cleft and the formation of mature MHC class II/antigen complex is complete (Alfonso and Karlsson 2000).

The HLA DO protein is DM-dependent in terms of trafficking, as it travels through the cell complexed with DM. This dimer contains a multiple targeting motif (tyrosine-based of DM and tyrosine and di-leucine in the DO tail; van Lith *et al.* 2001). Both proteins incorporate into MIIC membranes, ensuring proximity to MHC class II/Ii complexes and enabling lateral interaction (van Lith *et al.* 2001). DM and DO molecules are only expressed by cells that are APCs. The former are abundant in all Antigen Presenting Cells, while the latter is only expressed by B cells (Geuze 1998; Alfonso and Karlsson 2000; Karlsson 2005). Although B cells express both molecules, so it could be assumed that the peptide editing process will be increased, this is actually not the case. In fact, DO expression decreases peptide editing in B cells. It was therefore proposed that DO itself does not facilitate editing, but inhibits it (Denzin *et al.* 1997), to ensure that a response is evoked against the most relevant, BCR-internalized antigen (Alfonso and Karlsson 2000).

After the antigen processing and editing, mature antigen–containing complexes are sent to the plasma membrane. They can reach the surface by means of small vesicles detaching from tubular extensions of the early MIICs or by fusion of the limiting membrane of MVBs with the plasma membrane. Alternatively, MHC class II/peptide complexes can be also released from the cell to the external *milieu*, as secreted exosomes. It was shown recently that these small vesicular bodies can mediate antigen presentation on their own (Geuze 1998), as they also contain molecules capable of delivering a second signal.

1.2.2. Presentation of lipid antigens

1.2.2.1. CD1-mediated presentation

Before CD1 molecules were discovered it was assumed that antigen presentation generated primarily protein–derived epitopes. However, there is also a range of lipids and glycolipids that can trigger immune responses, via the CD1 system (Porcelli and Modlin 1999). Five genes are found within the CD1 gene complex in humans (CD1A-E) situated outside the MHC complex on chromosome 1. Despite the different localization within the genome, these show an MHC class I molecule-like intron/exon structure and the proteins have significant homology to MHC class I and II molecules (Porcelli and Modlin 1999). As the genes are similar, yet nonpolymorphic, there is a hypothesis that the CD1 complex originated from a primordial ancestral antigen presenting molecule. This gene had undergone a series of duplication events and then the daughter genes evolved into a group of MHC class I/II and CD1 genes after multiple point mutations and duplications (reviewed in Bontrop 2006).

CD1 isoforms are functionally divided into three groups: group I (CD1a, b, c), group II (CD1d) and an intermediate group III (CD1e). The genes of the human CD1 complex have homologues among different species; reports have been published describing combination of various CD1 isoforms in Old World monkeys, sheep, cattle, rabbit and guinea pig (reviewed in Brigl and Brenner, 2004). Overall, the conservation of the CD1 system is remarkable. For example, there is over 95% similarity between CD1d α1-2 domains of between humans and rhesus macaques (Kashiwase *et al.* 2003), while CD1a, CD1e and multiple CD1b molecules are present in the cattle (Van Rhijn *et al*, 2006). However, extrapolating results from experiments with rodents must be done with caution. Due to a relatively recent deletion event these animals lack all group I genes. In addition, group II genes in these animals comprise two genes instead of one, CD1d1 and CD1d2 and of these only the former is functional (Porcelli and Modlin 1999; Rhind 2001).

The structure of CD1 isotypes was recently solved (Gadola *et al.* 2002; Giabbai *et al.* 2005; Koch *et al.* 2005). As mentioned before, the architecture of all the CD1 molecules is

strikingly similar to class I molecules. As in the latter three α domains (α1, α2, α3) are seen which form a complex with β_2-microglobulin (reviewed in Porcelli and Modlin 1999). However, it seems that β_2-microglobulin is not always necessary for the expression distinguishing CD1d from class I molecules. For example, CD1d is also expressed in a non–complexed form on intestinal cells (Balk *et al.* 1994) and larynx (Rees *et al.* 2008). The other significant difference is observed in the groove which is larger and deeper than in class I molecules. Moreover, the residues forming this groove are mainly hydrophobic, thus the molecule is capable of accommodating non-polar alkyl chains.

Despite the fact that CD1 molecules are similar to one another, there are also significant differences between the isotypes. For example, the CD1a groove has two large pockets (A' and F'). The A' pocket is very narrow and curved and can accommodate a C_{23} long alkyl chain while a shallower F' pocket can fit a second chain and a polar headgroup. CD1a is also suitable for single-tailed epitopes; the F' pocket is then occupied by the headgroup alone (reviewed in Hava *et al.* 2005). In contrast, the groove of CD1b also contains these pockets, but two additional channels can be observed. First of them (T') connects the A' and F' pockets so the tunnel forms a loop. Because of this the molecule can accommodate very long alkyl chains of up to C_{80} (Porcelli and Modlin 1999; Moody and Porcelli 2003; Hava *et al.* 2005). The additional C' channel not only provides extra space for a third chain, but, as it is open to the outside, it allows a larger antigen to protrude from the side of the molecule while it is bound (Porcelli and Modlin 1999). Overall, the structure of the groove provides flexibility in the length and shape of lipids, resulting in a wide range of presented antigens despite the largely conserved architecture of the molecules.

The part of the groove which faces the TCR recognition surface is formed by more hydrophilic, charged amino acids which shape the space for a polar headgroup of the antigen. These charged amino acids have a very important role in anchoring the presented molecule into the groove and preventing its escape from the complex (Koch *et al.* 2005; Zajonc *et al.* 2005). The overall structure of the molecule is ideal for glycolipid antigens, as these consist of an alkyl chain (or chains) and a polar sugar moiety.

1.2.2.2. CD1 ligands

The examples of bound ligands vary between isotypes which is a reflection of their diverse structures. Group I is capable of presenting microbial lipids and derivatives such as fatty acids, glycolipids and phospholipids. These molecules are also capable of complexing lipopeptide antigens (including mycolic acid and lipoarabinomannan LAM found in mycobacterial walls; Moody *et al.* 1997).

Group II is functionally different. The CD1d molecule forms complexes not only with external, microbial antigens, but also with self-derived molecules (Chiu *et al.* 1999). These include sphingolipids and diacylglycerols (Porcelli and Modlin 1999; Prigozy *et al.* 2001; Godfrey *et al.* 2004). There are also exogenous ceramides and other glycolipidic compounds that can be presented by CD1d. These will be described later.

The group III molecule (CD1e) is different from the others as it does not participate in ligand presentation, but assists in the loading of antigens into the other molecules of the CD1 family (Angenieux *et al.* 2000; de la Salle *et al.* 2005), in which it resembles the non–classical class II molecules, HLA-DO and HLA-DM.

1.2.2.3. Antigen processing and CD1 trafficking

To encounter their corresponding antigens, CD1 molecules are targeted to various compartments of the endocytic system. They do not acquire peptide ligands in the endoplasmic reticulum (ER) so they are TAP-independent. There is a tyrosine-containing motif in the tail of the CD1 molecules that is responsible for trafficking from the ER to the endocytic system. Although these routes vary between isotypes, all molecules are first directed towards the plasma membrane and then recycled to the appropriate compartments (De Libero and Mori 2005). Trafficking routes of CD1 molecules are illustrated in Figure 1.5.

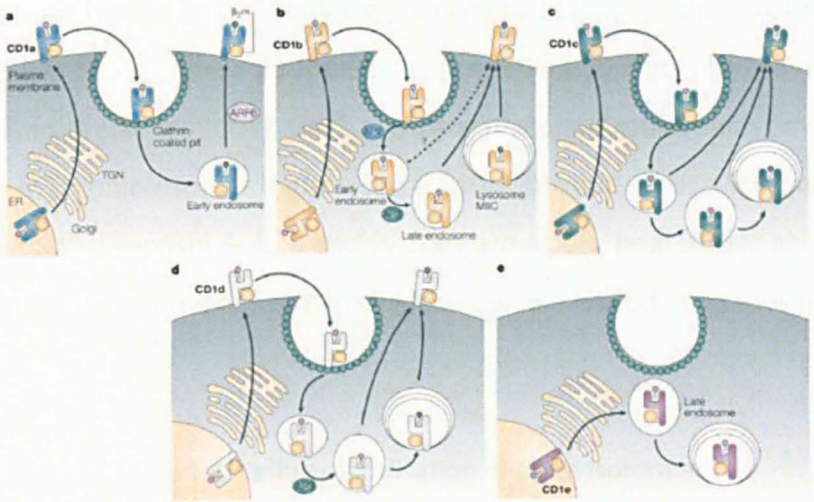


Figure 1.5 Trafficking of CD1 isotypes (from De Libero and Mori 2005). Various isotypes adapt different pathways of intracellular trafficking from the plasma membrane to the appropriate vesicles of the endocytic system, where the optimal conditions for loading of their antigens are maintained. A) CD1a is most abundant in early endosomes while B-D) all other isotypes are present predominantly in late endosomes or lysosomes. E) CD1e molecule is not directed to the plasma membrane and immediately localizes to the late endosomal/lysosomal compartment where it mediates the function of antigen loading.

The groove of a CD1 molecule is not empty when trafficking. Instead, there is a self-lipid (e.g. phosphatidylinositol), that occupies the groove (De Silva *et al.* 2002), which is acquired by the molecule in ER. This is in agreement with the molecular structure as the presence of a "spacer" in the A' pocket has been observed (Zajonc *et al.* 2005).

For CD1 molecules transiently expressed at the surface before acquiring an antigen in the endocytic system, this complexed self-ligand can be transiently seen at the membrane (Hava *et al.* 2005). The process of trafficking is complicated and requires multiple chaperones. Adapter Proteins (AP1, AP2, AP3) control the intracellular movements of CD1 (reviewed in Moody and Porcelli 2003). Different isotypes of CD1 are localised to different parts of the endocytic system, characterised by a pH which is most suitable for the acquisition of an appropriate antigen. As summarised in Table 1.2 the isotypes can be found in various endocytic vesicles where the acidic environment is required for the conformational change enabling the groove entrance to be wider and more accessible for the loading of glycolipid antigens (Moody and Porcelli 2003). Moreover, additional lipid transfer proteins (LTPs) or

lipid-binding proteins (Kolter *et al.* 2005) are involved to assure specificity of antigen loading into CD1 molecules. These accessory molecules are SAPs (sphingolipid activator proteins = saposins, (Kang and Cresswell 2004), GM2 activator protein (Conzelmann *et al.* 1982; Kolter *et al.* 2005) and MTP (microsomal triglyceride transfer protein (Brozovic *et al.* 2004). There are four different SAPs: saposin A, B, C and D. They are acidic, heat-stable glycoproteins of a conserved structure, all derived from a single molecule called prosaposin (Kolter *et al.* 2005).

Table 1.2. Cellular localisation of CD1d isoforms

CD1d ISOTYPE	INTRACELLULAR LOCALIZATION	pH (approx.)
CD1a	early endosomes	6
CD1b	all system, mostly lysosomes	4.8
CD1c	all endocytic system	6 – 4.8
CD1d	late endosomes and lysosomes	5 and 4.8
CD1e	late endosomes and lysosomes	5 – 4.8

Various CD1 isotypes primarily localise to different cellular compartments to provide optimal pH conditions for loading of most suitable antigens into their binding grooves (based on Moody and Porcelli 2003; De Libero and Mori 2005).

These molecules release lipids or glycolipids from limiting and intraluminal bilayers in the endocytic system, by a membrane-perturbing detergent-like action (Figure 1.6). These membranes are sources of both self- and exogenous ligands and saposins (especially SAP B; Yuan *et al.* 2007) function as facilitators of their digestion. Moreover, saposins are also characterised by a unique ability to assist the exchange of relatively low affinity antigens e.g. self-ligands bound to the groove (Kang and Cresswell 2004).

Another molecule, GM2 activator, is active in the process of ganglioside degradation. Its active domains form open loops which penetrate the bilayer where its lipid recognition site identifies a ganglioside substrate. After this interaction the loops change conformation and remove the ganglioside from the membrane (Kolter *et al.* 2005) for digestion by hexaminidase A (Hex A). Inherited defects or deficiencies of these proteins cause various

lipid storage diseases, which can be fatal (e.g. in neural gangliosidosis) so the functional activity of these "lipid releasers" seems to be also essential in other lipid metabolic pathways.

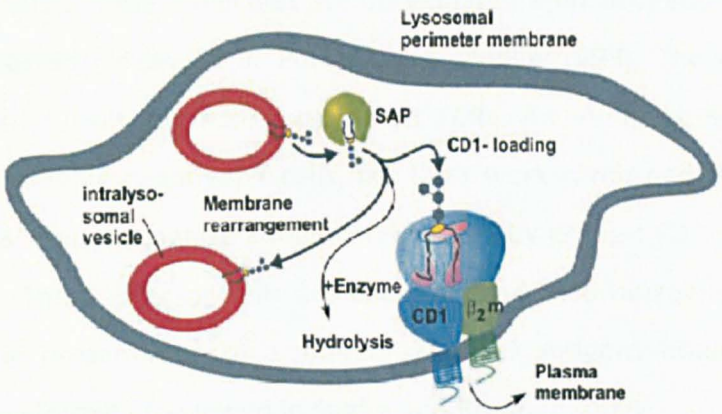


Figure 1.6 Model of function of sphingolipid activator protein (SAP); (from Kolter *et al.* 2005). SAPs posses the ability of extracting lipids from the membranes of vesicles of the endocytic system and loading them into the groove of CD1 molecules. The complexes are then directed to the plasma membrane for antigen presentation.

An additional molecule, microsomal triglyceride transfer protein (MTP), is also involved in CD1 lipidation and the exchange of lipids between vesicles of the endocytic system. Showing around 86% identity between mouse and human (Dougan *et al.* 2005), it is a highly conserved protein. It has a recognised role as a molecule specifically transferring ligands to suitable vesicles. Recently it has been suggested that it is involved in the transfer of self-lipids (especially phospholipids; Dougan *et al.* 2005) into the CD1d groove during CD1d assembly in the ER (Brozovic *et al.* 2004; Dougan *et al.* 2005). Chemical inhibition of MTP proved to influence CD1d-mediated presentation of both endogenous and exogenous ligands in various murine APCs (splenocytes, $CD11^+$ dendritic cells, BM-derived DCs). Moreover, silencing of gene expression affected CD1d function in human professional APCs (MDDCs) and APC–derived cell lines. In addition, the inhibition of this chaperone protein influenced the membrane expression of the CD1d molecule itself, probably due to reduced ER output (Dougan *et al.* 2005).

1.2.2.4. Expression and recognition of CD1 group I molecules

Group I CD1 molecules are expressed by immature cortical thymocytes and APCs of both lymphoid and non-lymphoid localization (such as lymphocytes and Langerhans cells respectively) (Liu 2001). These molecules are upregulated after activation and they are not constitutively expressed (reviewed in Porcelli and Modlin 1999). The effector cells that recognise them are T cells expressing αβ or γδ TCRs. An initial observation suggested recognition by DN (double negative) T cells, but later work confirmed that all T cells (e.g. both $CD4^+$ and $CD8^+$ cells) recognise antigens presented by group I CD1 molecules (Porcelli and Modlin 1999). These effector cells are characterised by a narrow spectrum of TCRs, consistent with the presentation of a limited range of antigens (Rhind 2001) and TCR activation leads to cytotoxity (reviewed in Sugita and Brenner 2000).

1.2.2.5. Expression of CD1e

As mentioned above, the CD1e molecule facilitates the process of antigen editing (Angenieux *et al.* 2000), in which it resembles the non-classical class II molecules, DM and DO. It is therefore abundant in the endocytic system, especially in late Golgi and endosomal compartments of dendritic cells (Angenieux *et al.* 2000) and probably other APCs.

1.3.1. Expression of the CD1d molecule

Human CD1d is expressed constitutively by various cells of the body, especially APCs of myeloid and lymphoid origin. Examples include monocytes, mature and immature dendritic cells, circulating B lymphocytes (Exley *et al.* 2000) and a subset of NKT cells (Hameg *et al.* 2000). Although the majority of resting T cells are $CD1d^-$, expression can be upregulated (Exley *et al.* 2000), as it is on macrophages (Skold *et al.* 2005). In the thymus the majority of cortical thymocytes express the molecule and this drives positive selection of CD1d-restricted cells (Bendelac 1995). In addition, the molecule can be found in abundance on mantle zone B cells, but it is lacking from germinal centres (Exley *et al.* 2000). Apart from bone marrow-derived cells, expression was also found in liver, gastrointestinal tract (Blumberg *et al.* 1991), larynx (Rees *et al.* 2008), skin, placenta (Boyson *et al.* 2002) and other tissues (reviewed in Porcelli and Modlin 1999). Surprisingly however, expression is not seen on Langerhans cells (Ronger-Savle *et al.* 2005).

1.3.2. CD1d structure

The structure of the CD1d molecule follows the same principles as group I CD1 molecules, with three α domains and a non-covalently associated β_2–microglobulin (Figure 1.7 A). The α_1 and α_2 helices are situated closer to each other than in class I molecules and cover the sheet of six anti-parallel β strands. As mentioned before association with β_2m is not an absolute requirement for plasma membrane expression, as non-complexed CD1d molecules have been observed on the cell surface (Balk *et al.* 1994; Kim *et al.* 1999; Rees *et al.* 2008). Compared to class I the antigen-binding hydrophobic groove of the CD1d molecule is much deeper and forms 2 long pockets/channels, called A' and F' pockets, the latter in humans is also referred to as C'. The channels diverge from each other as they extend deeper into the CD1d molecule and have closed ends. Thus, any ligand has to access the groove via a narrow opening on the top. The large volume of the CD1d molecule's groove, 1400 $Å^3$ and 1650 $Å^3$ for a loaded and empty groove respectively (Koch *et al.* 2005; Zajonc *et al.* 2005), and the

structure of the binding cavity, surrounded by hydrophobic residues, enables the accommodation of lipid ligands characterised by two alkyl chains.

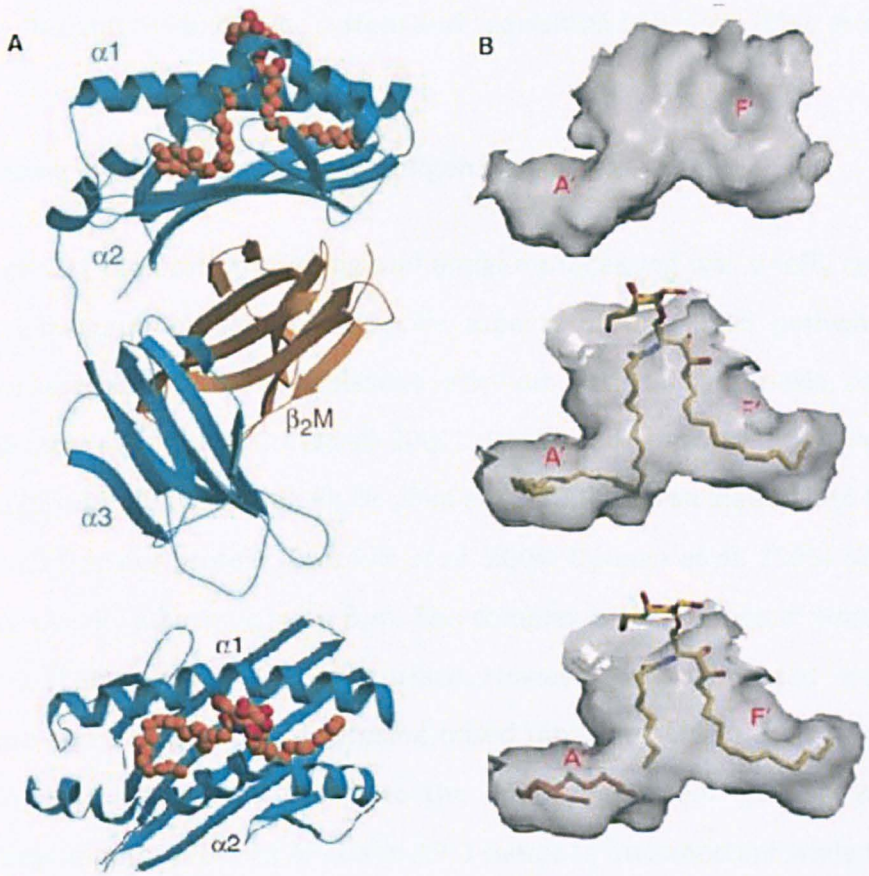


Figure 1.7 Crystal structure of CD1d molecule A) hCD1d accommodating αGalCer, from Koch *et al.* 2005, modified B) groove of mCD1 molecule; upper panel: empty molecule, middle panel: with αGalCer (yellow), lower panel: with PBS-25 (yellow) and a spacer (orange), from Zajonc *et al.* 2005, modified

The A' channel is longer and can accommodate acyl chains of up to 26 carbon atoms in humans (24 in mice), while the shallower F' pocket provides space for part of a ligand headgroup and a chain of up to 18 carbon atoms in both species (Koch *et al.* 2005; Wu *et al.* 2006). There is an additional small linear hydrophobic compound observed in the A' pocket of a murine CD1d molecule loaded with a ligand with a relatively short acyl chain (Zajonc *et al.* 2005) as shown in Figure 1.7 B, recently identified as a molecule of palmitic acid (Wu *et al.* 2006). This "spacer" most likely plays a role in stabilising the structure of the A' channel and prevents it from collapsing, thus maintaining the conformation of the whole molecule (Zajonc *et al.* 2005).

Apart from the extracellular part, CD1d also contains a transmembrane domain and a cytoplasmic chain. The former anchors the CD1d-ligand complex in a lipid raft of the plasma membrane. The latter contains a tyrosine-based motif which is essential for trafficking of this molecule through the endocytic system and acquisition of ligands (Chiu *et al.* 2002).

1.3.3. Trafficking of CD1d molecule and antigen processing

The subject of CD1 molecule trafficking and antigen processing was briefly commented on before; this paragraph outlines the specific aspects of the CD1d pathway. The CD1d molecules are assembled in the endoplasmic reticulum, assisted by calnexin, calreticulin and the ERp57 protein (Kang and Cresswell 2002). During this process a self ligand, a short phospholipid (phosphatidylinositol, PI; De Silva *et al.* 2002) is also loaded into the groove by the microsomal transfer protein (Brozovic *et al.* 2004; Dougan *et al.* 2005). Afterwards the molecule also usually associates with β_2m. The complex is then directed through the trans-Golgi network (TGN) towards the cell surface. However, it is expressed only for a short period of time and, facilitated by a tyrosine-based motif present in the cytoplasmic tail of the molecule, it is internalised back into the endocytic system (Chiu *et al.* 2002). The interaction with adapter proteins AP-2 and AP-3 seems to be important while the CD1d-self-lipid complex traffics into the appropriate vesicle (Cernadas *et al.* 2003; Elewaut *et al.* 2003; Godfrey and Berzins 2007). In addition, a fraction of CD1d molecules associates with class II/Ii chains which enhances the recycling process because the Ii chain contains a di-leucine trafficking motif (Chen *et al.* 2007, reviewed in Moody and Porcelli 2001). In the late endosomes/lysosomes, where ligands requiring processing by α-galactosidase A are digested (Prigozy *et al.* 2001), the low affinity ligand is replaced with a final antigen. The ligand editing is facilitated by the engagement of the CD1e molecule (de la Salle *et al.* 2005) and various saposins (Kang and Cresswell 2004). The primary importance of saposin B was shown (Yuan *et al.* 2007), but evidence was also presented that the efficiency of saposins depends on the substrate specificity (Zhou *et al.* 2004). Moreover, the role of a dimeric form of Niemann-Pick type C2 protein (NPC2) in ligand editing was also recently substantiated (Schrantz *et al.* 2007), while the Niemann-Pick type C1 protein (NPC1) apparently acts in trafficking of lipids between vesicles of the endocytic system (Sagiv *et al.* 2006). The

involvement of the endocytic system in the expression of the CD1d molecule-ligand complexes extends further to the formation of lipid rafts. These membrane microdomains or platforms contain important regulatory molecules and therefore play a role in providing a basis for various cellular functions, including the arrangement of immunological synapses (Simons and Ikonen 1997). It was demonstrated that CD1d molecules are found exclusively in lipid rafts (Park *et al.* 2005) and that the formation of lipid rafts is essential for CD1d-mediated presentation of antigen (Simons and Ikonen 1997; Park *et al.* 2005).

1.3.4. CD1d ligands

α–galactosylceramide (αGalCer, Figure 1.8) was the first ligand found to stimulate NKT cells when presented by APCs expressing the CD1d molecule (Kawano *et al.* 1997; Nieda *et al.* 1999). The compound was first derived from an Okinawan Sea sponge, *Agelas mauritianus*. Researchers from Kirin Brewery discovered the antitumor properties of the extract from the sponge and, through a series of proliferation experiments, proved that α-galactosylceramide is an active CD1d ligand (Nieda *et al.* 1999). The optimal lengths of both acyl and sphingosine chain of this ligand show a perfect fit in the CD1d pockets as illustrated by crystallization studies (Koch *et al.* 2005). The important feature of the αGalCer structure is an α-linkage between the ceramide and sugar moieties. This linkage is not normally present in mammals, which cannot synthesise this bond (reviewed in Brigl *et al.* 2003). Thus, αGalCer involvement in the process of CD1d-restricted NKT cell development is very unlikely. Furthermore, the glycoceramide structure is regarded by some researchers as a pathogen-associated molecular pattern (PAMP) (Zajonc *et al.* 2005; Mallevaey *et al.* 2009).

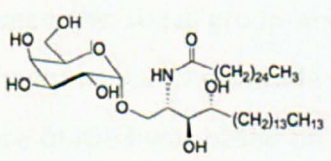


Figure 1.8 Structure of a potent, most commonly used CD1d ligand, α-galactosylceramide (αGalCer, KRN7000). (Taylor et al. 2003). For chemical structures of additional ligands see Appendix.

Moreover, αGalCer resembles other ligands for NKT cells, compounds such as monoglycosylceramides (Mattner *et al.* 2005), phosphatidylinositol mannoside (Fischer *et al.* 2004) and acidic glycosphingolipids (Nozaki *et al.* 2008) found in some bacterial and fungal species, which can be presented by CD1d. Therefore it has been speculated that originally obtained αGalCer was in fact isolated from bacteria colonising *A. mauritianus* rather than the sponges themselves (Zajonc *et al.* 2005). Irrespective of its origin, the compound still remains an important tool in NKT cell research and was included in phase I clinical trial in malignancies (Okai *et al.* 2002; Nieda *et al.* 2004).

In addition, a spectrum of endogenous cellular lipids (Park *et al.* 1998; Gumperz *et al.* 2000; De Silva *et al.* 2002; Salio *et al.* 2007) can be CD1d-presented, as even in assays where no external self-derived ligands are introduced (Gumperz *et al.* 2000; Salio *et al.* 2007) the proliferation of NKT cells can be observed. This illustrates the reactivity of CD1d-restricted cells towards self-derived ligands present in mammalian cells. Since the discovery of this feature of CD1d-restricted cells, a few endogenous ligands have been identified for mice and human CD1d i.e. glycosyl-/phosphatidylinositol (Joyce *et al.* 1998; De Silva *et al.* 2002), human melanoma cell line-derived GD3 (Wu *et al.* 2003), phosphatidylcholine (Giabbai *et al.* 2005) and sulfatide (Zajonc *et al.* 2005) with crystal structures solved in many cases.

However, it is the identification of a selecting thymic ligand which represents the biggest challenge. In 2004 Zhou *et al.* presented the work suggesting that isoglobotrihexosylceramide (iGb3) fulfils this role in the generation of CD1d-restricted T cells (Godfrey *et al.* 2004; Zhou *et al.* 2004). Following this, the synthesis process and compound's derivatives were also evaluated (Keusch *et al.* 2000; Xia *et al.* 2006; Xia *et al.* 2006; Yao *et al.* 2006; Xia *et al.* 2007). Comparing αGalCer with iGb3 the main difference is the presence of a β linkage between the sugar group and ceramide in the latter. As this compound can be synthesised by mammals, this molecule would fit the description of a self ligand. The claim on the importance of iGb3 was based on the reduced population of CD1d-restricted *i*NKT cells in mice suffering from a lysosomal storage disease due to the lack of an enzyme required in the iGb3 synthesis pathway (hexosaminidase B; HexB). However, soon it became clear that, although being expressed in some mammals (Keusch *et al.* 2000; Taylor *et al.* 2003; Milland *et al.* 2006; Speak *et al.* 2007), iGb3 cannot be detected with standard

methodology in human tissues (Speak *et al.* 2007). Moreover, the genes encoding the enzyme responsible for primary iGb3 generation, i.e. α-1,3 galactosyltransferase, (α (1,3)GT; (Taylor et al. 2003) and iGb3 synthase (iGb3S; Keusch *et al.* 2000) are both non-functional pseudogenes in higher primates (Galili *et al.* 1988; Milland *et al.* 2006). As a consequence, all these mammals produce anti-α-(1,3)Gal antibodies against the sugar epitope of iGb3 (Hamadeh *et al.* 1995; Sandrin *et al.* 1997; Duk *et al.* 2003) as an immunological response to lipopolysaccharides and polysaccharides from bacteria of the gastrointestinal tract (Galili *et al.* 1988). The importance of these antibodies is highlighted by the fact that the high titres observed in humans result in a strong barrier for pig-to-human xenotransplants (Milland *et al.* 2005; Milland *et al.* 2006). As an additional argument, Gadola *et al.* presented evidence that other lysosomal storage diseases, which cause disruption at the level of the endocytic system of the cell, result in a similar reduction of NKT cell frequencies (Gadola *et al.* 2006). In agreement with this is the observation of rising *i*NKT cell numbers in Gaucher storage disease patients treated with enzyme replacement therapy (Balreira *et al.* 2005). Moreover, Porubsky *et al.* showed that iGb3-deficient mice have perfectly normal NKT cell development (Stronge *et al.* 2007), while Brigl *et al.* suggest that non-*i*NKT cells do not require the compound for their selection (Brigl *et al.* 2006).

Finally, the use of IB4 lectin in blocking experiments by Zhou *et al.* must be interpreted with caution, as this lectin is rather promiscuous in terms of carbohydrate structure recognition (Sandrin *et al.* 1997; Duk *et al.* 2003). Therefore it is unlikely that iGb3 drives positive selection of NKT cells or peripheral activation of this cell population in humans (Speak *et al.* 2007), which was suggested (Fischer *et al.* 2004; Mattner *et al.* 2005; Niemeyer 2005). Thus, the identity of the endogenous ligand, capable of driving NKT cell selection in the thymus is still elusive.

In addition to the antigens mentioned above a broad range of synthetic compounds were generated to date, e.g. αGalCer C20:2, OCH, PBS-25, PBS-57, SGL-S23, thio-iGb3 (Zajonc *et al.* 2005; Lee *et al.* 2006; Liu *et al.* 2006; Xia *et al.* 2006; Forestier *et al.* 2007; Kaieda *et al.* 2007; Xia *et al.* 2007), derivatives of phosphoethanolamine (Rauch *et al.* 2003) to name a few. These glycolipids exhibiting various physical and biological properties, can be used for both activation and detection of NKT cells (Liu *et al.* 2006; Silk *et al.* 2008) and could

potentially be employed as therapeutic agents for CD1d-restricted T cell stimulation. Moreover, recent data showed that CD1d ligands may also be found amongst non-glycosidic ceramide families, as exemplified by threitolceramide; ThrCer; (Silk *et al.* 2008), long-chained hydrophobic peptides with aromatic rings (Castano *et al.* 1995) and even, surprisingly, amongst chainless benzofuran sulphates (Nozaki *et al.* 2008). The biological activity of such diverse compounds increases the range of possible chemical structures of CD1d antigens further, giving a scope to search for both natural ligands and pharmacologically active compounds for disease treatment. Chemical structures of a few selected ligands are shown at the back of this thesis (see Appendix).

Furthermore, a differential NKT cell response was seen to some CD1d ligands investigated to date (Beaudoin *et al.* 2002; Zajonc *et al.* 2005; Forestier *et al.* 2007). For example, OCH, which has a truncated sphingosine chain has an apparent ability to direct CD1d-restricted responses towards a Th2 profile, by stimulating IL-4 production (Lee *et al.* 2006). Although it provides a much weaker stimulus compared to aGalCer, it seems to act as a potent "selective activator". This has suggested the possible applicability of this compound in diseases with insufficient IL-4 production (Chiba *et al.* 2004), including RA.

The summated data on the outcome of CD1d-mediated presentation of various compounds suggested that the nature of a given ligand influences the response observed from CD1d-restricted cells. This observation led to the formulation of a theory that ligands with truncated chains are presented for a shorter time, leading to a secretory pattern dominated by IL-4. In contrast longer ligands, with a higher affinity for CD1d, characterised by longer stimulation times (Castano *et al.* 1995), cause a prolonged TCR stimulation time and induce IFN-γ production (Oki *et al.* 2004). Interestingly, similar cytokine bias was found a few years ago for peptide presentation by MHC class II molecules (Kumar *et al.* 1995). This, so called "structure–activity relationship" (SAR, Oki *et al.* 2004)) proposes a unique "cytokine fingerprint" for every ligand (Goff *et al.* 2004; Lee *et al.* 2006), which led to speculations that in the future constructing tailor-made compounds with desired characteristics for a given purpose will be possible (Gadola *et al.* 2006). However, this differential cytokine secretion pattern was recently questioned by McCarthy *et al.* (McCarthy *et al.* 2007).

1.4. CD1d-restricted cells

1.4.1. The discovery of CD1d-restricted NKT cells

The differences between CD1 groups I and II extend further than the specificity of ligand recognition. The other important feature is the cell population towards which the stimulation is targeted. While in the former group the cells which respond are classical αβ T cells, in the case of the latter this recognition of antigen presentation is mediated by a novel subset of lymphocytes. These cells were first found in mice, where T cells with highly skewed TCR chain usage (Vα14-Jα281, now known as Vα14-Jα18) were detected (Arase *et al.* 1992; Hayakawa *et al.* 1992; Dellabona *et al.* 1994; Lantz and Bendelac 1994). This suggested that the population in question recognises a largely conserved antigen species. At the same time, similar TCR bias was also discovered in a subset of human T cells, where a rearrangement between Vα24 and JαQ (now known as Vα24-Jα18) without random N-nucleotide additions was observed. In addition, limited Vβ chain usage (Porcelli *et al.* 1993), with the majority of cells expressing a Vβ11 chain (Porcelli *et al.* 1993), was initially identified. However, with time it appeared that the usage of both strictly defined Vα and Vβ chains is not a stringent requirement and that only the Vα24 chain is uniquely utilized within the invariant TCR (Porcelli *et al.* 1996), although it is still not necessary for the recognition of αGalCer (Gadola *et al.* 2002).

At the same time a population of T cells characterised by the co-expression of an NK cell marker, NK1.1 (NKRP1C or CD161c) was discovered in mice. Due to their mixed characteristics these cells were named "NK1.1$^+$ (like) T cells", "Natural T cells (NTs)" or "Natural Killer T cells" (NKTs) (Godfrey *et al.* 2004), of which the latter name is presently most widely used. Consecutive studies revealed that they belong predominantly to a DN subset in humans (Porcelli *et al.* 1993) and the CD4$^+$ subset in mice (Arase *et al.* 1992; Hayakawa *et al.* 1992; Arase *et al.* 1993; Lantz and Bendelac 1994) and are characterised by a potent cytokine secreting ability (Arase *et al.* 1993; Yoshimoto and Paul 1994; Yoshimoto *et al.* 1995; Bendelac *et al.* 1996; Sato *et al.* 1996). Moreover, a deficiency in this cell population was also identified in NOD mice, a model of autoimmune, insulin-dependent

diabetes (Gombert *et al.* 1996; Baxter *et al.* 1997; Godfrey *et al.* 1997), providing a hint about the regulatory properties of these cells.

The breakthrough happened in 1995 when Bendelac *et al.* demonstrated that the murine NK1.1$^+$ T cell population is CD1d-restricted (Bendelac *et al.* 1995). In 1997 Exley *et al.* reported that the two simultaneously described human cell populations represent the same subset of T cells (Exley *et al.* 1997). In addition, this study confirmed that these cells are similarly CD1d-reactive. Later that year Kawano *et al.* identified that a group of glycolipids, α-galactosylceramide (αGalCer) being the most potent, is presented to murine NKT lymphocytes (Kawano *et al.* 1997); two years later this was proved for human cells (Nieda *et al.* 1999). This discovery revealed for the first time that not only peptides can be presented for TCR-mediated recognition, but lipid-derived compounds can also be antigenic. Moreover, this novel pathway seemed to be conserved amongst the investigated mammalian species (Brossay *et al.* 1998; Kashiwase *et al.* 2003).

1.4.2. The definition and classification of CD1d-restricted NKT cells

The definition of NKT cells has evolved with time, leading to some confusion in interpreting data from previous studies. As mentioned above, initially this definition included the co-expression of the CD3 molecule and the NK cell marker (CD161 in humans and NK1.1 in mice). When the limited TCR chain usage was realised, the classification evolved and encompassed Vα24/Vβ11 chain expression. These so called "invariant NKT cells (*i*NKTs)" typically represent a maximum of 1% of PBLs in a healthy donor. Soon it appeared that not all *i*NKT cells co-express the NK cell marker CD161. Therefore a newer classification divided NKT cells into 3 types; type I: T cells expressing both invariant TCR (*i*TCR) and the CD161 molecule; type II: Vα24$^+$ cells without this NK marker and type III: CD161$^+$ cells, bearing non-invariant TCRs (MacDonald 2002). However, since the availability of soluble CD1d molecules and CD1d tetramers (Benlagha *et al.* 2000; Karadimitris *et al.* 2001; Gadola *et al.* 2003) it has become clear that other cells which express variable TCR receptors and respond to the CD1d-mediated presentation of ligands can be detected (Gumperz *et al.* 2000; Gadola *et al.*

2002; Van Rhijn *et al.* 2004). Consequently, the most recent classification divides the CD1d-restricted cell population into two/three subsets as illustrated in Table 1.3. Type I (classical NKT cells) represent *i*NKT cells and type II (non-classical NKT cells) comprising all other CD1d-restricted cells, which bear variable TCRs (Chiu *et al.* 1999; Gadola *et al.* 2002; Stenstrom *et al.* 2004; Brigl *et al.* 2006; Russano *et al.* 2007; Renukaradhya *et al.* 2008). TCR sequencing demonstrated that these latter cells can express one of several α chains other than Vα24 and one of numerous non-Vβ11 β chains (Gadola *et al.* 2002). Importantly, it is crucial to acknowledge that CD1d-restricted γδ T cells were also recently identified (Spada *et al.* 2000; Cui *et al.* 2005; Russano *et al.* 2006; Russano *et al.* 2007) and these are included in the non-*i*NKT cell subset.

Table 1.3. Recent classification of NKT and NKT-like cells

	Invariant (Type I/classical NKT cells)	Non-invariant (Type II/non-classical NKT cells)	NKT-like cells
CD1d dependence	Yes	Yes	No
αGalCer-reactivity	Yes	Yes/No	No
TCRα chain	Vα14-Jα18 (mice) Vα24-Jα18 (humans)	Diverse	Diverse
TCRβ chain	V8.2, Vβ7, Vβ2 (mice) Vβ11 (humans)	Diverse	Diverse
NK1.1/CD161	+ (resting mature) -/low (immature or post-activation)	+/-	+
Subsets	$CD4^+$ and DN (mice) $CD4^+$, DN, $CD8\alpha\alpha^+/\alpha\beta$ (humans)	$CD4^+$ and DN (mice) $CD4^+$ and $CD8\alpha\beta$ (humans)	$CD4^+$, DN, $CD8^+$
IL-4/IFNγ	+/+	+/+	-/+
CD69 expression	high	+/-	+/-

The generally recognised subsets are the invariant and non-invariant populations, while NKT-like cells are only included into classification for the reason of phenotypic characteristics and some functional features, excluding recognition of CD1d-mediated presentation of lipid antigens, based on Godfrey *et al.* 2004, Gadola *et al.* 2002, Gumperz *et al* 2002 and Castano *et al.* 1995.

Apart from the two subpopulations mentioned, reverting to a previous nomenclature, the third type, "NKT-like cells" is sometimes also recognised. These cells do not respond to

CD1d-mediated presentation; the main similarity between them and *i*NKT/non-*i*NKT cell subsets is the presence of TCRs and NK cell markers. This population includes a novel subset of lymphocytes, mucosal-associated invariant T cells (MAIT). These cells are restricted by yet another member of MHC class Ib, the MR1 molecule (Huang *et al.* 2005). Moreover, MAITs also express a limited TCR spectrum, i.e. Vα7.2-Jα33 paired with Vβ13 or Vβ2 in humans and Vα19-Jα33 paired with Vβ6 or Vβ8 in mice (Martin *et al.* 2009).

Furthermore, MR1-restricted cells also seem to respond to glycolipids as recently shown (Okamoto *et al.* 2005; Schumann and De Libero 2007) and that these responses include rapid secretion of IL-4, IL-5, IL-10 and IFN-γ (Kawachi *et al.* 2006). Although there are clear phenotypical and functional similarities to CD1d-restricted cells, the developmental pathways seem to be diverse for these two populations (Martin *et al.* 2009). In addition, the frequencies of MAIT cells observed in human and murine peripheral blood are different from those of NKT cells. Typically, these cells constitute up to at most 4% of the lymphocytes while murine studies showed much smaller numbers (Martin *et al.* 2009).

1.4.3. NKT cell phenotype

Although early publications suggested that NKT cells (defined as invariant TCR^+ or $NK1.1^+$ T cells) are exclusively DN in humans (Porcelli *et al.* 1993) and $CD4^+$ in mice (Arase *et al.* 1992; Hayakawa *et al.* 1992; Arase *et al.* 1993; Fong *et al.* 1994; Lantz and Bendelac 1994), more recent studies identified other subpopulations as well. Presently it is recognised that while murine *i*NKT cells have CD4/DN phenotypes (Godfrey *et al.* 2004), human *i*NKT cells can be found amongst $CD4^+$, DN or $CD8\alpha\alpha/\alpha\beta^+$ lymphocytes (Takahashi *et al.* 2002; Ho *et al.* 2004); the latter two subsets are often analysed together as $CD4^-$ cells. In terms of cytokine secretion, the $CD4^+$ *i*NKT subpopulation was shown to be capable of IL-4/IFN-γ production, therefore representing a Th0 secretion profile. In contrast, the $CD4^-$ subset is characterised by the selective release of IFN-γ and hence it represents a Th1 secretory pattern (Kim *et al.* 2002; Takahashi *et al.* 2002). Moreover, varying functional properties of these subsets may be explained by the differential expression of certain proteins (Lin *et al.* 2006). For example,

$CD4^-$ *i*NKT cells seem to express perforin, 2B4, CD94 and CD161 markers as well as NKG2A/NKG2D receptors, which result in Natural Killer-like cytotoxicity (Kim *et al.* 2002; Lee *et al.* 2002; Takahashi *et al.* 2002; Lin *et al.* 2006). On the other hand, KIR receptors CD158a/b are not expressed by any of the subsets, while CD56 seems to be abundant on both CD4 subpopulations (Lee *et al.* 2002). In addition, other receptors which may be important for *i*NKT cell biology include CD45RO, CD45RB and CD69. All these molecules seem to be widely expressed by both subsets of fresh and activated *i*NKTs (Lee *et al.* 2002; Ikarashi *et al.* 2005) and is interpreted as an activated/memory phenotype of these cells.

Not surprisingly, some of the findings presented above were also reproduced for human CD1d-restricted NKT cell subsets (defined as CD1d tetramer-αGalCer$^+$ cells; *i*NKT cells and non-*i*NKT cells), which similarly comprise $CD4^+$ and $CD4^-$ (DN, $CD8\alpha\alpha/\alpha\beta^+$) subsets (Gumperz *et al.* 2002). In addition, it seems that both cytokine expression (Gadola *et al.* 2002; Gumperz *et al.* 2002) and perforin production (Gumperz *et al.* 2002) are distributed between the subpopulations in a similar fashion as for the *i*NKT cells. The CD8 population is again relatively small, so often described as a fraction of the DN subset. However, an unusually high proportion of these $CD8^+$ CD1d-reactive cells was found in one study (Castano *et al.* 1995). A striking phenotypical difference between non-invariant CD1d-restricted cells from *i*NKT cells is the relative lack of CD161 and CD69 expression by the former (Gadola *et al.* 2002; Gumperz *et al.* 2002), see table 1.3.

Gadola *et al.* recently demonstrated that non-*i*NKT cells are never found amongst DN or $CD8\alpha\alpha^+$ lymphocytes (Gadola *et al.* 2002). In addition, these cells seem to differ in terms of functional distribution between subsets, e.g. both $CD4^+$ and $CD4^-$ subsets of $V\alpha24^-$ have cytotoxic capacity (Gadola *et al.* 2002). These non-invariant cells are also largely devoid of CD161 expression and their TCRs may show different affinity towards the same ligands when compared to the *i*NKT cells. Collectively these observations point at the possibility that, although similar to the invariant counterparts at certain aspects, non-*i*NKT cells represent a functionally separate population. Consistently with this, opposite roles of these two subsets have been recently demonstrated in lymphoma (Renukaradhya *et al.* 2008) and autoimmune hepatitis (Halder *et al.* 2007), which will be presented later in detail.

The differential cytokine production by different NKT cell subsets is an important finding. It gives some clues to how these cells are able to regulate a variety of physiological and pathological processes, i.e. through selective recruitment of a given subset to the inflammation site and controlling the secondary responses locally (Kim *et al.* 2002; Kim *et al.* 2002). In agreement with this, it seems that the subpopulations also vary in terms of chemokine receptor expression. While the majority of NKTs (both invariant and non-invariant CD1d-restricted cells) express a non-lymphoid, effector homing pattern, i.e. CCR2, CCR5, CXCR3 and CXCR4 abundance, there are subtle differences between the CD4 subpopulations (Gumperz *et al.* 2002; Kim *et al.* 2002; Kim *et al.* 2002; Lee *et al.* 2002). For example, $CD4^+$ cells of the *i*NKT subset express more CCR4 and therefore are more likely traffic to Th2 type inflammatory sites, while $CD4^-$ cells express more CCR1, CCR6 and CXCR6, indicating preferential recruitment to sites of Th1 inflammation (Kim *et al.* 2002; Kim *et al.* 2002; Lee *et al.* 2002; Olson and Ley 2002). Moreover, accessory/adhesion molecules are also distributed in varying patterns, e.g. α_1 $integrin^+$ cells are found exclusively within the $CD4^+$ $V\alpha24^+$ subset, but α_L integrin (LFA-1) is abundant on the $CD4^-$ subpopulation. While the former directs the cells to collagen/laminin-rich tissues, the latter seems to enhance adhesion to endothelium (Kim *et al.* 2002). In contrast, however, Lin *et al.* did not find differences in the status of ICAM adhesion molecules (Lin *et al.* 2006), which seem essential for the formation of immunological synapses by *i*NKT cells (McCarthy *et al.* 2007).

1.4.4. NKT cell development

Despite recent suggestions that iGb3 is required for thymic selection in mice (Zhou *et al.* 2004), this compound is unlikely to be the endogenous ligand which rules the selection of any of the NKT subsets, as argued in Paragraph 1.3.4. However, although the natural selecting ligand is still unknown, some of the particulars of the NKT development process have been clarified based on *i*NKT cell research in rodents and these will be briefly described next.

The development of NKT cells is thymus dependent (Tilloy *et al.* 1999). The first detectable cell in the NKT lineage is the $CD4^+CD8^+$ lymphocyte, which is also identified in the classical T cell development pathway as the common precursor ($CD4^+CD8^+$ DP thymocyte) (Figure 1.9). At this stage, cells highly express the CD24 molecule (Heat Stable Antigen; HSA). These NKT precursors subsequently undergo random rearrangement of their TCR chains, leading to expression of *i*TCRs. There are two proposed sequences of events around the developmental stage of TCR rearrangement, described as precommitment and instructional models. While the former suggest that early NKT precursors are predetermined for the rearrangement resulting in *i*TCR expression, the latter proposes that the expression of this receptor "instructs" the cell to acquire unique properties of NKT cells (reviewed in Kronenberg 2005).

After the rearrangement a process of positive selection by $CD1d^+$ DP cortical thymocytes takes place (Bendelac 1995). This is considerably different from that of classical T cells, which are selected by thymic cortical epithelial cells (Jenkinson *et al.* 1980). In addition, it seems that the selecting ligand must be acquired by the molecule in the endocytic system, as mice with various lipid storage diseases causing its disruption lack *i*NKT cells (Gadola *et al.* 2006). This argues that low-affinity ER-acquired compounds cannot fulfil this role.

Having encountered the CD1d-mediated presentation, the cells then lose the majority of CD8 expression and function as $CD4^+$, $CD69^{high}$ thymocytes, which gradually downregulate CD24 and undergo three consecutive stages of the maturation process. These are: the "naïve" thymocyte stage ($CD44^{low}NK1.1^{neg}$), a memory stage ($CD44^{high}NK1.1^{neg}$), and the, so called, NK stage ($CD44^{high}NK1.1^{pos}$) (Bendelac *et al.* 2007). At these respective time points in the development of NKT precursors represent differential cytokine secretory abilities, progressing from exclusive IL-4 producers, through a Th0 phenotype to cells which secrete more IFNγ than IL-4 (Arase *et al.* 1992; Hayakawa *et al.* 1992; Arase *et al.* 1993; Bendelac *et al.* 2007). The cells also undergo a series of proliferation post naïve stage and generate a $CD4^+$ and a DN population (Bendelac *et al.* 2007; Godfrey and Berzins 2007).

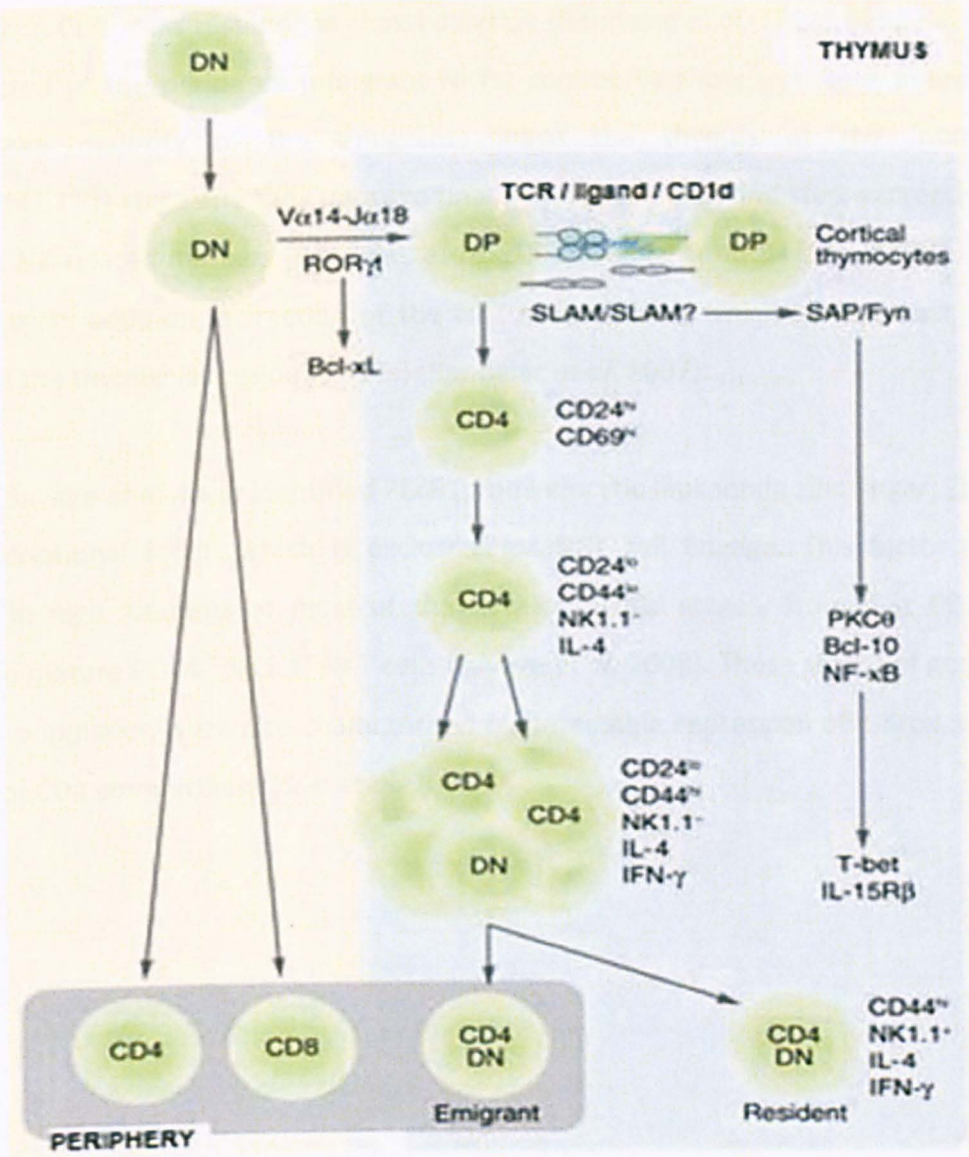


Figure 1.9 NKT cell thymic development, from Bendelac *et al.* 2007, modified

*i*NKT cells are a positively selected by cortical epithelial cells in the thymus and subsequently undergo phenotypical and functional changes known as the "naïve" thymocyte stage ($CD44^{low}NK1.1^{neg}$), memory ($CD44^{high}NK1.1^{neg}$) and NK ($CD44^{high}NK1.1^{pos}$) stages of their development. At the latter stage the majority of the cells emigrate from the thymus and only a fraction remains as resident thymocytes.

There is also a hint of a negative selection step mediated by thymic stromal cells, as the presence of αGalCer (high affinity ligand) blocks the development of the cells in this lineage (Pellicci *et al.* 2003). Moreover, CD8 molecule expression also seems to influence the fate of NKT precursors, as all murine (but not human) $CD8^+$ cells are deleted during thymic development, which has been explained by the excessive affinity of the TCR-CD1d

interaction in $CD8^+$ cells resulting in clonal deletion (Bendelac *et al.* 1994). While in mice the cells directed to the periphery (emigrant NKTs) commonly show a mature phenotype, in humans the majority of the emigrants leave the thymus at the intermediate ($CD44^{high}NK1.1^{neg}$) stage and they undergo final maturation steps and start expressing NK1.1 and other NK-related markers (Bendelac *et al.* 2007; Godfrey and Berzins 2007) outside of the thymus. In addition, a fraction of the NKT cells in mice which survive past this step remains in the thymus (as resident NKTs) (Bendelac *et al.* 2007).

Recently, Savage *et al.* have identified PLZF (promyelocytic leukaemia zinc finger, Zbtb16) as the transcriptional factor which is exclusive for NKT cell lineage. This factor could be detected in high amounts at most of the developmental stages, from first $CD24^{lo}$ cells through to mature $CD44^{hi}$ $NK1.1^+$ NKT cells (Savage *et al.* 2008). These stages of general NKT precursor population were also characterised by detectable expression of c-Krox, which is a hallmark of CD4 commitment (Sun *et al.* 2005).

1.5.1. Ligand binding

Many research groups have suggested that functional responses from CD1d-restricted cells are affected by the nature of the antigen loaded onto the CD1d molecule. It was substantiated for the murine molecule where unsaturation and cis-isomerisation of the alkyl chains of a given ligand improves the process of loading into the groove and the stability of the complex (Rauch *et al.* 2003). Although one group reported that the half life of CD1d-αGalCer complex is less than 1min (Rauch *et al.* 2003), other groups provided evidence of much higher stability (Benlagha *et al.* 2000; Cantu *et al.* 2003). Recently McCarthy *et al.* elegantly demonstrated that both acyl and sphingosine chains influence the half life of the hCD1d-ligand complex. In addition, the study presents a model which shows that although modification of an acyl chain in the A' pocket does not interfere with the recognition of a formed complex, the truncation of the sphingosine chain results in partial collapse of the CD1d grove. This in turn alters the 3D structure of the surface detected by the TCR, affecting the recognition of the ligand and the activation of NKT cells *in* vitro (McCarthy *et al.* 2007). Therefore the effect of hCD1d-ligand presentation is affected by the ligand's polar headgroup (directly) and the sphingosine chain (indirectly)(McCarthy *et al.* 2007; Stronge *et al.* 2007). Moreover, the authors showed that the functional outcome i.e. immunological synapse formation, polarisation of cytotoxic granules and overall efficiency of CD1d-dependent lysis, are all affected by changes within the ligand structure (McCarthy *et al.* 2007).

The crystal structure of the CD1d-ligand complex have been solved for several antigens (Rauch *et al.* 2003; Giabbai *et al.* 2005; Koch *et al.* 2005; Zajonc *et al.* 2005; Zajonc *et al.* 2005; Wu *et al.* 2006) and therefore some general principles which influence loading can be identified. As already described, the CD1d molecule is suited to present antigens characterised by two alkyl chains and a headgroup. While being loaded into the groove of the CD1d molecule the acyl chain is inserted in a counter clockwise manner (Giabbai *et al.* 2005; Koch *et al.* 2005; Zajonc *et al.* 2005) as it follows the curvature of the A' pocket. The other chain (e.g. sphingosine in the case of αGalCer) inserts into the F' channel in a relatively

straight line towards the pole of the binding groove. The hydrophobic moiety of a ligand forms the part of the antigen that protrudes between the CD1d α helices for TCR recognition. The localisation of the groove channels supports a firm orientation of this headgroup towards the recognition surface (Koch *et al.* 2005; Zajonc *et al.* 2005), which seems to be crucial for the interaction with the TCR as the lack of a rigid position affects a ligand's potency (Mannami *et al.* 1989; Giabbai *et al.* 2005; Wu *et al.* 2006).

It also seems that the structure of the pockets is flexible and can adapt to a given ligand, adjusting the position of residues forming the groove in both humans and mice (Koch *et al.* 2005; Zajonc *et al.* 2005). Moreover, this structural alteration is reflected in the overall configuration of the CD1d molecule. When empty, the channels partially collapse. This causes a conformational shift within the molecule, which opens up the entrance of the channels. Therefore the molecule adapts an "open" structure, characterised by a more accessible binding groove. However, when containing a ligand, this conformation changes into a "closed" one which does not favour ligand loading (Koch *et al.* 2005; Zajonc *et al.* 2005; McCarthy *et al.* 2007). Importantly, therefore, not only the conformation of the channels is flexible, but the surface of CD1d alters following the groove adjustment to the ligand. This model of conformational changes propagated from within the groove to the TCR surface recognition (Koch *et al.* 2005; McCarthy *et al.* 2007) suggests a large spectrum of potential ligands that CD1d is capable of binding.

For human CD1d-mediated antigen presentation hydrogen bonds were found to form links between parts of αGalCer and some residues of the CD1d groove (Figure 1.10). These anchor the sugar headgroup (2'-OH) to Asp151, the glycosidic α-linkage to Thr154 and the sphingosine chain (3'-OH) to Asp80, while the acyl chain is bound to CD1d only via van der Waals' interactions (Giabbai *et al.* 2005). As a consequence, the sugar moiety protrudes from the groove between the α_1 and α_2 helices, which exposes the headgroup for the TCR recognition (Koch *et al.* 2005), Figure 1.10. These findings are therefore complementary to the data which showed the importance of a headgroup (Naidenko *et al.* 1999; Fischer *et al.* 2004) in TCR recognition.

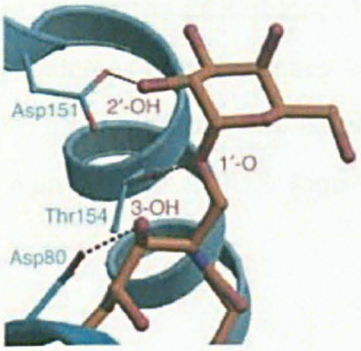


Figure 1.10 Hydrogen bonds linking αGalCer (orange) to hCD1d molecule (blue); from Koch *et al.* 2005. Hydrogen bonds are shown: the sugar headgroup is linked to Asp151, the glycosidic α-linkage to Thr154 and the sphingosine chain to Asp80 of hCD1d molecule.

1.5.2. TCR recognition of the CD1d-ligand complex

The predominance of certain α and β TCR chains (Vα24 and Vβ11 in humans, Vα14 and predominantly Vβ8.2, Vβ7 or Vβ2 in mice) was recognised early in the history of NKT cell research, suggesting that both these chains are crucial for antigen recognition. However, in 1996 Porcelli *et al.* identified some donors in whom other Vβs were paired with the Vα24 chain (Porcelli *et al.* 1996), implying that Vβ11 may not be as important as previously thought. Moreover, from that study it appeared that even if a Vβ11 chain was the exclusive Vβ chain expressed by NKT cells in a given donor, it was not *invariant*. Instead, multiple Vβ11 CDR lengths were identified (Porcelli *et al.* 1996) giving rise to the term "semi-*invariant* TCR". On the other hand, Vα24$^-$ cells recognising αGalCer, which predominantly express a Vβ11 chain were recently identified (Gadola *et al.* 2002; Brigl *et al.* 2006). Many other non-invariant CD1d cell populations, varying in Vα and Vβ chain usage and ligand specificity, have also been detected since (Chiu *et al.* 1999; Godfrey *et al.* 2004; Stenstrom *et al.* 2004; Renukaradhya *et al.* 2008).

The study of Gadola *et al.* solved the crystal structures of three hCD1d-αGalCer-specific T cell receptor (Gadola *et al.* 2006) complexes. The results indicate that the CDR3α, CDR3β and CDR1β loops are involved in the recognition of the headgroup moiety of αGalCer (Figure 1.11 A), while the CDR1α and CDR2α loops interact with the α_1 and α_2 helices of CD1d molecule, respectively. An additional point of contact, formed by a protrusion of Arg79 from

the α_1 helix of the CD1d molecule towards the CDR2β loop, has also been identified (Gadola *et al.* 2006). Moreover, the study indicates that the space between the CDR3α and β loops of the TCR is where the headgroup of the CD1d ligand is recognised, and suggests that the positively charged Lys32 side chain of the CDR1β loop plays a crucial role in ligand recognition.

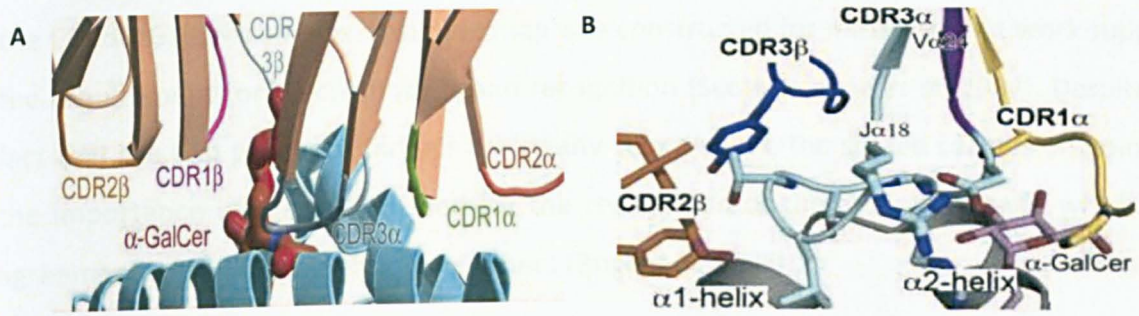


Figure 1.11 Existing models of TCR/CD1d-αGalCer interaction A) diagonal B) perpendicular; figures modified, from Gadola *et al.* 2006 and Wun *et al.* 2008, respectively. The former model assumes similar position of the approaching to CD1d molecule TCR as in the case of classical MHC class I presentation, while the latter proposes an utterly different picture of the interaction.

The authors propose that this binding cavity is characterised by a certain degree of flexibility allowing for the recognition of various ligands and imply that a Vα24 chain is not an absolute requirement for the recognition of antigens presented via CD1d. This notion is also supported by SPR studies that detected similar affinities of TCRs expressed by invariant and non-invariant clones (Gadola *et al.* 2006). The authors propose a docking model for these three TCRs that resembles the recognition of peptide presented in the MHC class I/II context (Garboczi *et al.* 1996; Garcia *et al.* 1996), where the TCR approaches the complex centrally and is positioned diagonally over the recognition surface.

However, in 2007 another group proposed a strikingly different model (Borg *et al.* 2007; Wun *et al.* 2008) in a study based on extensive mutagenesis experiments. The results suggested a very different picture of TCR binding to a hCD1d-αGalCer complex, i.e. an involvement of CDR1α exclusively in the recognition of the ligand's headgroup, CDR3α in

antigen recognition and contact with CD1d helices and CDR2β/CDR3β providing interaction with the CD1d molecule (α_1 and α_2 helices, respectively) as depicted in Figure 1.11 B (Wun *et al.* 2008). The alanine substitutions described in this study enabled a detailed map of critical residues in the centre of the recognition surface (Wun *et al.* 2008). The proposed configuration suggests a displacement of the TCR from the mid-point of the binding groove, centred over the F channel, with the TCR α chain providing most of the interaction footprint (Borg *et al.* 2007). The authors assume an unusual perpendicular position of the TCR over the CD1d-αGalCer complex. A similar map was constructed for mice and that work supports the requirement for Vβ chain for ligand recognition (Scott-Browne *et al.* 2007). Despite the fact that the two proposed models are clearly very distinct, the shared conclusion points to the importance of the CDR3α loop for the recognition of the antigenic ligand which is in agreement with the observations of others (Brigl *et al.* 2006).

However, recent data revealed that the relative frequency of *i*NKT cell clones expressing various Vβ chains in mice *i*NKT positively correlates with the avidity of the TCRs (containing these various chains) to the CD1d-αGalCer tetramer (Mallevaey *et al.* 2009). In addition, when responses of hybridomas differing in Vβ chain expression were compared, it was clear that TCR β chain usage influenced the recognition of various compounds (Mallevaey *et al.* 2009). These results argue that the expression of a given β chain, especially the residues within the CDR2β loop, might affect *i*NKT cell selection and ligand specificity and is therefore important *in vivo* (Mallevaey *et al.* 2009).

1.5.3. Co-receptors and co-stimulatory molecules

The activation of conventional T cells recognising peptide antigens requires more than the interaction between the MHC class I or class II/peptide complex and the TCR/CD3 complex. Firstly, the CD4 and CD8 molecules expressed on the T cell subsets make contact with a non-peptide binding area of the class II and class I molecules respectively. This links the molecules on the side and increases overall avidity of the bond. However, there are also

exceptions to this co-receptor requirement as it was shown that at least some CTL subsets can recognise their target peptide in a CD8-independent way (Cerundolo *et al.* 1991).

To effectively activate a T cell additional interactions are required. These co-stimulatory molecules provide "the second signal". In contrast to the TCR recognition, this is nonspecific and does not depend on an antigen. Classically, the interaction between CD28 expressed on a T cell and CD80/CD86 (B7-1/B7-2), abundant on antigen presenting cells, is required. This system also provides a negative feedback mechanism via the upregulation of an inhibitory molecule CTLA-4 (Cytotoxic T Lymphocyte Antigen-4; CD152) by recently activated T cells. Similarly to CD28, CTLA-4 also binds CD80/CD86 molecules, but the affinity of this bond is higher than that of the CD28-CD80/86 interaction. However, this complex does not result in the transduction of an activating signal towards the T cell, resulting in the eventual inhibition of T cell function.

Recognition of a glycolipid ligand presented via CD1d follows the same principles, such as the dependence on antigen presenting molecules and signalling via the TCR/CD3 complex as well as the involvement of co-receptors and co-stimulation. Similarly to classical T cells, the involvement of the CD4 or CD8 molecules is recognised. Both of these seem to be important in NKT cell physiology; CD8 was shown to determine the developmental fate of NKT cell precursors in mice (Bendelac *et al.* 1994), while CD4 expression influences the outcome of the stimulation (Chen *et al.* 2007; Thedrez *et al.* 2007). However, it is also clear that the expression of these TCR co-receptors is not an absolute requirement, which is reflected in the relative abundance of a DN subset.

From early studies NK1.1 (CD161) seemed to be the obvious candidate as a mediator of the second signal in *i*NKT cells (Exley *et al.* 1998). Exley *et al.* showed that this molecule co-stimulates *i*NKT cell proliferation and cytokine secretion, and differences in the secretory patterns and stimulatory potential of CD161/NK1.1 were found for murine and human cells. In mice crosslinking this molecule alone was sufficient to activate NKT cells. However ligand binding by CD161 had no influence on CD1d-dependent cellular cytotoxicity (Exley *et al.* 1998). Apart from CD161, the authors also investigated the role of CD28 in NKT cell activation, but did not observe any substantial effect of the latter (Exley *et al.* 1998). In

contrast, other studies found that both a secretory (Kaneda *et al.* 2005) and a proliferatory (Uldrich *et al.* 2005) response to αGalCer stimulation in mice depended on signalling via CD28. Moreover, the latter study illustrated that CD40/CD40L interaction has similar effects (Uldrich *et al.* 2005). On the other hand, secretion of IL-12p70 by APCs was increased when anti-CD40 antibodies were used to co-stimulate dendritic cells (Yue *et al.* 2005), indicating that the cytokine *milieu* can be potentially influenced by this axis *in vivo*, where other cells are present. The importance of CD40/CD40L co-stimulation was also identified by Kitamura *et al.* (Kitamura *et al.* 1999).

Other molecules which were proposed to provide co-stimulatory signals for NKT cells include ICOS, GITR, OX40 and 4-1BB and their ligands. ICOS, a member of the CD28 family, which interacts with B7-related protein-1 (B7RP-1) was shown to co-operate with the CD28 axis for cytokine production and cytotoxic activity (Kaneda *et al.* 2005). Glucocorticoid-induced TNF receptor (GITR) is also capable of enhancing cytokine secretion, upregulating activation markers and influencing NKT cell proliferation by inducing nuclear translocation of certain transcription factors (Kim *et al.* 2006). The role of OX40 as an important NKT cell co-stimulant was recently suggested in antitumor responses (Zaini *et al.* 2007), while 4-1BB (CD137), a member of the TNF family, was identified as a factor aggravating airway hyperresponsiveness (AHR) and lung inflammation in murine studies (Kim *et al.* 2008).

1.5.4. TCR signal transduction in NKT cells

Little is known about the signalling pathways naturally occurring in NKT cells, with the majority of information generated in the context of the lineage development. It is assumed that these cells adopt at least a part of the TCR signalling pathway that can be observed in classical T cells (Figure 1.12). Therefore, the association of the CD3 complex (γ, δ, ε and ζ CD3 chains) containing ITAM (immunoreceptor tyrosine-based activation motif) sequences with the TCR is observed. The phosphorylation of ITAMs after TCR engagement is mediated by two protein tyrosine kinases, Lck and Fyn. The former is associated with CD4/CD8 co-receptors, hence it is probably functional in the appropriate CD1d-restricted cell subsets, and activates the ZAP-70 (zeta-associated protein of 70kDa) protein complex. The Fyn

kinase, on the other hand, seems to be linked to signalling through the SLAM receptor family, mediated through SAP (SLAM-associated protein), which proved to be crucial for CD1d-restricted NKT cells, as SAP knock-out animals had numerical and functional deficiencies in these cells (Chung *et al.* 2005).

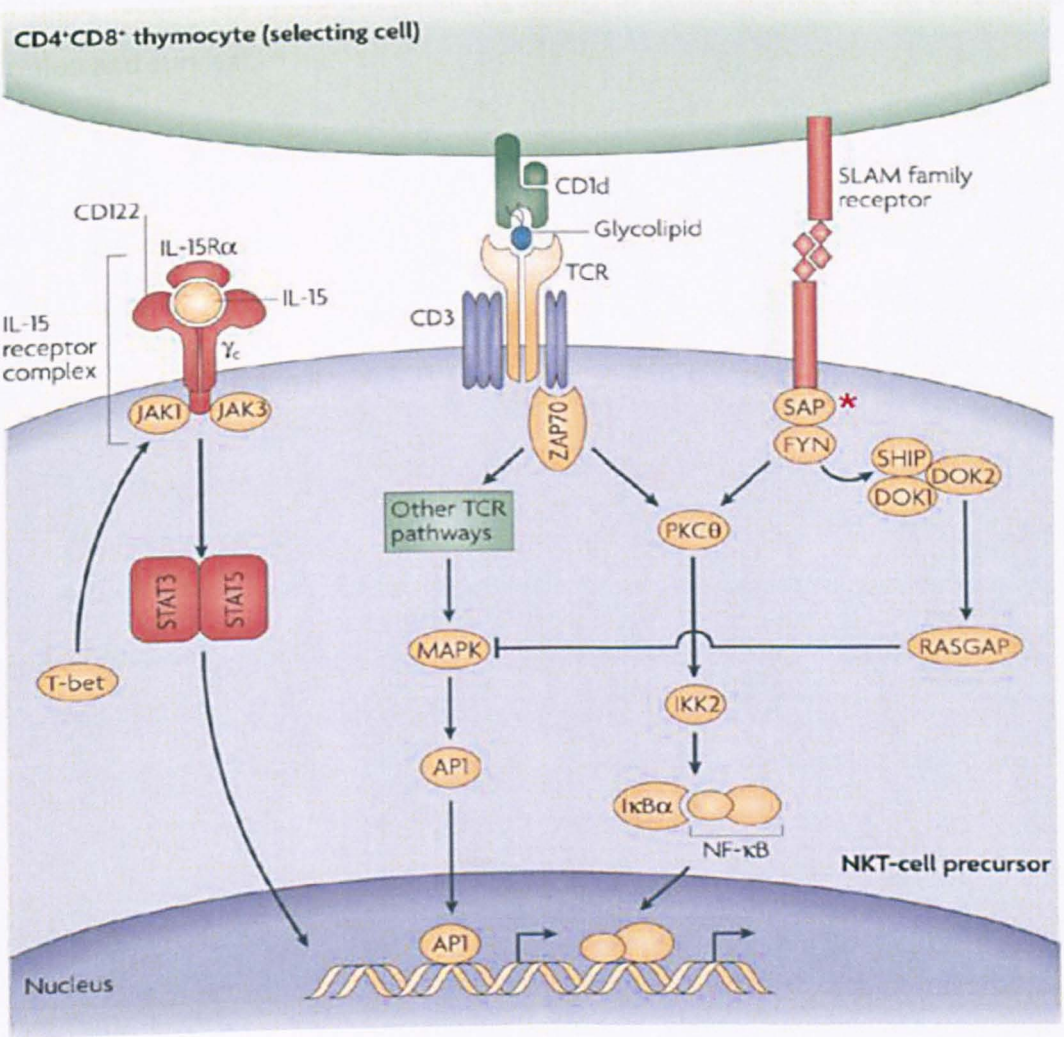


Figure 1.12 Intracellular pathways in NKT cell development; from Godfrey and Berzins 2007; modified
The main signalling pathway is similar to the classical T cell signalling associated with CD3 complex chains and ZAP-7 protein, leading to the activation of cell differentiation and survival genes through activation of MAPK and PKC and generation of important nuclear factors: AP1 and NFκB. * denotes SAP recently investigated in relation to NKT cells; Lck not depicted.

Both pathways mentioned above similarly activate protein kinase Cθ, leading to the activation of nuclear factor κB (NFκB). However, they also act antagonistically, as ZAP-70 leads to the activation of mitogen-activated protein kinase (MAPK), while SLAM/SAP involvement inhibits it, providing a negative feedback loop (Godfrey and Berzins 2007). The outcome of activation of these two pathways is reflected by the generation of transcription factors AP-1 and NFκB, respectively, which results in the initiation of DNA transcription. Both of these factors are multifunctional regulators of genes related to cell differentiation, proliferation and survival.

1.5.5. Tools to visualise NKT cells and CD1d presentation of a ligand

As outlined above, the population of CD1d-restricted cells is characterised by a skewed TCR usage, with preferential expression of Vα24 and Vβ11 chains in humans and Vα14 and Vβ8.2, Vβ7 or Vβ2 in mice. This fact was utilised in early NKT cell experiments for the purposes of identifying this cell population. However, this approach is obviously not applicable for the detection of non-*i*NKT cells, characterised by a variable TCR. Following the development of MHC class I tetramers, new soluble CD1d molecule-based reagents: CD1d fusion protein, CD1d dimers and tetramers, were engineered. While the former reagent is generated in mammalian cell line (CHO) (Gumperz *et al.* 2000), three main methods are used for the dimers and tetramers, i.e. oxidative refolding chromatography (Karadimitris *et al.* 2001), detergent-assisted refolding (Gadola *et al.* 2003) and generation in insect cells (Benlagha *et al.* 2000). The great advantage of these reagents is that they can be loaded with various ligands, increasing the flexibility of experimental design. Moreover, the multivalent nature of these reagents increases sensitivity of the detection, enhancing the binding of the molecules to CD1d-reactive cells. The availability of these new reagents also enabled the discovery that CD1d-restricted NKT cells can react to more than just one antigen. The limitation is however, that cell-derived ligands occupying the CD1d groove can hamper the process of loading the experimentally introduced ligands and have an impact on the specificity of NKT cell detection (Stronge *et al.* 2007). Despite this, multiple publications have shown successful adaptation of these new tools (Joyce *et al.* 1998; Benlagha *et al.* 2000; Gadola *et al.* 2002). These reagents have been utilised in a variety of techniques, including flow cytometry (Gumperz *et al.* 2002), magnetic sorting (Wu *et al.* 2003) or antigen-specific stimulation assays (Fischer *et al.* 2004).

In addition to soluble CD1d-based reagents, antibodies specific for hCD1d-αGalCer complexes (Yu *et al.* 2007) and soluble invariant TCR molecules (McCarthy *et al.* 2007) were also generated. The former were employed to detect APCs capable of presentation to CD1d-restricted NKT cells. The latter allowed for the description of the binding kinetics of *i*TCRs to CD1d complexes containing various antigens.

1.6.1. Direct effect on CD1d-restricted NKT cells

Having encountered a specific antigen stimulation, the CD1d-restricted cell population becomes activated and responds. This can be observed as proliferation, cytotoxic killing or cytokine secretion. If the activation is incomplete, the cells can also become anergic. The proliferation of *i*NKT cells or of CD1d-responsive clones can be detected by staining with anti-Vα24 antibodies and CD1d tetramers loaded with a desired ligand and is crucial for efficient functioning of this small lymphocyte population. The expansion phase was observed *in vitro* (Kawano *et al.* 1997; Nieda *et al.* 1999; Wilson *et al.* 2003) and *in vivo*, both in murine and human studies (Okai *et al.* 2002; Ikarashi *et al.* 2005). In the majority of *in vitro* experiments αGalCer was used together with exogenous cytokines (especially IL-2, IL-7, IL-12, IL-15 or IL-18) in order to obtain sufficient cell numbers for further manipulations, as these cytokines can increase the yields several-fold. However, it was recently confirmed that the addition of cytokines can differentially alter the expansion, by promoting proliferation of certain NKT subsets more than the others as well as influencing their secretory and cytotoxic capacity (Lin *et al.* 2004).

The expansion phase is characterised by a transient downregulation of T cell receptors and CD161 (NK1.1) on the surface of NKT cells (Wilson *et al.* 2003; Ikarashi *et al.* 2005) which was the reason for the erroneous assumption in the past that these cells undergo clonal deletion (as reviewed in Van Kaer 2004). It is now clear that NKT cells remain viable and may increase in numbers (Wilson *et al.* 2003). However, the timing of antigen exposure has an effect on NKT cell activation. In mice repeated exposure to αGalCer over a short period of time (3 days) enhances the response, while priming with the same ligand a week before re-stimulation severely decreases the secretory outcome (Ikarashi *et al.* 2005). Similar results were obtained by others in both rodent and human studies (Parekh *et al.* 2005; Uldrich *et al.* 2005) suggesting that antigen exposure can lead to the anergy of these cells if NKT cells are exposed to antigen and re-stimulated after a longer period of time.

Apart from the common expression of some cell surface markers NKT and NK cells also share the ability to act as cytotoxic effector cells. Cytotoxicity involves the release of granules containing perforin (Kawano *et al.* 1998; Kawano *et al.* 1999; Metelitsa *et al.* 2003) and granzyme B, as well as FasL, TNFα and TRAIL pathways (Metelitsa *et al.* 2003). Moreover, subsets of CD1d-responsive cells seem to differ in their cytotoxicity, with more potent activity observed in $CD4^-$ cells, due to their substantial production of perforin (Gumperz *et al.* 2002). NKT cells seem to recognise CD1d-expressing cancer cells (Kawano *et al.* 1999; Metelitsa *et al.* 2001; Haraguchi *et al.* 2006; Renukaradhya *et al.* 2008), although some researchers argue that CD1d expression is not required for efficient killing (Kawano *et al.* 1998). In addition, various groups presented somewhat conflicting results about the identity of the recognised target cell lines (Kawano *et al.* 1999; Metelitsa *et al.* 2001; Moody and Porcelli 2001; Chamoto *et al.* 2004). Although one initial study suggested that several cell lines were susceptible to NKT-mediated lysis, e.g. K562, Daudi, HeLa, etc. (Kawano *et al.* 1999), other results contradict these findings and argue that NK cell contamination could have affected the earlier experiments (Metelitsa *et al.* 2001).

The most prominent feature of NKT cells, however, is their potent secretory ability, recognised in early studies (Hayakawa *et al.* 1992; Arase *et al.* 1993; Yoshimoto *et al.* 1995; Bendelac *et al.* 1996; Sato *et al.* 1996). Multiple publications have shown that this cell population is able to produce Th1, Th2 and Th17 cytokines (Yoshiga *et al.* 2008 and reviewed in Godfrey and Kronenberg, 2004), both in combination or separately. Although CD1d-restricted NKT cells can clearly secrete a variety of active compounds (Gadola *et al.* 2002; Wu *et al.* 2003; Lin *et al.* 2006) the majority of studies investigating CD1d-mediated NKT cell activation focuses on the secretion of only two cytokines, IFN-γ and IL-4. NKTs release large amounts of these cytokines rapidly (Yoshimoto and Paul 1994), facilitated by the acetylation of histones associated with the promoter regions of these transcripts (Stetson *et al.* 2003).

The precise mechanism which governs the secretory outcome in particular circumstances (infection, cancer, autoimmunity, etc.) remains unclear, although there are multiple suggestions and hypotheses. As mentioned before (Paragraph 1.3.4.) the structure-activity-

relationship of the presented ligands or the predominance of a certain NKT subset, may influence Th1 or Th2 cytokine release patterns.

1.6.2. Effect of NKT cell activation on the ongoing immune response

IFN-γ and IL-4 are able to polarise the following adaptive immune responses in a given direction. Therefore NKT cells recruited at the early (innate) stages of an inflammatory process can potentially influence the course of the resulting immune response via the secretion of these cytokines. The early recruitment, the recognition of a conserved ligand/group of ligands and the immunoregulatory role places NKT cells between innate and adaptive responses (Niemeyer 2005; Van Kaer and Joyce 2005; Nagarajan and Kronenberg 2007; Mallevaey *et al.* 2009). Moreover, non-specific activation of these cells has been shown to boost immunity and increase the response of other cell populations (Metelitsa *et al.* 2001; Hermans *et al.* 2007; Nagarajan and Kronenberg 2007) leading to the use of αGalCer as an adjuvant.

Probably the most prominent effect of NKT cells is the result of their cross-talk with dendritic cells. The importance of IL-12 for NKT function has been widely demonstrated (Cui *et al.* 1997; Kitamura *et al.* 1999; Shin *et al.* 2001; Yue *et al.* 2005). The data suggest the existence of a positive feedback loop between dendritic cells and NKT cells, commencing with the CD40/CD40L interaction between these cells (Hermans *et al.* 2003; Yue *et al.* 2005). This results in the expression of IL-12 receptors (IL-12R) on NKT cells and enhanced secretion of the cytokine by dendritic cells (Kitamura *et al.* 1999). Moreover, αGalCer-stimulated *i*NKT cells seem to "condition" dendritic cells for efficient activation of $CD4^+$ and $CD8^+$ T cells resulting in potentiated responses to peptide antigens (Fujii *et al.* 2003; Hermans *et al.* 2003). Moreover, αGalCer stimulation of NKT cells augments both $CD8^+$ T cell-mediated immunity and humoral responses when co-administered with Toll-like receptor (TLRs) ligands (Hermans *et al.* 2007).

B cells are also recipients of NKT cell help, as shown by Galli *et al.* (Galli *et al.* 2003) who identified *i*NKT cells as efficient promoters of proliferation of both memory and naive B

lymphocytes *in vitro*, affecting immunoglobulin production. This enhancement can also be observed without the addition of exogenous CD1d ligands, suggesting the recognition of an endogenous ligand(s) presented by CD1d on B cells (Galli *et al.* 2003). Moreover, recent data illustrate that this help extends further and involves augmentation of specific antibody responses directed towards particulate ligands internalised via BCR uptake (Barral *et al.* 2008).

In addition, communication between NKT and NK cells has also been described. An early study by Carnaud *et al.* demonstrated that NKT cell stimulation led to a rapid, IFN-γ-dependent activation of NK cells. Moreover, it seems that this interaction initiated a network of consecutive activation events, as subsequently both B cells and $CD8^+$ T cells also showed upregulated CD69 expression (Castano *et al.* 1995). Other studies also found an enhancement of NK cytolytic function, where the data indicated that NKT-secreted IL-2 provided the activating signal (Castano *et al.* 1995; Metelitsa *et al.* 2001).

In contrast to the examples given above, where NKT cells enhance immune responses, it seems that this lymphocyte population is also involved in suppressing the generation of $CD4^+$ effector T cells (Beaudoin *et al.* 2002), effectively controlling potentially autoaggressive T cell clones. Recently it was identified that the $CD8\alpha\alpha^+$ *i*NKT subset possesses cytotoxic properties which control expansion of activated T cells (Ho *et al.* 2004). This immunoregulatory role was first observed in a murine model of type 1 diabetes, which will be described later.

CD1d-restricted lymphocytes appear to interrelate with other regulatory cells. The foremost example is the reciprocal interaction with the $CD4^+CD25^+$ T_{reg} population (La Cava *et al.* 2006). These cells are characterised by an anergic phenotype and modulate immune responses mainly through suppression of effector T cells. It was demonstrated that *i*NKT-derived secretion of IL-2 activates these cells, which in turn proliferate, retaining their functional (suppressive) properties (Jiang *et al.* 2005). Reciprocally, it was shown that T_{regs} inhibit both proliferation and cytokine secretion by *i*NKTs in a cell-cell contact-dependent manner (Azuma *et al.* 2003), providing negative feedback for this CD1d-restricted population.

In addition, NKT cells control another population which have a regulatory function, namely myeloid-derived suppressor cells (MDSCs). This heterogeneous population comprises cells of myeloid origin and includes myeloid progenitor cells and various immature myeloid-derived cells (macrophages, granulocytes and dendritic cells), characterised by activated, granulocytic or monocytic phenotype. Functionally they are potent direct suppressors of T-cells which is mediated by an action of reactive oxygen and nitrogen species and arginase 1. Indirectly, they were also shown to promote T_{regs} (Gabrilovich and Nagaraj 2009). The interaction between NKT cells and MDSCs was recently demonstrated in influenza A virus infection (IAV). This study identified a CD40-dependent mechanism in which *i*NKT cells are able to control MDSC-mediated suppression of virus-specific $CD8^+$ T cell responses (De Santo *et al.* 2008). In agreement with this, a study on NKT cells in lymphoma recognised that $J\alpha18^{-/-}$ mice have elevated numbers of these myeloid-derived suppressor cells which is correlated with poorer anti-tumour response (Renukaradhya *et al.* 2008). According to the authors, the increase in the MDSC population is responsible for the inhibition of antitumor responses and promoting tumour growth seen in these animals.

1.6.3. CD1d-independent activation

Apart from TCR-mediated activation CD1d-restricted NKT cells can also be triggered via other mechanisms. In 2006 Kim *et al.* documented that engagement of one of the Fc receptors, FcγIIIR (CD16), also provides an activating signal to $NK1.1^+TCR\beta^+$ cells in mice (Kim *et al.* 2006), with the majority of these cells being αGalCer-responsive. This study demonstrated that NKT cells constitutively express the CD16 receptor that detects aggregated IgG antibodies. This interaction results in TCR-independent cell activation as judged by CD69 and CD25 upregulation and cytokine secretion. This activation sequence could not be reproduced with non-complexed antibodies, suggesting a requirement for cross-linking of CD16 receptors (Kim *et al.* 2006). The authors of the study also examined antibody-induced arthritis in a serum transfer model. The data showed that adoptive transfer of NKT cells from $FcγIIIR^{-/-}$ animals could not restore joint inflammation in $CD1d^{-/-}$ mice (Kim *et al.* 2006). In contrast, when $FcγIIIR^{+/+}$ animals were used as a source of NKT cells, the arthritis developed as normal, which required a IL-4/IFNγ-induced decrease of

TGF-β secretion (Kim *et al.* 2006). The authors concluded that FcγIIIR engagement on NKT cells is the primary mechanism involved in the initiation of the disease in this model.

Moreover, three separate groups reported that TCR-independent NKT cell activation can also be achieved by the use of a combination of specific cytokines, i.e. IL-12 and IL-18 (Uldrich *et al.* 2005; Nagarajan and Kronenberg 2007) or IL-12, IL-18 and IL-15 (Salio *et al.* 2007). These cytokines can be secreted by a number of cells, especially by activated APCs, which argues for the role of these cells in a positive feedback loop in boosting the immune response. The cytokine-dependent stimulation of NKT cells seems to result in proliferation (Uldrich *et al.* 2005; Nagarajan and Kronenberg 2007) and the produced cells predominantly secrete IFN-γ (Nagarajan and Kronenberg 2007). In addition, another group successfully used IL-2 alone to drive the expansion of CD1d tetramer-αGalCer$^+$ cells (Ikarashi *et al.* 2005), showing that other cytokines can also trigger this population. These results indicate that a TCR-mediated "first signal" is not obligatory to activate CD1d-restricted NKT cells, which is especially important in conditions resulting in a chronic inflammatory state, such as RA, where levels of various cytokines are altered both in the peripheral blood and at the site of inflammation.

1.7.1. NKT cells in cancer

The recognition of the importance of CD1d-restricted cells in tumour immunity extends to the beginning of NKT cell research, when scientists from the Kirin Brewery discovered anti-tumour properties of agelasphins, glycosphingolipids extracted from *Agelas mauritianus* (Natori *et al.* 1997). Following this, reports demonstrated the NKT-mediated prevention of melanoma, erythroleukemia and lung carcinoma metastases by αGalCer injection in wild type (WT), but not in Jα18$^{-/-}$ mice (Kawano *et al.* 1998), suggesting that *i*NKT cell activation is translated into functional outcome. Although challenging *i*NKT cells with various tumour cell lines *in vitro* showed some inconsistency in the outcome as mentioned before, the experiments in the *in vivo* model clearly indicate that CD1d-restricted cells play a role in controlling the growth and metastasis formation of various tumours (Cui *et al.* 1997; Shin *et al.* 2001).

Recently, a CD1d^{+} human melanoma cell line was shown to express an abundance of GD3 ganglioside, which is cross-presented to murine CD1d-restricted CD3^{+}NK1.1^{+} cells and induces proliferation and cytokine secretion (Wu *et al.* 2003). Moreover, several studies highlighted the importance of IL-12 in activating these cells for killing (Cui *et al.* 1997; Shin *et al.* 2001). In addition, as mentioned above, NKT cells also contribute to the control of neoplastic cells indirectly, by enhancing NK cell activity through IL-2 production (Metelitsa *et al.* 2001). As a consequence, it was proposed that the indirect regulatory/stimulatory effect of NKT cells is more important in anti-tumour immunity than their direct cytotoxic activity (Chamoto *et al.* 2004).

Importantly, recently published work suggested that the invariant and non-invariant subsets of CD1d-restricted cells have different roles in tumour immunity (Renukaradhya *et al.* 2008). This study showed that *i*NKT cells protected from the development of a B cell lymphoma, as Jα18$^{-/-}$ mice had an increased morbidity compared with CD1d knock-outs and the adoptive transfer of sorted *i*NKT population protected the Jα18$^{-/-}$ animals. This protection was probably mediated via the reduction of the population of myeloid-derived suppressor cells

(MDSCs). On the other hand, the authors suggest that the non-invariant cells prevent effector populations, such as NK cells or tumour-specific CTLs, from killing (Renukaradhya *et al.* 2008), hence their suppressive influence in this model.

1.7.2. Role of CD1d-restricted cells in infection

The role of NKT cell activation in various infections was also recognised (Brigl and Brenner 2004). It seems that bacterial wall components, such as LPS, can activate NKT cells either directly or indirectly. Direct NKT cell activation is induced by the presentation of antigens such as glycosylceramides, which provide rigidity to cellular walls of Gram (+) bacteria (such as Ehrlichia and Sphingomonas; (Mattner *et al.* 2005), resulting in secretion of both IFNγ and IL-4 (Mattner *et al.* 2005; Van Kaer and Joyce 2005; Nagarajan and Kronenberg 2007). In contrast compounds derived from Gram (-) Salmonella, and Mycobacteriae skew cytokine secretion towards a Th1 pattern (Brigl *et al.* 2003; Fischer *et al.* 2004). The indirect pathway includes the activation of APCs by LPS, which results in the presentation of endogenous ligands (Salio *et al.* 2007). This latter pathway elicited predominantly IFNγ production in all experiments (Mattner *et al.* 2005; Van Kaer and Joyce 2005; Nagarajan and Kronenberg 2007; Salio *et al.* 2007). In addition, APC derived IL-12 plays a role in enhancing the activation status of NKT cells to combat infectious agents (Brigl *et al.* 2003).

There is little data published for human bacterial or mycobacterial infections. Reports suggested the decrease of *i*NKT cell population in patients with pulmonary tuberculosis (Barcelos *et al*, 2006; Snyder-Cappione *et al*, 2007), suggesting the role of this population in the disease.

The course of animal viral infections, for example Herpes simplex virus, Influenza A and HIV-1, is also influenced by CD1d-mediated presentation (Raftery *et al.* 2006; De Santo *et al.* 2008; Li and Xu 2008). In general, NKT cell activation seems to be protective, as $CD1d^{-/-}$ animals develop more severe disease, probably due to a decrease in the frequency of virus-specific T cells, which are promoted by NKT cells (reviewed in Brigl and Brenner 2004).

Moreover, the virus-dependent disruption of CD1d presentation (Raftery *et al.* 2006; Li and Xu 2008) or the stimulation of myeloid-derived suppressor cells (MDSCs) block the anti-viral response (De Santo *et al.* 2008).

In addition, changes in human NKT cell function have also been reported in viral infections. Especially HIV-1 infection has been widely investigated and a decrease in NKT cell frequencies and Th1 responses were demonstrated (Snyder-Cappione et al, 2009 and reviewed in Li and Xu, 2008). Similar results, indicating a positive role of NKT cells in the course of viral infections, were also obtained for other viral pathogens, such as human T-lymphotropic virus type 1 (HTLV-1, Azakami et al, 2009) and influenza A (IAV, De Santo *et al,* 2008).

Finally, the course of inflammation in parasite infection also involves NKT cells (reviewed in Brigl and Brenner 2004). For example, NKT cells seem to effectively kill immature dendritic cells infected with Leishmania infantum in human *in vitro* studies (Campos-Martin *et al,* 2006). A differential response was observed in *Schistosomiasis* in $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mouse strains (Mallevaey *et al.* 2007). The former, lacking both invariant and non-invariant CD1d-restricted NKT cells, secreted dramatically less IL-4 after infection, while IFN-γ levels remained unchanged. In sharp contrast, $J\alpha18^{-/-}$ mice, devoid of *i*NKTs only, exhibited the opposite pattern, severely decreased IFN-γ and maintained IL-4 secretion. Functionally these two mice strains differed in the involvement of humoral responses and extent of fibrosis in the liver. The study suggested that while *i*NKT cells played a role in clearing the parasites through the involvement of Th1 cells, the non-invariant subset of CD1d-restricted cells, by promoting Th2 pathways resulted in a reduced parasite clearance in $J\alpha18^{-/-}$ animals (Mallevaey *et al.* 2007).

In addition, the findings of Duthie *et al.* in *Trypanosoma cruzi* infection also imply a negative role for the non-*i*NKT cell subset. The data from this study show that these cells, by supporting the inflammation, cause excessive infection-induced damage, resulting in increased morbidity and mortality of the infected animals (Duthie *et al.* 2005).

1.7.3. NKT cells in tolerance, allergy and asthma

There are a number of situations where NKT cells seem to function as regulators, preventing excessive inflammation that would result in deleterious consequences for the organism, such as allergic reactions, asthma and immunotolerance. For example, *i*NKT cells have a role in the placenta, which can be regarded as a semi-allogeneic graft. These cells were found to accumulate at the maternal-foetal interface in humans, most likely being responsible for suppressing immune responses towards the foetus (Boyson *et al.* 2002). However, a repeated NKT activation (by means of αGalCer exposure over time) inevitably leads to abortion in pregnant mice (Ito *et al.* 2000).

The majority of studies on NKT cell role in transplantation suggest that these cells participate in eliciting tolerance towards an allograft (as reviewed in Jukes *et al.* 2007). For example, activation of NKT cells with αGalCer increased the survival of allogeneic islet graft in mice (Yang *et al.* 2007). The observed modulation of anti-graft response in this model was mediated via the TGF-β pathway. Interestingly, the cytokine was not secreted, but transiently upregulated on the cell membrane. Consequently, the effective protection from graft rejection was mediated through cell-cell contact as demonstrated by transwell experiments (Yang *et al.* 2007).

Similarly, in atopic dermatitis (AD), *i*NKT cells were found to be markedly reduced in the peripheral blood of patients (Takahashi *et al.* 2003; Ilhan *et al.* 2007), suggesting a protective role of this cell population in humans. The $CD4^+/CD4^-$ *i*NKT subset balance seemed to be disturbed, with $CD4^+$ cells dominating in the patients (Takahashi *et al.* 2003). This altered subset contribution is believed to partly explain the Th2 deviation observed in atopic dermatitis.

In contrast, a report denying NKT cell involvement in allergic skin inflammation in murine model has also been published (Elkhal *et al.* 2006). Surprisingly, the authors found the importance of this cell population in promoting Th2-dependent airway inflammation in these mice (Elkhal *et al.* 2006). This latter result seems to be in agreement with other studies, where NKT cells appear pro-inflammatory via the overproduction of IL-4 and IL-13

(as reviewed in Oki and Miyake 2007). Two independent groups have shown that adoptive transfer of sorted *i*NKT cells into Jα18$^{-/-}$ mice restores airway hyperreactivity (AHR), which is normally impaired in these animals (Akbari *et al.* 2003; Lisbonne *et al.* 2003). The deleterious impact *i*NKT cells have on the course of asthma has been especially linked to their IL-4 production (Oki and Miyake 2007).

Furthermore, it has been shown that in both CD1d$^{-/-}$ and Jα18$^{-/-}$ mice contact sensitivity (CS) fails to develop (Campos *et al.* 2003) suggesting a proinflammatory role for the NKT cell population.

1.7.4. NKT cells and autoimmunity

An initial clue regarding the involvement of NKT cells in autoimmune processes was provided in 1993, in a study by Takeda *et al.* in a lupus-prone C57BL/6 *lpr* mouse model (Takeda and Dennert 1993). The results illustrated the inverse correlation between the prevalence of NK1^{+} cells – a population which also included CD3-expressing cells - in this murine model and the development of autoimmunity, suggesting that these cells disappeared from the animals before disease onset. Based on measurements of autoantibody production and allograft rejection in these mice, the authors proposed a suppressive or immunoregulatory function of these cells (Takeda and Dennert 1993).

A further confirmation of the role of NKT cells in prevention of autoimmunity came with the identification of numerical and functional defects in this population in a non-obese diabetic (NOD) mouse strain. These mice are susceptible to the inflammatory disease which resembles type 1 diabetes observed in humans. The animals develop insulitis spontaneously at a relatively early age, often by 10 weeks from birth, which is caused by the autoimmune T cell-mediated inflammation which destroys insulin-producing β cells in pancreatic islets. This results in a severe insulin deficiency which prevents efficient glycaemic homeostasis.

In 1996 Gombert *et al.* investigated the NOD mouse model and identified a decrease in the population of NK1.1$^+$ thymocytes, apparent already at 3 weeks of age (Gombert *et al.* 1996). This deficiency was also accompanied by a functional defect in IL-4 secretion. Following this, all thymic development stages of the lineage were also investigated and the decrease in DN NK1.1$^+$ cells was confirmed (Godfrey *et al.* 1997). Later, experiments involving adoptive transfer of NKT-enriched thymocytes were carried out (Baxter *et al.* 1997), which revealed that this population has the ability to prevent the development of diabetes if administered before the onset of the disease (Baxter *et al.* 1997), indicating the immunoregulatory role of these cells.

Consequently, other animal models of human autoimmune conditions were investigated (reviewed in Van Kaer 2004) and similar findings were published for lupus (SLE) (Yang *et al.* 2007), autoimmune hepatitis (Takeda *et al.* 2000), experimental autoimmune encephalomyelitis (EAE) (Singh *et al.* 2001; Mars *et al.* 2002) and others. A proposed mechanism for the involvement of this cell population in the prevention of these diseases is the suppression of autoreactive CD4$^+$ T cell clones (Beaudoin *et al.* 2002), which CD1d-restricted cells seem to control by the secretion of IL-4 (Mendiratta *et al.* 1997; Araki *et al.* 2003; Yang *et al.* 2007) or direct killing (Ho *et al.* 2004).

The findings in rodents were followed by parallel observations in humans. Especially, the first human study which investigated type 1 diabetes in discordant twins showed the importance of maintaining the IL-4/IFN-γ cytokine balance by CD4$^-$CD8$^-$ *i*NKT populations in the prevention of disease progression (Wilson *et al.* 1998). Since then more studies presented results which advocated similar defects in *i*NKT populations in other human autoimmune conditions, such as multiple sclerosis (MS,(Araki *et al.* 2003), Sjögren's syndrome(Kojo *et al.* 2001; van der Vliet *et al.* 2001), systemic sclerosis (Kojo *et al.* 2001; van der Vliet *et al.* 2001), psoriasis (van der Vliet *et al.* 2001), and Crohn's disease (van der Vliet *et al.* 2001). Moreover, CD1d-mediated activation seemed to have a therapeutic potential in these autoimmune disorders (Brossay *et al.* 1998; Hong *et al.* 2001; Sharif *et al.* 2001; Singh *et al.* 2001). Taken together, the evidence pointed at the crucial protective role of the CD1d–*i*NKT cell axis in a broad range of autoimmune diseases.

However, recently contradictory data on *i*NKT cells in diabetes was also published. A study of a Japanese diabetic population reported higher frequencies of $V\alpha24^{+}V11\beta^{+}$ *i*NKT cells in patients with type 1 diabetes compared to both healthy donors or diabetic patients (suffering from type 2 diabetes or latent autoimmune diabetes) (Oikawa *et al.* 2002). Finally, the study by Lee *et al.*, which also included discordant twins, failed to detect numerical or functional differences in CD1d-tetramer^{+} $V\alpha24^{+}$ cell populations in IDDM patients (Lee *et al.* 2002). These latter reports contradict the importance of $V\alpha24^{+}$ cells in the disease and warrant readdressing the question on the role of this population.

Importantly, to date there are no studies which report the prevalence and functional activity of non-invariant CD1d-restricted cells in human autoimmunity. Interestingly however, a recent study in murine EAE identified a sulphatide-reactive population of CD1d-restricted T cells. These accumulate in the central nervous system (CNS) of the animals in the MOG_{35-55} peptide-induced model (Jahng *et al.* 2004). Although chemically sulphatide is a sulphonated galactosylceramide, hence very similar to αGalCer, the cells studied did not respond to the presentation of the latter compound. This can be partially explained by the fact that the invariant Vα14 chain is not utilized by these cells and the expression of only the Vβ8 chain was shared with *i*NKT cells (Jahng *et al.* 2004). Importantly, the treatment of mice with sulphatide significantly improved the outcome of the disease by inhibiting MOG_{35-55}-reactive T cell clones (Jahng *et al.* 2004). Unfortunately, the authors did not investigate the mechanism for this inhibition. However, it is likely to be cytokine driven, as the sulphatide-reactive population reverses its secretory profile from Th1 to Th2 in a recall response to this compound (Jahng *et al.* 2004).

In addition, a recent study on NKT cell involvement in concanavalin A-induced (ConA) hepatitis also identified a beneficial effect of the stimulation of sulfatide-reactive CD1d-restricted cells (Halder *et al.* 2007). The authors concluded that while *i*NKT cells participate in inducing liver damage in the ConA-induced model of liver disease, non-*i*NKT cells seem to protect the mice. Sulfatide-activated cells interacted with plasmacytoid DCs which, in turn, released IL-12 and MIP-2 that recruited *i*NKT cells to the inflammation site. However, these

incoming cells appeared anergic, which was attributed to the presence of IL-12 (Halder *et al.* 2007).

While these two papers indicate the importance of non-*i*NKT cells in autoimmune processes, the existence of a similar CD1d-restricted non-αGalCer reactive NKT cell population in human autoimmunity remains to be demonstrated.

1.7.5. The CD1d-NKT axis in RA

In murine models of inflammatory arthritis the supposed role of NKT cells depend on the model used. NKT cells appear protective in both collagen (Ohnishi *et al.* 2005; Coppieters *et al.* 2007) and *Chlamydia trachomatis*-induced (CtIA) arthritis models (Bharhani *et al.* 2009). Moreover, in the former model, treatment with OCH, a αGalCer analogue with a truncated sphingosine chain, was found to be beneficial, presumably due to induced selective Th2-type cytokine secretion (Chiba *et al.* 2004). In addition, IL-10 was also shown to participate in αGalCer-induced protection in these mice (Miellot *et al.* 2005).

Similarly, αGalCer treatment in the CtIA reactive arthritis model, where inflammation is induced by intra-articular injection of *Chlamydia trachomatis*, reduces both the histological scores and pathogen load leading to alleviated symptoms of the disease (Bharhani *et al.* 2009). The protection seems to be mediated by an increase in IFN-γ, IL-4 and IL-10 secretion and downregulation of macrophage inflammatory protein-2 (MIP-2; CXCL1) and IFN-gamma-inducible protein-10 (IP-10; CXCL10). The authors of the study conclude that these responses enable efficient clearance of *Chlamydia* and reduce inflammation in the joints.

Surprisingly however, activation of CD1d-restricted cells with the same ligand in the antibody-induced (serum transfer) model of arthritis resulted in a decrease in TGFβ secretion which caused a deterioration of arthritis in the animals (Kim *et al.* 2005). In addition, a TCR-independent model of NKT cell activation in antibody-induced arthritis was also proposed by the same group (Kim *et al.* 2006), as mentioned in the previous chapter.

Numerical and functional differences in *i*NKT cells were also described in humans with RA. In 1999 Maeda *et al.* observed decreased numbers of DN *i*NKT cells in the synovial tissue of RA sufferers (Maeda *et al.* 1999). The same group also investigated the numbers and function of *i*NKT cells in the peripheral blood in several autoimmune diseases and found that, apart from a reduction in the initial frequencies of *i*NKT cells in these patients, the functional outcome (proliferation) was also diminished or even completely abolished in the majority of the affected individuals (Kojo *et al.* 2001). Later that year another study found that a spectrum of autoimmune diseases characterised by tissue damage (including RA, systemic lupus erythematosus and systemic sclerosis) are characterised by a similar reduction in peripheral blood *i*NKT cell frequencies (van der Vliet *et al.* 2001). These results were reproduced by Linsen *et al.*, who illustrated that the functional *i*NKT cell defect in RA is limited to the circulation as cells isolated from synovial fluid were functional as judged by both proliferation and cytokine secretory capacity (Linsen *et al.* 2005).

All of these studies were carried out before the discovery of the non-*i*NKT cells. As demonstrated earlier in this chapter the functional role of these non-invariant cells appears to oppose the action of *i*NKT cells in the studied animal models (Halder *et al.* 2007; Renukaradhya *et al.* 2008). In addition, an interaction between the two NKT cell subsets has also been proposed (Halder *et al.* 2007).

The treatment of autoimmunity (including RA) by stimulating CD1d-restricted NKT cells with αGalCer or its structural analogs has been widely advocated in human autoimmunity. Given the lack of information on the presence of non-*i*NKT cells in human autoimmunity the result of this experimental therapy is still unpredictable.

Aims

Publications addressing this issue of NKT cell involvement in RA patients are few and have notable limitations. Moreover, these early reports on NKT cell populations describe numerical and functional deficiencies in the invariant subset of NKT cells (*i*NKTs) only. The aim of the study presented in this thesis was to confirm previous findings on iNKT cells and extend these by studying the whole CD1d-restricted T cell population, including non-*i*NKT cells, in this condition.

The tasks were therefore three-fold. First of all, the prevalence of *i*NKT cell needed to be confirmed in both the blood and the synovial fluid of the patient population. The results of these experiments are described in Chapter 3, while Chapter 4 describes investigations into the proliferative responses of these cells following in vitro antigen exposure.

Secondly, measurements of the abundance of the total NKT cell subset in RA were planned, using tetrameric CD1d molecules. These experiments were potentially very interesting as the assessment of this NKT cell subpopulation has not been carried out by other investigators. The combined data on the total NKT cell pool and the *i*NKT cell pool would also allow the calculation of the frequency of non-*i*NKT cells, which, as described in the introduction, may oppose *i*NKT cell mediated T cell regulation. The results of this part of the study are presented in Chapter 5.

Finally, the evaluation of some functional properties of the CD1d-restricted population in RA was intended. This was planned by assessment of a comprehensive Th1/Th2 cytokine profile in peripheral blood and synovial fluid-derived cell cultures in response to the CD1d-restricted NKT cell-specific antigen, αGalCer. These experiments were also hoped to provide an opportunity to test the potential of this compound as a therapeutic agent for treatment of the disease in humans.

Chapter 2: Materials & Methods

Peripheral blood and synovial fluid samples were obtained from RA patients attending the Injection Clinic and Rheumatology Outpatient Clinic at Aintree University Hospitals NHS Foundation Trust in Fazakerley, Liverpool. Every donor was presented with the project information sheets and gave informed consent, after having the chance to ask questions. All the individuals were aware that the participation in the study was voluntary and would not affect the treatment given to them at the Clinic. Simultaneously, where possible, data was collected regarding the duration of the disease (since diagnosis) and treatment, with special attention paid towards biological drugs. Of all the individuals who provided samples 8 patients were undergoing anti-TNF therapy at the time of sampling.

Healthy volunteers were recruited into the control group from members of staff and individuals who were named on the list of donors available in the Medical School. Blood samples were donated after giving informed consent. Personal and family history was reviewed to ensure no autoimmune diseases in anamnesis. A logistical problem in relation with sample collection resulted in an imperfect gender match between patient and control groups, however, data available so far indicates that there are no sex-related differences in the NKT population (Jing *et al.* 2007). Table 2.1 provides age and gender characterisation of healthy controls and RA patients who kindly donated a sample for the study.

Ethical approvals for the study were obtained from the Liverpool Research Ethics Committee and the South Sefton Ethics Committee.

Table2.1 Age and gender information for donors who provided samples for the experiments

CHAPTER	EXPERIMENTS		NUMBER OF DONORS	MEAN AGE	SD	FEMALE/ MALE	ANTI-TNF THERAPY
3	Prevalence of *i*NKT cells	H	20	56.2	6.4	10/10	N/A
		RAPB	22	57	12.47	16/6	3
		RASF	12	57.25	14.73	9/3	3
4	*i*NKT cell expansion assays	H	20	56.2	6.4	10/10	N/A
		RAPB	22	57	12.47	16/6	3
		RASF	12	57.25	14.73	9/3	3
5	CD1d tetr-αGalCer staining	H	10	57.1	6.66	4/6	N/A
		RAPB	10	47.67	8.84	7/3	1
		RASF	10	57.4	16.19	8/2	3
6	Cytokine assessment	H	10	56.6	6.29	5/5	N/A
		RAPB	10	56.7	12.99	8/2	1
		RASF	10	59.4	9.05	9/1	3

The appropriate donor numbers are presented chapter by chapter as the groups varied between experiments. Abbreviations: healthy peripheral blood (H), patients' blood (RAPB) and patients' synovial fluid (RASF).

2.2.1. Preparation of peripheral blood samples

All the samples were collected into syringes containing a drop of heparin (CP Pharmaceuticals Ltd) or green vacutainers (BD). The blood was transferred into 30 ml universals, mixed with PBS (at a ratio of 1:1) to achieve better cell separation and then slowly layered on top of 10 ml Ficoll (Ficoll Paque, Amersham Pharmacia) in 50 ml conical tubes. The density gradient centrifugation was carried out in a 5810 R Centrifuge (Eppendorf) for 25 min at 400 g at room temperature with the brake off.

Peripheral blood mononuclear cells (PBMC) accumulating at the interface were harvested into a universal, washed with 10 ml of phosphate buffered isotonic saline (PBS; pH 7.4, 0.5% FCS, 2mM EDTA) and centrifuged at 400g for 5 min in a Megafuge 1.0 R centrifuge (Heraeus Instruments) at room temperature. The cell pellet was resuspended in 3 ml of PBS and cells were counted under an inverted microscope in a hemocytometer (counting chamber). 0.4% Trypan blue (Sigma) in PBS was used to visualise dead cells.

2.2.2. Preparation of synovial fluid samples

Synovial fluid was collected by an experienced clinician by needle and syringe aspiration from a swollen joint space. The samples were then transferred into heparinised universals to prevent coagulation. At this stage the samples were transferred to the laboratory. The samples were then transferred into a conical tube and made up to 50ml with PBS. After mixing they were processed by 5 min centrifugation at 400g, at room temperature. Cell pellets were then resuspended with

various volumes of PBS depending of their size. 20 µl of DNAse (Sigma, diluted to 300 µg/ml in PBS) was added for every 10 ml of cell suspension to enable digestion of clumps and release of cells. The samples were vortexed and placed on a roller for 20 min. After this incubation a gradient centrifugation and consecutive steps of the protocol were carried out as described for peripheral blood samples.

2.2.3. Cell freezing

Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were counted and resuspended in freezing solution, containing 10% of dimethyl sulphoxide (DMSO, Sigma) in Fetal Calf Serum (FCS, Roche). The samples were placed into 2 ml cryovials (Corning) and frozen slowly at -80°C for a day. The following day they were transferred into liquid nitrogen storage.

2.2.4. Cell thawing

Samples were taken out of the liquid nitrogen storage when sufficient numbers had been collected. To begin with, 10 ml of RPMI medium (Sigma), supplemented with 10% FCS (Roche), 0.2mM Glutamine (Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen) (= complete medium, CM) was transferred into universals which were then placed into a water bath at 37°C.

Frozen cell suspensions were slowly thawed by mixing with small amounts of warm medium and subsequently transferred into the universals containing medium. The samples were then centrifuged at 400g for 5 min at room temperature to wash the cells free of the DMSO-containing solution used for freezing. After the wash cell pellets were resuspended in PBS or medium as required and cells were counted under an inverted microscope.

2.3.1. PBMC expansion assays: identification of key time points

The initial part of the expansion experiment included identification of appropriate time points for the best detection of the αGalCer-evoked $V\alpha24^+$ cell enrichment in the cultures. Therefore, to begin with, a peripheral blood sample from one healthy donor (donor H9), a blood sample from RA patient (patient P10) and a synovial fluid sample from an RA patient (patient P11) were cultured.

First of all, mononuclear cells were resuspended in CM. The CD1d ligand α-glucosylceramide (αGalCer, Alexis Biochemicals) was first resuspended in DMSO and then added to the samples at a concentration of 100 ng/ml for $V\alpha24^+$ cell stimulation. Aliquots of 200,000 PBMCs or SFMCs were seeded into 96 well plates and the remaining empty wells were filled with PBS to prevent evaporation. The plates were then placed in an incubator (37°C, 5% CO_2, humid atmosphere) for 14 days. The cells were then sampled every day for 14 days and stained.

2.3.2. PBMC expansion assays with αGalCer and iGb3

In a similar way to the preliminary experiment, αGalCer and iGb3 expansion assays were conducted on three groups of samples: peripheral blood from healthy controls (H), peripheral blood from RA patients (RAPB) and synovial fluid from RA patients (RASF). The numbers of samples in the groups were: 20, 22 and 12, respectively. The protocol for staining was adopted from that used in the preliminary cultures. Apart from α-glucosylceramide stimulated mononuclear cell cultures also isoglobotrihexosylceramide (iGb3, Alexis Biochemicals) was used in a separate set of plates. Prior to that, this reagent was also resuspended in DMSO. Samples from six

donors from each group were used for these assays. iGb3 was used at the same concentration as αGalCer, as recommended by the manufacturer.

On days 4, 7, 10 and 14 the cells were pooled into FACS tubes (Falcon) and centrifuged for 3 min at 400g at room temperature. Supernatant was collected and stored in a freezer at -80°C. Cell pellets were resuspended with 3 ml of PBS and the medium was washed off by spinning at 400g for 5 min at room temperature. Flow cytometry staining with anti-Vα24, PE conjugated antibody (Immunotech or Beckman Coulter) and anti-CD4, FITC conjugated antibody (BD) was carried out.

2.4.1. Anti-Vα24 Staining for Flow Cytometry at Day 0

Peripheral blood and synovial fluid mononuclear cells were used to assess the frequency of $V\alpha24^+$ cells by antibody staining and flow cytometry analysis. The cells were collected into universals and counted as described before. Aliquots of 500,000 cells per sample were transferred into FACS tubes (Falcon) and used for staining. The cells were subsequently resuspended in 3 ml of PBS and centrifuged at 400g for 5 min as a wash. Staining with anti-CD4 monoclonal antibody, FITC conjugated (BD) and anti-Vα24, PE conjugated monoclonal antibody (Immunotech or Beckman Coulter) was conducted. 5 µl of each reagent was used per sample in a total volume of 200 µl of PBS (antibody concentration of 1:40). Double colour staining was introduced to assess the population of $CD4^+$ and $CD4^-$ $V\alpha24^+$ cells. The samples were vortexed and the staining was carried out on ice in a dark cold room for an hour. After that time 3 ml of PBS was poured into the tubes and the samples were spun at 400g for 5 min at 4°C to wash off all unbound antibodies. After centrifugation cell pellets were resuspended in PBS again and vortexed well to obtain the required single cell suspension.

At this time the samples were ready for flow cytometry, and they were acquired on a CyAn Flow Cytometer (DakoCytomation) and the results were analyzed with Summit software, Version 4.3.

2.4.2. Staining with conjugated antibodies: expansion assay

The protocol for staining of samples in the expansion experiments closely followed the protocol for antibody staining on day 0. However, due to a large reduction in the number of cells to be stained per sample (to 100,000), the concentrations of antibodies were appropriately reduced to 1:100 (i.e. 2 µl of a given antibody in 200 µl PBS).

2.4.3. CD1d tetramer staining to assess CD1d–restricted NKT cell populations

Recombinant CD1d tetramers, lacking a ligand in the peptide binding groove, were purchased from Proimmune (Oxford). To begin with, α-galactosylceramide (Alexis) loading was conducted at 37°C for 24 – 48 h. As before, αGalCer was resuspended in DMSO-containing solution. The final concentration of DMSO was 10% as recommended by Watarai *et al.* (Watarai *et al.* 2008), which prevented the detrimental effect of this solvent on the tetramer molecules. According to the literature, the loading of CD1d tetramers with this ligand requires at least 10 molar excess (Cantu *et al.* 2003), but 40 molar excess of this ligand was chosen as it has successfully been used previously (Gumperz *et al.* 2002). The tetramers were freshly loaded prior to the experiments. The reagent was stored in the fridge and used for staining within two weeks, according to the manufacturer's instructions.

Thirty samples were used for this experiment: 10 samples of peripheral blood from healthy donors along with 10 samples of peripheral blood and 10 samples of synovial fluid from RA patients. PBMCs and SFMCs were used at 500,000 per staining and aliquoted into FACS tubes (Falcon). Staining protocol optimization was carried out for triple staining with APC conjugated CD1d tetramers-αGalCer, anti–CD4 FITC (BD) and anti–CD19 PE antibodies (BD). The latter antibody was added as the manufacturer

suggested conducting the analysis on $CD19^-$ cells. This step ensured that non-specific binding of the tetramers by B cells was avoided. All the reagents were used at the concentration of 1:40 in PBS and the staining was conducted in a single–step protocol, for 1 hour on ice.

After the incubation period, the cells were washed with PBS and centrifuged for 5 min at 400g. Then the samples were acquired on a CyAn Flow Cytometer (Dako Cytomation) and the results were analyzed using Summit software, Version 4.3, ensuring that $CD19^+$ cells were excluded from the analysis.

2.5.1. Technique description – Multi bead assay

The multi bead assay is a fairly new technique which enables the simultaneous detection of a number of analytes in the same sample. This number varies and depends on the products available from various manufacturers. Typically, there are kits which enable the detection of many cytokines, chemokines and metabolites (such as cardiovascular markers for example), giving a researcher the option of identifying the secretory pattern in a given sample.

More than that, many additional analytes can be measured as well, as kits are also offered for the detection of single chemical compounds (e.g. simplex kits) which can be combined, allowing for flexible experimental design. A similar sort of assay is also manufactured by Luminex (xMAP technology), however the latter requires specialised equipment to analyse the results (i. e. a Luminex machine). Samples processed with the multi bead assay described here (FlowPlex, UK Bio) can be acquired on a standard flow cytometer which is a great advantage, as many laboratories are equipped with this.

The main advantage of the technique is, however, the fact that it enables the measurement of the concentrations of many analytes from an individual sample of very small volume. Only 25 µl is usually enough to run the assay and obtain satisfactory results. Proceeding with the analysis of multiple cytokines from the same sample also greatly reduces the risk of an error when handling the reagents. Depending on the number of samples, there is an option to run the assay in round bottomed flow cytometer tubes or in a 96 well filter plate using a vacuum manifold, the latter simplifying the technique to a great extent.

The assay is based on ELISA principles, i. e. forming antibody/analyte pairs on the surface of a mix of fluorescent beads. These beads are coupled to antibodies with the specificity for a given analyte, for example, a cytokine. These antibodies bind analyte in the assay mixture. The secondary antibodies, which are biotinylated, are then added to the mixture. These recognise different epitopes of the same molecules, so the complex forms an ELISA sandwich. Finally, streptavidin–phycoerythrin (PE) is introduced to give the required labelling, observed on FL-2 Log scale. The concentration of the final product can then be measured by the lasers in a standard flow cytometer acquisition (Figure 2.1).

To allow for better discrimination, there are two populations of beads in a bead mixture which are characterized by two different sizes. These can be identified on the forward/side scatter histogram when acquiring the samples. The beads are internally dyed with various concentrations of the fluorescent dye, which enables the laser to distinguish it from the others and, therefore, to characterize the particular population of beads on FL-4 Log scale. This principle is very similar to the principle of the Luminex assay (Figure 2.2), however the latter uses a combination of two different dyes to characterise up to 100 bead populations.

For the purpose of the research described here, a Th1/Th2 11-plex kit from UK Bio (FlowPlex) was used. The kit enabled the measurement of human: IFN-γ, IL-1β, IL–2, IL–4, IL–5, IL–6, IL–8, IL–10, IL–12p70, TNF-α, and TNF-β. This enabled the recognition of the cytokine patterns downstream of NKT activation in the peripheral blood of healthy donors and in the peripheral blood and synovial fluid of RA patients.

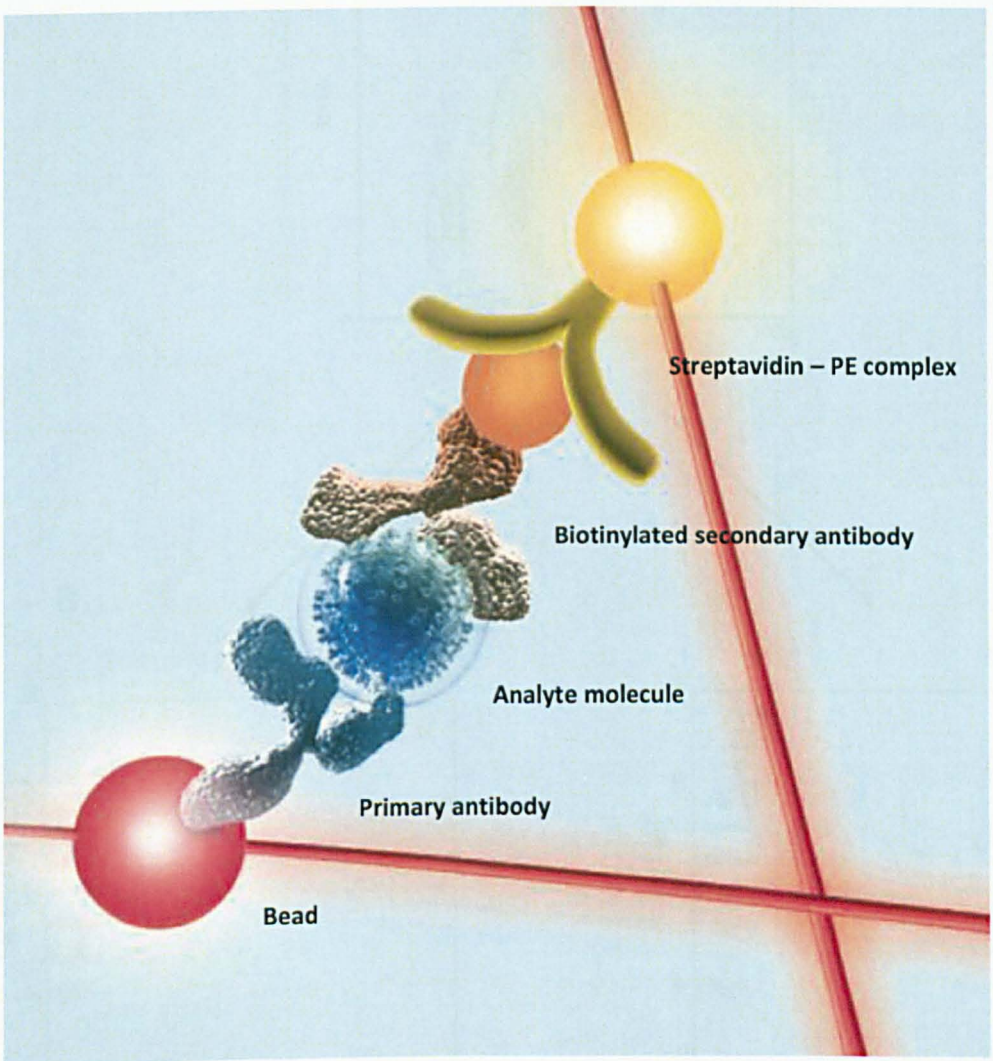


Figure 2.1 ELISA sandwich complexes are formed on the surface of fluorescently- labelled beads in the assay mixture. This complex consists of a primary antibody, an analyte present in a sample, a secondary, biotinylated antibody and streptavidin-PE conjugate. From Bender MedSystems website (www.bendermedsystems.com), modified.

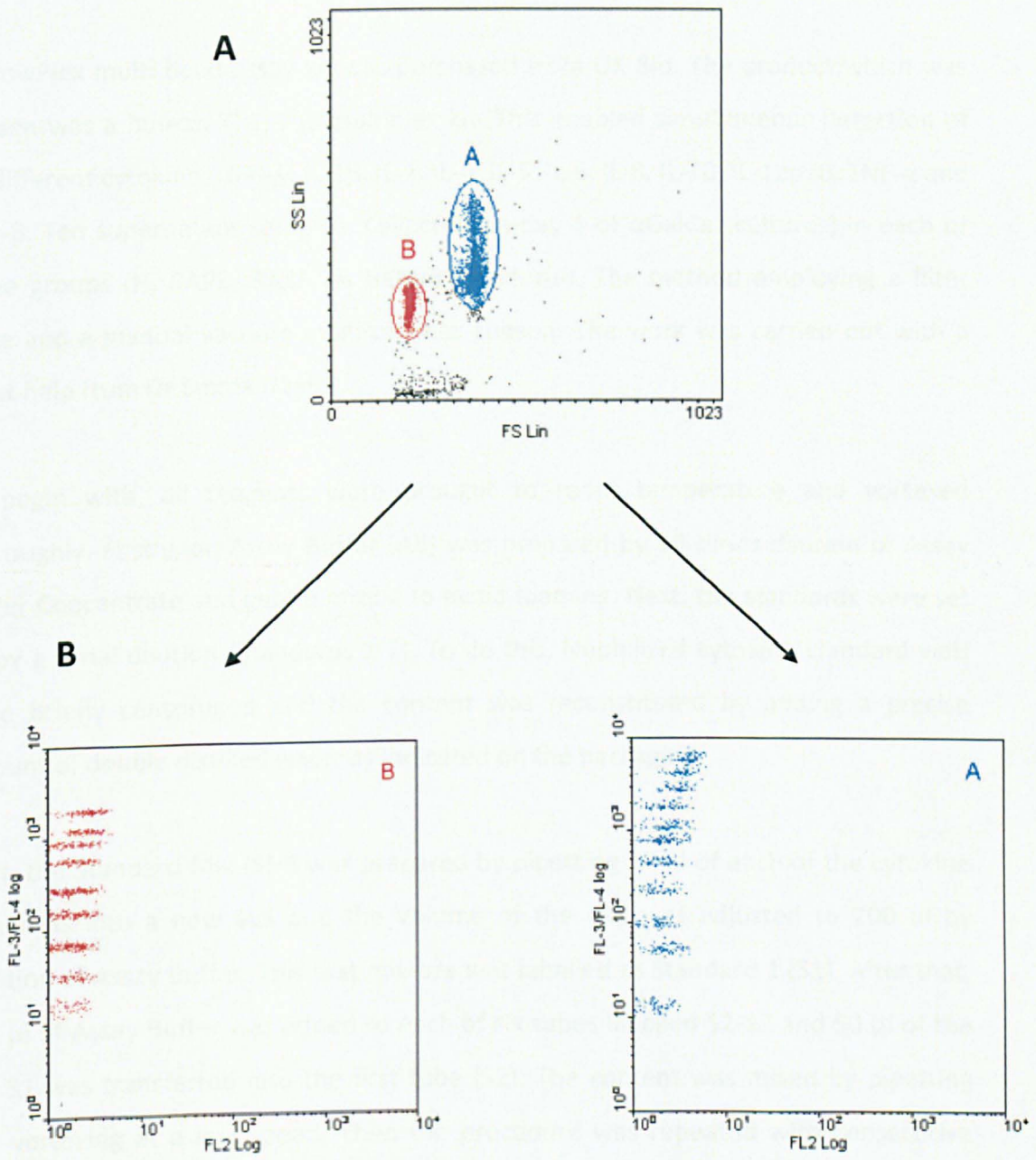


Figure 2.2 A) Two populations of fluorescently labelled beads on FS/SSC histogram B) determination of bead subpopulations in the FL3 or FL4 direction (PECy5). The concentration of a given analyte is measured based on the brightness of beads in the FL2 (PE) direction. Figures from UK Bio, manufacturer website www.ukbio.co.uk, modified.

2.5.2. FlowPlex multi bead assay – the kit and preparation of reagents

A FlowPlex multi bead assay kit was purchased from UK Bio. The product which was chosen was a human Th1/Th2 multiplex kit. This enabled simultaneous detection of 11 different cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α and TNF-β. Ten supernatant samples (collected on day 4 of αGalCer cultures) in each of three groups (H, RAPB, RASF, as before) were run. The method employing a filter plate and a manual vacuum manifold was chosen. The work was carried out with a great help from Dr Emma Naylor.

To begin with, all reagents were brought to room temperature and vortexed thoroughly. Firstly, an Assay Buffer (AB) was prepared by 10 times dilution of Assay Buffer Concentrate and gentle mixing to avoid foaming. Next, the standards were set up by a serial dilution (Standards 1-7). To do this, lyophilized cytokine standard vials were briefly centrifuged and the content was reconstituted by adding a precise amount of double distilled water as indicated on the packaging.

Next, the Standard Mix (SM) was prepared by pipetting 10 µl of each of the cytokine standards into a new vial and the volume of the mix was adjusted to 200 µl by addition of Assay Buffer. This first mixture was labelled as Standard 1 (S1). After that, 100 µl of Assay Buffer was added to each of six tubes labelled S2-S7 and 50 µl of the mix S1 was transferred into the first tube (S2). The content was mixed by pipetting and vortexing at a low speed. Then the procedure was repeated with consecutive tubes to ensure 1:3 dilution in the series.

Next, the required amount of the Bead Mixture was calculated by multiplying a number of tests to be run by 25 µl. This final amount of the prepared mix was 1500 µl, enough for 30 samples, two standard curves (7 samples each), setup beads and pipetting reservoir. The vials containing beads were centrifuged before use and 75 µl

of every bead mixture was transferred into a new tube. Then, the volume was adjusted to 1500 µl with Reagent Dilution Buffer (RDB).

Following this, a required volume of the Biotin Conjugate was calculated and 3000 µl of the mix was prepared by combining 150 µl of each of biotin-conjugates together with 1350 µl of the RDB buffer. Finally, Streptavidin-PE solution was also made by diluting 88 µl of the concentrated reagent in 2662 µl of Assay Buffer. When all the reagents needed were ready, they were kept at room temperature until required.

2.5.3. FlowPlex multi bead assay: the protocol

As aforementioned, due to the number of tests run, a protocol for the filter plate was chosen. This greatly simplified the procedure, reducing the length of time required and likeliness of introducing errors. The 1.2µm pore size Beadlyte filter plate used in this experiment was purchased from Millipore. The number of wells needed was determined and an unused part of the plate was covered with adhesive film to ensure proper vacuum filtration. All the remaining wells were pre-treated by adding 50 µl of Assay Buffer, to pre-wet the filters. The excess was aspirated with a manifold and the bottom of the plate was lightly tapped on absorbent paper, ensuring that filtration membranes remained intact.

Next, 25 µl of standard dilutions S1-S7 were added into the wells on both sides of the uncovered plate, in opposite directions. The eight wells were left as blank and only 25 µl of Assay Buffer was added to them. Well number A2 of the plate was used for flow cytometer setup beads and 25 µl of Standard mixture 1 was added to it. The remaining wells were designated for actual experimental tests and 25 µl volumes of culture supernatant samples were added to each of those wells.

When all the wells contained appropriate standards and experimental samples, 25 µl of Bead mixture was added to all of them, followed by 50 µl of Biotin-conjugate mixture. The multichannel pipette was used when appropriate. The wells were then covered with an adhesive film and protected from light by an aluminium foil. The samples were incubated at room temperature for two hours on a shaker. After the incubation, the protective adhesive film was gently removed for the time being and the wells were emptied by manifold aspiration as before.

As the next step, 100 µl volume of Assay Buffer was added to every well followed by 50 µl of Streptavidin-PE solution. The plate was covered again and left on the rotating shaker at room temperature. As before, the samples were light-protected by aluminium foil. After an hour of incubation the protective film was removed and the wells were emptied with manifold aspiration. Two washing steps were introduced. These included adding of 100 µl of Assay Buffer to each well followed by manifold aspiration and light tapping of the plate on the absorbent paper.

To pool the samples 200 µl of Assay Buffer was added to a given well and the contents were mixed by repeated pipetting. The samples were then collected into standard flow cytometer tubes and an additional 300 µl of the same buffer was added to every tube in order to increase the volume of each sample. According to the protocol, the samples could have been left at this stage in a dark cold room for a maximum of 24 h. In this case, however, they were acquired straight after the setup of flow cytometer (Cyan, Dako Cytomation).

2.5.4. Flow cytometer setup

To prepare the equipment and save a protocol file setup beads were acquired and FS/SS parameters were adjusted. The plot identified two populations of beads varying in size which were gated onto separate FL2/FL4 histograms. This resulted in a separation of all the bead subpopulations. Compensation was applied to ensure that these bead subpopulations form horizontal lines on the plots, to enable further analysis. After the acquisition of all the samples the results were analysed with the software provided by UK Bio.

2.6. Statistical analysis of the results

Each data analysis commenced with normality check of a given dataset, which was tested with InStat version 3.06 (GraphPad Software Inc.). Depending on the required test the results were analysed with SPSS for Windows V17, (SPSS Corp, US) or InStat version 3.06 software. Based on the result of normality check an appropriate test was used. Significance was accepted at the 5% level throughout (p value < 0.05).

The comparison of total *i*NKT cell prevalence between sources (Chapter 3) and expansion experiments was performed using one-way ANOVA analysis. Initial analysis of cell proliferation studies (Chapter 4) was carried out with Student T test, followed by two- and three- factor within subjects ANOVA. The comparisons were carried out with and without post-hoc Bonferroni adjustments applied.

The comparison of CD1d-restricted cell prevalence (Chapter 5) was carried out with one-way ANOVA. The overall and single cytokine secretion (Chapter 6) was compared using Mann-Whitney test as these data were non-normally distributed. The IL-4/IFNγ and IL-10/TNFα ratios were compared with one-way ANOVA test.

The statistical analysis was carried out with a great help from Prof Martin Birchall and Dr Aleksandra Mandecka.

Chapter 3: Prevalence of cells expressing Vα24 TCR chain in rheumatoid arthritis

It is widely accepted that the pathology observed in many autoimmune diseases is a result of an uncontrolled activation of T cells. It is unclear at present whether these lymphocytes recognize self-antigens, expressed by affected tissues, or if the activation is non-specific, driven by the presence of cytokines in the affected joints. One of the theories on the mechanisms responsible for disease induction proposes that while the proliferation of self-reactive T cells in healthy people is suppressed by various regulatory cell populations, in patients these are dysfunctional, leading to uncontrolled T cell activation resulting in autoimmunity.

So far two regulatory lymphocyte subsets have been identified with a probable role in RA, regulatory T cells (T_{reg}) and NKT cells. While the former have been extensively tested for their ability to control T cell activation in human autoimmunity, the immunoregulatory properties of NKT cells have been studied much less extensively.

As mentioned in the Introduction, the first observation which suggested a regulatory role for this lymphocyte subset was made by Takeda *et al.* in 1993 in lupus-prone C57BL/6 *lpr* mice where the disappearance of $NK1.1^+$ lymphocytes correlated with development of autoimmunity (Takeda and Dennert 1993). The study showed that the mice which develop the disease had a reduced prevalence of this population and this inversely correlated with B cell-mediated specific responses (anti-DNA antibody production). In 1996 Gombert *et al.* published the first study on the autoimmunity-prone non-obese diabetic (NOD) mouse model (Gombert *et al.* 1996). The authors detected selective $NK1.1^+$ thymocyte defects: both a quantitative reduction and a functional impairment (decreased IL-4 secretion). Since then, these findings have been confirmed by multiple groups and more work has been carried out to investigate these cells in NOD mice (Baxter *et al.* 1997; Godfrey *et al.* 1997; Lehuen *et al.* 1998; Hong *et al.* 2001; Naumov *et al.* 2001; Sharif *et al.* 2001; Beaudoin *et al.*

2002). Other autoimmune disease models were studied (Singh *et al.* 2001; Mars *et al.* 2002; Yang *et al.* 2007) and similar conclusions were drawn.

In these animal models the evidence supporting the immunoregulatory role of NKT cells is very convincing. Critically, the disappearance of *i*NKT cells from the circulation prior to disease onset has been observed (Takeda and Dennert 1993). In addition, accelerated disease progression in CD1d-deficient animals lacking NKT cells has been shown by Shi *et al.* (Shi *et al.* 2001). This latter study identified that disease progression was increased in $CD1d^{-/-}$ NOD mice compared with both $CD1d^{+/-}$ and $CD1d^{+/+}$ animals. Moreover, the infiltrate observed in fast progressing mice contained an increased number of activated memory T cells, which suggested that CD1d-restricted NKT cells are able to control these (Shi *et al.* 2001). In addition, activation of NKT cells using αGalCer (Hong *et al.* 2001; Naumov *et al.* 2001; Sharif *et al.* 2001; Chen *et al.* 2005) or its analogue αGalCer C20:2 (Forestier *et al.* 2007) as well as transgene-driven over-expression of *i*NKT cells (Lehuen *et al.* 1998) seems to prevent or delay clinical disease.

The first study which reported on the *i*NKT cell population in type I diabetes in humans found that the population of double negative (DN) subset of these cells was reduced in this disease (Wilson *et al.* 1998). This study investigated the frequencies of these cells in discordant twins and triplets, and demonstrated that individuals who suffered from type 1 diabetes had lower cell numbers compared with their at-risk non-progressing siblings.

Consequently, the possible involvement of the *i*NKT cell population was investigated in other human autoimmune diseases and similarly reduced *i*NKT cell numbers were observed in systemic lupus erythematosus (SLE) and multiple sclerosis (Demoulins *et al.* 2003; Yang *et al.* 2007). Another study identified a decrease in the frequency of circulating *i*NKT cells in individuals with autoimmune inflammation leading to tissue

damage (van der Vliet *et al.* 2001). The results suggested that in the majority of these conditions, i.e. polymyositis/dermatomyositis, RA, Sjögren syndrome, systemic lupus erythematosus, Crohn's disease, autoimmune hepatitis, systemic sclerosis, bullous dermatoses and psoriasis this population was underrepresented (van der Vliet *et al.* 2001). According to van der Vliet *et al.* the only two diseases of autoimmune etiology which did not share this characteristic feature were Graves' disease and celiac disease, where Vα24$^+$ cell numbers were preserved. Although the numbers of individual samples in some of the experimental groups were rather low (e.g. 4 patients) the overall picture suggested the importance of *i*NKT cells in protection against autoimmunity. This would support the initial human study on type 1 diabetes. However, recently data regarding NKT cell numbers in diabetes, which is contradictory to the results of Wilson *et al.* was published (Lee *et al.* 2002; Oikawa *et al.* 2002), while findings of smaller studies in other conditions remain unconfirmed.

Studies investigating NKT cell prevalence in RA are scarce. An animal model of arthritis developing spontaneous autoimmune inflammation is lacking. This makes it impossible to assess the role of Vα14$^+$ cells in a true autoimmune model. This forces the use of murine models of reactive arthritis, where the inflammation is elicited by various manipulations, e.g. injection of a compound (such as type II collagen) or an infectious agent (*Chlamydia trachomatis*) into an animal. While these mice develop arthritis which shares some features of RA and are invaluable to understand the immunopathology of the disease, it is important to remember that the pathological mechanism in these models is likely to differ substantially from that of human RA.

Only four studies on NKT cell prevalence in RA have been conducted to date. While these studies provide some information on the involvement of this cell population in the disease, they also have limitations. The first publication which reported on NKT cells in RA was a short letter to Rheumatology written by Maeda *et al.* (Maeda *et al.* 1999). In this study reduced TCR Vα24 chain mRNA expression was found in

magnetically separated DN lymphocytes isolated from the synovial tissue of RA patients compared with PBLs from both healthy donors and the patients. The study was carried out on samples of synovial tissue and peripheral blood from four RA patients and three healthy controls and the results were based on polymerase chain reaction (PCR) and western blotting experiments.

Two years later the same group published a study which described the frequencies of $CD4^-CD8^-$ $V\alpha24^+V\beta11^+$ cells in five autoimmune diseases, namely RA, systemic lupus erythematosus (SLE), systemic sclerosis (SSc), Behçet's disease (BD) and Sjögren's syndrome (Kojo *et al.* 2001). The results presented showed that all these autoimmune diseases, with the exception of Behçet's disease, are characterized by a reduction in DN $V\alpha24^+V\beta11^+$ cells. The data on RA were based on a cohort of 20 patients. Only peripheral blood from these patients was sampled, there was no information on changes in the affected tissues. Most importantly, the authors of the study examined only the double negative (DN) subset of *i*NKT cells, so a sizeable proportion of the true *i*NKT population was not assessed. This was due to the historical definition of the NKT cell subset at the time. However, this approach resulted in an incomplete assessment.

Later that year another group also published a study on the prevalence of $V\alpha24^+V\beta11^+$ DN in various autoimmune diseases in which inflammatory damage can be observed (van der Vliet *et al.* 2001). Again, various conditions were characterized for the frequencies of circulating *i*NKTs and a decrease in cell frequencies was noted. The cohort of RA patients consisted of 15 individuals. This study also suffered from exactly the same limitations as the former, i.e. lack of synovial fluid or synovium samples and assessment of the DN cells only.

More recently, in 2005, a group from Belgium carried out some work to measure *i*NKT cells in RA patients (Linsen *et al.* 2005). The first set of experiments included flow

cytometry assessment of $V\alpha24^{+}V\beta11^{+}CD3^{+}$ cell frequencies in 23 samples of peripheral blood and 7 paired samples of synovial fluid. The results confirmed a reduction in circulating *i*NKT frequencies as seen before and demonstrated a similar change in the samples of synovial fluid. However the authors failed to identify a clear trend of peripheral blood/synovial fluid change in RA patients. Unfortunately, the samples collected were not assessed for $CD4^{+/-}$ subset composition. These data could be invaluable, due to the possibility of disturbed contribution of the CD4 subsets, with distinct functional roles.

Although providing some ideas about *i*NKT cell biology in RA, these early studies suffered from the highlighted limitations. Especially, the lack of a unified definition of NKT cell populations and overly restrictive TCR chain requirement makes the interpretation of these studies difficult as discussed in Chapter 1. TCR usage by NKT cells is presently a subject of discussion (Gadola *et al.* 2006; Borg *et al.* 2007; Mallevaey *et al.* 2009), but it is certain that experiments described above failed to capture some CD1d-restricted *i*NKT cells. Thus, due to the limitations of these studies, comprehensive information on *i*NKT cell prevalence in RA is still lacking, prompting the experiments described here.

3.2. Experimental setup

In order to investigate *i*NKT cell prevalence the frequency of these cells was measured in 20 samples of peripheral blood from healthy donors (H) as well as 22 samples of peripheral blood and 12 samples of synovial fluid from RA patients (RAPB and RASF, respectively).

Mononuclear cells (PBMCs and SFMCs) were isolated and 500,000 cells from each donor were double stained with anti-Vα24PE and anti-CD4FITC antibodies. Cells were analysed as described in Materials & Methods. For analysis a gate ("a lymphocyte gate") was set on the FSC/SSC profile and then the $CD4^+$ and $CD4^-$ populations were assessed as shown in Figure 3.1.

*i*NKT cells observed in humans can be found amongst $CD4^+$, double negative (DN) and $CD8^+$ cell types. However, the latter population is extremely small which generates additional difficulties in assessing it both qualitatively and quantitatively. Therefore, for the purpose of this and also Chapter 4, the $CD8^+$ population and DN cells were evaluated together as a single $CD4^-$ subset. These experiments were based on the flow cytometric analysis of fresh cells isolated from the peripheral blood and synovial fluid samples collected from RA patients and also peripheral blood withdrawn from healthy control individuals.

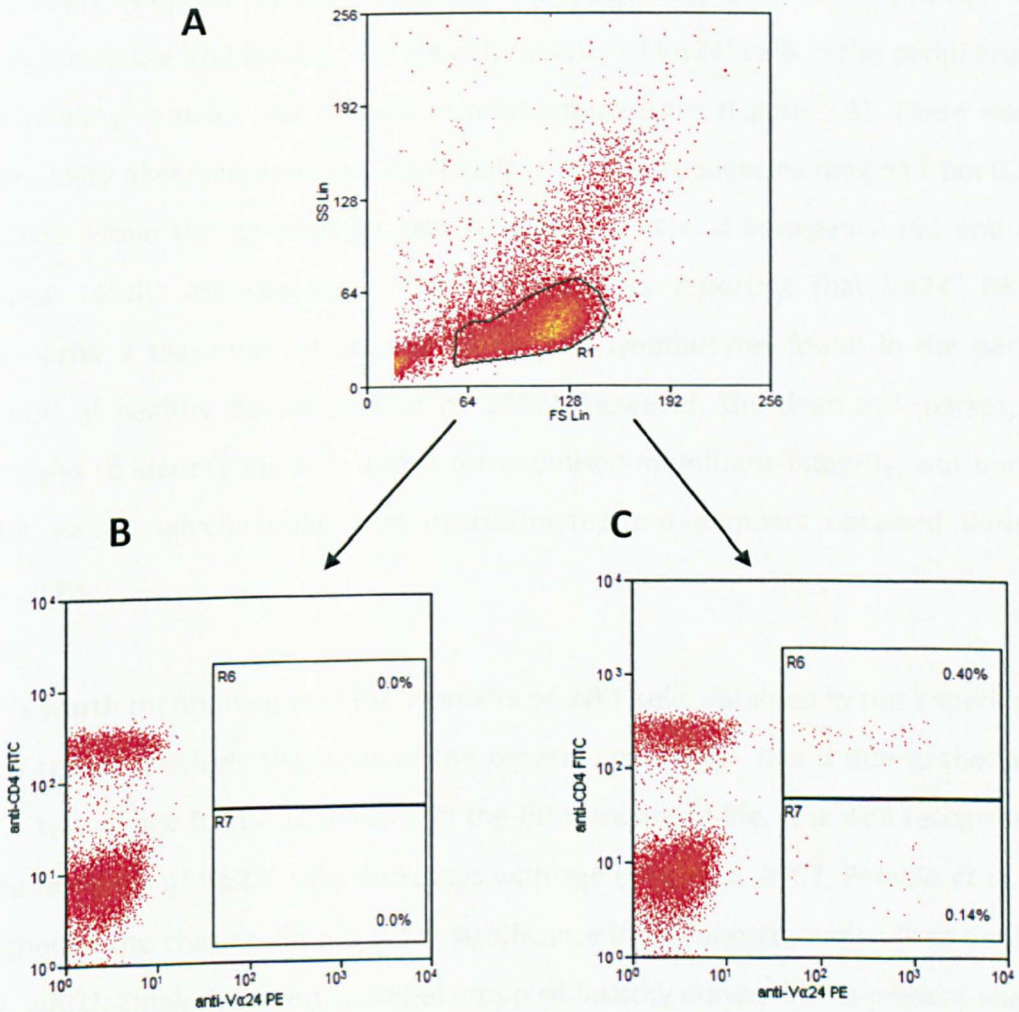


Figure 3.1 Gating used for the analysis of anti-Vα24/CD4 staining at Day 0 (as exemplified in a control sample; healthy donor). The samples were collected from the donors and mononuclear cells were isolated by gradient centrifugation. The staining was carried out for 1 h on ice. A) lymphocyte gate B) staining with anti-CD4 FITC C) staining with anti-Vα24 PE antibodies and anti-CD4 FITC antibodies to identify $CD4^+$ $V\alpha24^+$ and $CD4^-$ $V\alpha24^+$ populations.

The numbers of total *i*NKT cells in each sample was calculated by the addition of the numbers obtained for CD4$^+$ and CD4$^-$ subpopulations which were positive for TCR containing the Vα24 chain. The mean frequency of Vα24$^+$ cells in the peripheral blood of healthy controls was 0.362% of total lymphocytes (Figure 3.3). There was large variability observed between individuals, with the frequencies ranging from 0.16% to 0.93% within the lymphocyte gate (confidence interval between 0.293 and 0.431). These results are consistent with the literature reporting that Vα24$^+$ NKT cells comprise a maximum of around 1% of total lymphocytes found in the peripheral blood of healthy people (Lee *et al.* 2002). However, the dead cell marker, which enables to identify the cells with a compromised membrane integrity, was not used t the assays, which could have overestimated the numbers obtained during the analysis.

It is worth mentioning that the numbers of *i*NKT cells obtained in this experiment do not reflect precisely the mean of the general population. This is due to the fact that the typical age for RA patients is in the fifth decade of life. It is well recognised that the numbers of Vα24$^+$ cells decreases with age (Jing *et al.* 2007, Perablo *et al.* 2007), although the change did not reach significance in all cohorts studied (van der Vliet *et al.* 2001). Similarly, when an initial group of healthy donors in the present study was specifically assessed for the age-related change of *i*NKT cell frequencies, no significant difference was found despite the trend towards reduction observed amongst donors. However, to match the age with the patient population, these younger donors were not included in the analysis.

Flow cytometric analysis of peripheral blood samples from RA patients confirmed the results obtained in the study previously performed by the Japanese group (Kojo *et al.* 2001). The frequency of total Vα24$^+$ lymphocytes was found to be slightly reduced

compared with the frequency of these cells in peripheral blood of healthy donors, with the mean value of 0.355% of the cells found in the lymphocyte gate (Figure 3.1 and Figure 3.2). *i*NKT cell frequencies obtained for this group ranged between 0.14% and 0.73% (confidence interval between 0.289 and 0.420).

The decrease in the numbers of total Vα24$^+$ NKT cells isolated from the 12 samples of synovial fluid obtained from RA patients was slightly lower to that found in PBLs. The mean *i*NKT cell frequency observed in these samples was 0.319%, with a minimum of 0.08% and a maximum of 0.61% of the total lymphocyte number (confidence interval between 0.230 and 0.408).

Therefore, *i*NKT cell frequencies observed in the peripheral blood and synovial fluid from the patients appeared slightly reduced, reaching respective numbers of about 98% and 88% of the total Vα24$^+$ cell frequency noted in the blood of healthy donors. However, these differences did not reach statistical significance for the groups (pairwise comparison H vs. RAPB p=0.876; H vs. RASF p=0.449, RAPB vs. RASF p=0.524). However, the trend towards reduced frequency of *i*NKT cells in both patients' peripheral blood and synovial fluid was seen (Figure 3.3), supporting the results obtained by both research groups that investigated this issue.

While efforts were taken to collect peripheral blood and synovial fluid from the same individuals, due to the unexpected difficulties with the provision of clinical samples, samples from only three patients were paired and assessed as a part of the experiment. Two of them showed decreased frequencies of *i*NKT cells in the synovial fluid compared with the peripheral blood, while in the third patients the levels in PBL were lower. Therefore it was impossible to conclude unambiguously how these NKT populations change in the patients during the course of RA.

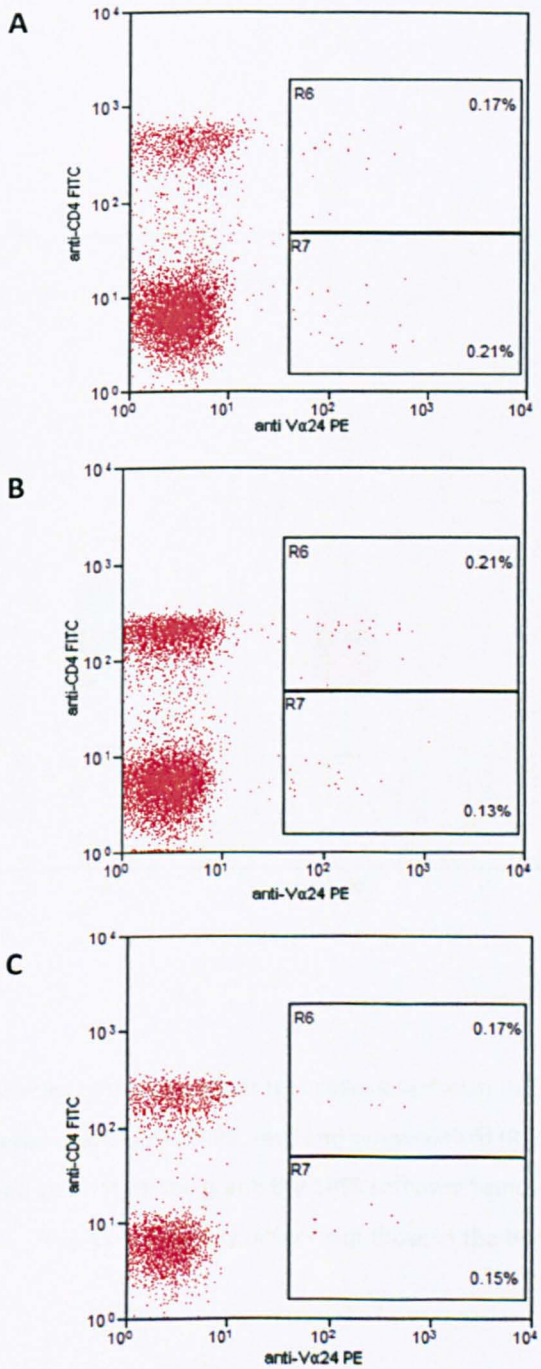


Figure 3.2. Representative FACS profiles from A) healthy donor (H) B) peripheral blood (RAPB) and C) synovial fluid (RASF) from RA patient. Horizontal axis represents anti-Vα24 staining; vertical axis represents anti-CD4 staining.

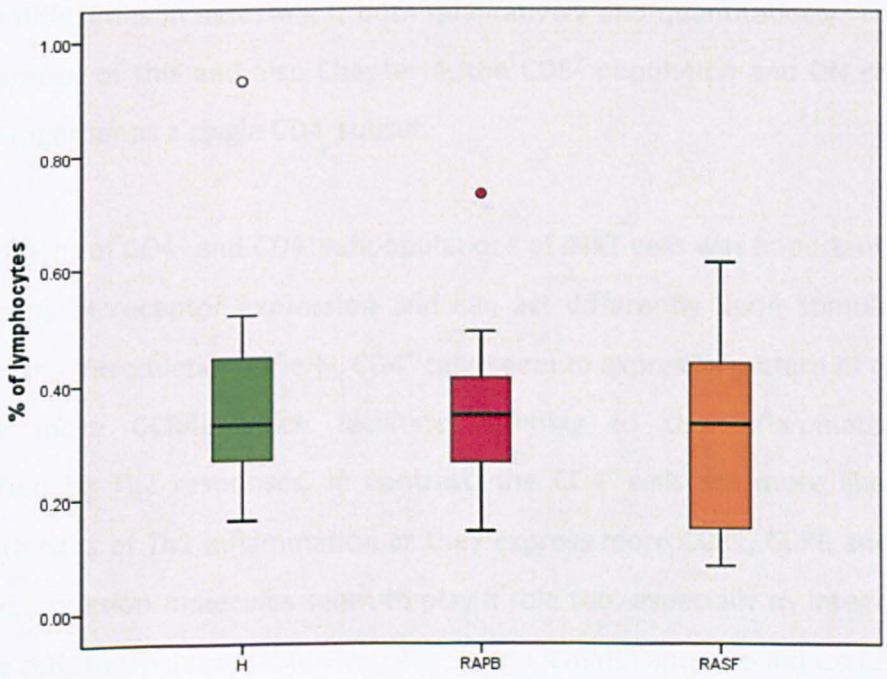


Figure 3.3 Mean frequencies of total invariant NKT cells detected in the peripheral blood of healthy donors (H, green), peripheral blood (RAPB, red) and synovial fluid (RASF) of RA patients by flow cytometry staining. When generating the graph the SPSS software removed the outliers, so the mean values can slightly differ from those in the text.

3.4. Composition of iNKT cells subpopulations in RA

*i*NKT cells observed in humans can have $CD4^+$, double negative (DN) and $CD8^+$ phenotypes. However, the latter population is extremely small which generates additional difficulties in assessing it both qualitatively and quantitatively. Therefore, for the purpose of this and also Chapter 4, the $CD8^+$ population and DN cells were evaluated together as a single $CD4^-$ subset.

The assessment of $CD4^+$ and $CD4^-$ subpopulations of *i*NKT cells was important, as they vary in terms of receptor expression and can act differently upon stimulation, as outlined in the Introduction. Briefly, $CD4^+$ cells seem to express a pattern of receptors (especially more CCR4) which facilitates homing to the inflammatory sites characterised by Th2 responses. In contrast, the $CD4^-$ cells are more likely to be recruited to sites of Th1 inflammation as they express more CCR1, CCR6 and CXCR6. In addition, adhesion molecules seem to play a role too, especially α_1 integrin which directs the cells to tissues enriched in collagen and laminin and is found exclusively on the $CD4^+$ $V\alpha24^+$ subset. In contrast, α_L integrin (LFA-1) is abundant on the $CD4^-$ subpopulation and enhances endothelial adhesion and extravasation.

In the present study it was found that $CD4^+$ cells that bear the Vα24 TCR chain constitute 67.4% of the total *i*NKT cell population in healthy people with the mean value of 0.244% (confidence interval between 0.205 and 0.283) of all lymphocytes (Figure 3.4 and Figure 3.5). The respective subpopulation in the peripheral blood of RA patients seemed to be of a similar frequency as in the controls (mean=0.219% in the lymphocyte gate; confidence interval 0.181 to 0.256), and also their contribution towards total *i*NKT cell numbers was similar, accounting for around 61.66% of all the cells stained with anti-Vα24 antibodies.

The respective mean value for synovial fluid samples was lower at 0.151% confidence interval between 0.100 and 0.202), resulting in 47.3% contribution of $CD4^+$ subpopulation to the total *i*NKT cell pool in the joints. While these changes seem small, there was a statistically significant difference between the means (p=0.005 between H and RASF groups, p=0.036 RAPB vs. RASF; respective confidence intervals for the difference between 0.026 and 0.158; between 0.05 and 0.131), as depicted in Figure 3.4.

The $CD4^-$ subpopulation in the healthy group formed around 32.6% of *i*NKT cells and also around 38% in the peripheral blood of the patients. A trend towards an increase in this subpopulation in the synovial fluid samples from RA sufferers was noted. These cells were more abundant than cells of the $CD4^-$ subset in the peripheral blood samples, accounting for 52.7% of the total *i*NKT cell pool. The differences in CD4 subset contribution, however, were not statistically significant.

The analysis of the results from the paired samples revealed decreased frequencies of $CD4^+$ cells in the synovial fluid; this was the case for all three individuals. However, no particular alteration in the pattern of distribution of the $CD4^-$ subset was found. In all of these cases $CD4^+$ cells constituted the majority (75.6%-85.7%) of the peripheral blood *i*NKT cells. The results obtained for synovial fluid from these individuals were not consistent and covered a much broader range of $CD4^+$ contribution (34.6%-78.6%).

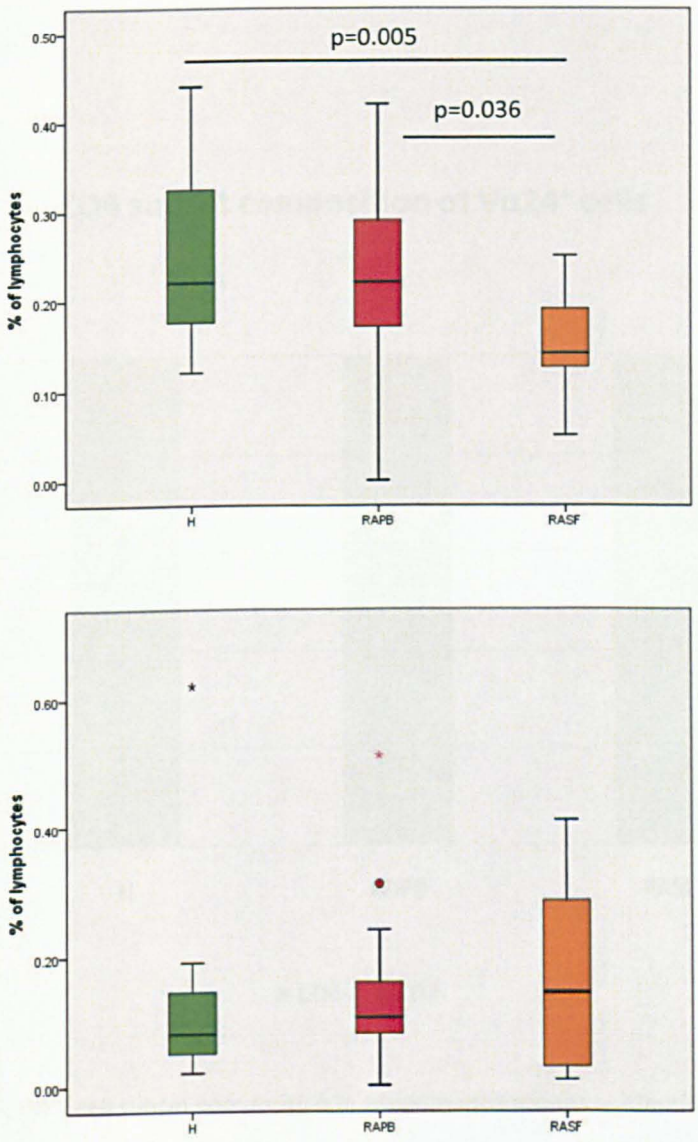


Figure 3.4. Mean frequencies of $CD4^+$ and $CD4^-$ invariant NKT cells observed in peripheral blood of healthy donors (H, green), peripheral blood (RAPB, red) and synovial fluid (RASF, yellow) of RA patients. When generating the graph the SPSS software removed the outliers, so the mean values can slightly differ from these in the text.

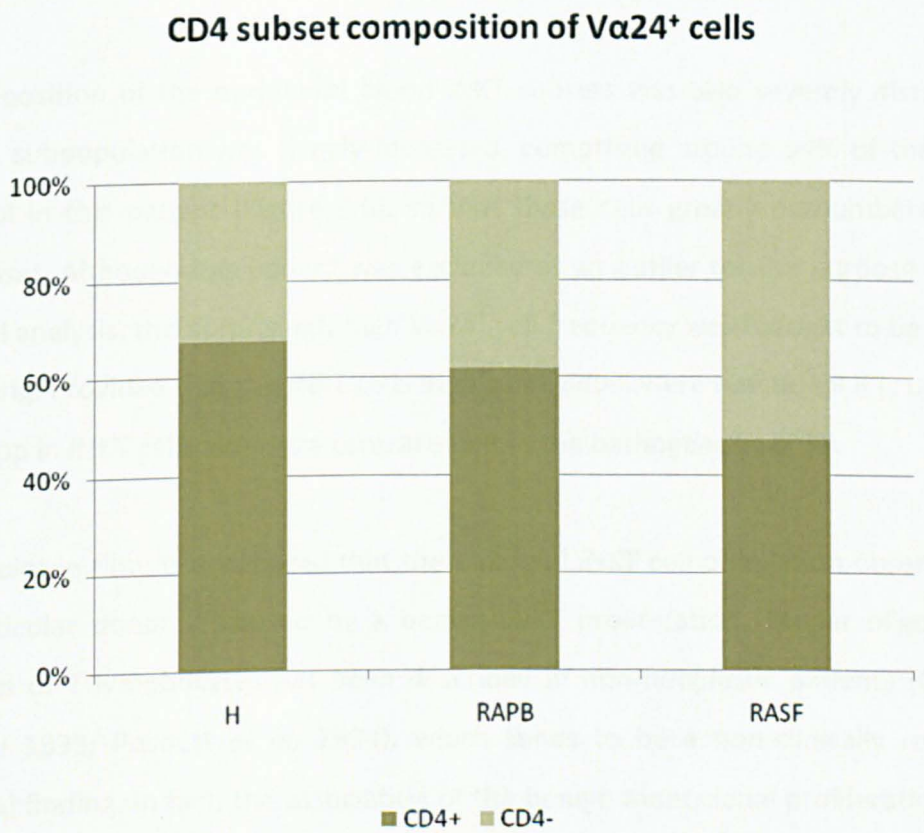


Figure 3.5. *i*NKT cell subset composition in experimental groups as identified by flow cytometry staining. $CD4^{+}V\alpha24^{+}$ cells (dark green), $CD4^{-}V\alpha24^{+}$ cells (light green).

Puzzling results were obtained from the peripheral blood sample of one patient (Patient 5, a woman, 50 years of age). When this sample was analyzed it appeared that the frequency of NKT cells expressing the TCR chain of interest was very high, totalling 7.5% of all lymphocytes (Figure 3.6).

The composition of the peripheral blood *i*NKT subsets was also severely disturbed; the $CD4^-$ subpopulation was largely increased, comprising around 94% of the total *i*NKT pool in this patient (Figure 3.6), so that these cells greatly outnumbered the $CD4^+$ subset. Although this patient was excluded as an outlier for the purpose of the statistical analysis, this surprisingly high $V\alpha24^+$ cell frequency was thought to be worth mentioning. Provided that the *i*NKT cells in this individual were functional it is unlikely that a drop in *i*NKT cells alone is a central event in the pathogenesis of RA.

At this point in time it is believed that the enlarged *i*NKT cell population observed in that particular donor is caused by a benign *i*NKT proliferation. Similar oligoclonal expansion of T lymphocytes has been described in non-neoplastic patients (Osame and Ijichi 1993; Posnett *et al.* 1994), which tends to be a non-clinically relevant accidental finding. In fact, the association of the benign monoclonal proliferation of T cells with RA has also been reported (Berliner *et al.* 1986).

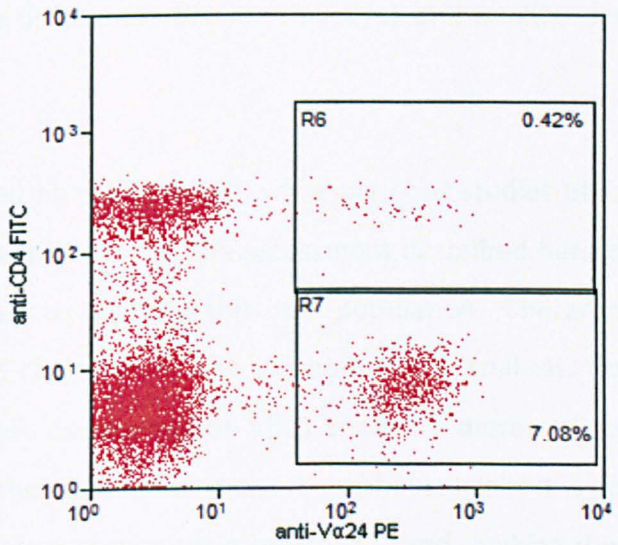


Figure 3.6 Atypical *i*NKT cell frequency observed in peripheral blood from Patient 5 on Day 0. The patient (a woman, 50 years of age) presented in the clinic with a largely expanded *i*NKT cell population. This expansion was especially noticeable in the $CD4^-$ subset, while $CD4^+$ subset was still above the mean frequency observed for RAPB group.

As mentioned in the Introduction and in the background section of this chapter a number of studies identifying a reduced number of *i*NKT cells in various autoimmune conditions, including RA, have been published. In contrast, while data obtained in the experiments presented in this chapter show a trend towards diminished *i*NKT cell numbers in RA, the differences between patients and healthy donors are much less pronounced.

This could be caused by the fact that, while previous studies utilized both Vα24 and Vβ11 chains to detect *i*NKT cells, the assessment described here permitted for a less restrictive definition to identify this cell population. Therefore the cells which expressed other Vβ chains were also included in the analysis. However, it could be possible that the cells expressing the Vβ11 chain are more severely compromised in the disease due to their distinctive properties. Theoretically it is possible that the TCR Vα24 Vβ11 combination recognises a selective ligand, making them especially prone to activation-induced cell death if this antigen was expressed in the disease, while other $V\alpha24^{+}$cells could be relatively spared. At this point in time it has not been established, however, whether this possibility is actually true or not.

The other likely explanation for the observed discrepancy is that, while the size of the patient and control cohorts was similar in presented work compared with the other studies, standard deviation is quite substantial. Therefore the lack of observed significance could be due to statistical issues, i.e. if the number of samples was increased substantially, the results could potentially reach significance

It is also important to reiterate that the initial data on type 1 diabetes in identical twins/triplets was refuted by the work of Lee *et al.* (Lee *et al.* 2002) and Oikawa *et al.* (Oikawa *et al.* 2002). However, the former study employed a very restrictive

definition for the assessment of *i*NKT cells, i.e. CD1d tetramer positivity as well as $V\alpha24^+$ and $V\beta11^+$ staining, narrowing the pool of the detected cells even further. The latter study on the other hand examined various disease manifestations (i.e. islet-associated autoantibody-positive type 1 diabetes, autoantibody-negative type 1 diabetes and latent autoimmune diabetes in adults, LADA), which could have influenced the results. Nonetheless, it could happen that the results presented here identified a similar lack of a substantial difference in *i*NKT cell abundance between healthy people and patients suffering from RA.

Although the reduction of *i*NKT cell frequency in RA is only observed as a trend in the present study, reasons which could be responsible for this phenomenon are worthwhile to consider. There are several possibilities that could explain, at least partly, the lower prevalence of *i*NKT cells detected in both peripheral blood and synovial fluid of patients diagnosed with the disease. Theoretical possibilities include activation-induced cell death (AICD), the downregulation of T cell receptors after antigenic encounter, low thymic output or competitive overgrowth of other T cell populations.

AICD seems the most obvious possible mechanism leading to the reduced levels of $V\alpha24^+$ NKT cells in patients with RA. In T cells, this is driven predominantly by the expression of Fas and Fas ligand which activates caspase–dependent signalling pathways, leading to consequent apoptosis of activated cells. These events lead towards the contraction (or even deletion) of a given T cell clone. The importance of the Fas pathway has been shown in murine NKT cells (Leite-de-Moraes *et al.* 2000) and the involvement of the caspase pathway in also likely in humans. However, other mechanisms, such as those involving c-Myc or tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) could be also involved (Green *et al.* 2003).

The reduction of anti–Vα24 staining in the samples from RA sufferers could indicate a downregulation event of the T cell receptors occurring after the activation of *i*NKT cells. It is well established that TCR ligation causes a loss of these receptors from the cell surface to avoid overstimulation and this also happens after *i*NKT cell activation (Crowe *et al.* 2003; Wilson *et al.* 2003). Historically, *i*NKT cells were erroneously regarded as dying after exposure to αGalCer as they lose Vα24 expression when analysed by flow cytometry. Intracellular staining for TCR chains or PCR to investigate expression of this TCR chain could help to verify this possibility in future work.

While these two explanations are plausible, currently there is no experimental or observational data supporting these mechanisms. It is also not easy to predict the localisation of this antigenic presentation. Given that the trend towards *i*NKT cell reduction can be observed in the peripheral blood this suggests that the antigenic stimulation takes place centrally, e.g. in the thymus, liver or intestines, rather than in the joints. Frequently seen extra-articular manifestations of the disease could argue for this possibility, although *i*NKT cell involvement in these is not known.

Another possible mechanism leading to lower *i*NKT cell prevalence in the patients is a premature decline of thymic output, years before the onset of RA. The thymus is mostly in involution by adolescence, hence reduced number of cells emigrating to the periphery could, at least partly, explain the decreased *i*NKT cell numbers observed in healthy adults with aging. In addition, although an accelerated reduction in T cell output has been observed in RA patients (Koetz *et al.* 2000), this does not satisfactorily explain the selective reduction of the *i*NKT cell pool reported by various investigators. Therefore selective impairment of $V\alpha24^+$ cell generation in the thymus must be considered. This could be for example caused by a decreased expression of a selecting ligand in RA, which cannot be tested at present.

Potential selective reduction in thymic output of *i*NKT cells could also be a result of the intrinsic properties of these lymphocytes. It is conceivable that patients with RA express a gene which causes some sort of a replicative block uniquely affecting this cell population during generation in the thymus or their proliferation in the periphery. This could be suggested by genetic predisposition to RA in families, although a genetic link could be also related with other mechanisms.

To compensate for the decline in the number of recent thymic emigrants (RTEs), Goronzy and Weyand propose an accelerated proliferation of naive T cells which is followed by a contraction of T cell clonal diversity (oligoclonality) (Goronzy and Weyand 2001). However, the extent of *i*NKT cell proliferation in the disease has never been investigated by the same measures, so there are no data regarding *i*NKT cell output and their consecutive fate. While this population can have a different rate of turnover than conventional T cells, it is likely that it undergoes similar expansion and consecutive contraction.

While the competitive exclusion of $V\alpha24^+$ cells cannot be completely ruled out, it is equally possible that the observed reduction in frequencies is not caused by "proliferative cell exhaustion", but instead the *i*NKT cell pool may appear to contract due to expansion of autoaggressive cells found in the classical T cell population or the non-invariant CD1d-restricted cells taking over their physiological niche.

It is also important to consider telomere erosion which could limit the proliferative potential of *i*NKT cells. Accordingly, it is worth mentioning, that premature telomere shortening has been found in both myeloid and lymphoid lineages in RA (Schonland *et al.* 2003). However, as telomerase activity can be detected in cells characterised by high rates of proliferation, such as lymphocytes (as reviewed in (Weng *et al.* 1997), it is easy to envisage that the enzyme functions in *i*NKT cells as well, enabling these cells to rebuild lost telomeric fragments, at least partly. The activity of telomerase in

peripheral blood lymphocytes has been demonstrated to be increased in RA and some other connective tissue diseases (Tarhan *et al.* 2008). Thus, it is hard to envisage a scenario that leads to a selective reduction of telomerase activity in NKT cells.

It would be possible to discriminate between these proposed explanations of *i*NKT cell reduction in RA. This would include a wide series of experiments, i.e. the examination of telomere lengths, telomerase activity and TCR excision circle (TREC) content. These experiments would be very informative, however they are extremely challenging in a lymphocyte population of this size.

Moreover, once the endogenous ligand is identified, its expression in the thymus (to assess the *i*NKT cell selection process) or in the blood and affected joints could be measured to compare peripheral activation sites. This set of experiments would include investigation of apoptotic death and assessment of Vα24 expression status over the course of time after the stimulation with this ligand to identify the role of AICD and TCR downregulation on the cells. Although some of these experiments are theoretically possible, they were beyond the constraints of what was feasible in the present study.

Apart from the general reduction in prevalence, the number of $CD4^+$ *i*NKT cells was found to be significantly reduced in RA peripheral blood and synovial fluid samples and a trend towards increased number of $CD4^-$ cells was observed (Figure 3.4). This could suggest preferential accumulation of the $CD4^-$ subset in the joints, which contrasts with the data from an earlier study (Maeda *et al.* 1999). This observed alteration in subset abundance could be potentially due to the pattern of activation and adhesion molecules expressed (Lin *et al.* 2006) as mentioned before. Therefore, it would be quite interesting to investigate the possibility of differences in the homing receptor profiles of *i*NKT subsets in synovial fluid compared to peripheral blood.

Another possibility is that the $CD4^-$ subset has slightly better *in situ* proliferative properties than the $CD4^+$ subset. It would be also informative to investigate expression of co-stimulatory molecules by the CD4 subpopulations and the role of the CD4 molecule itself in antigen–driven proliferation to explore this possibility.

Based on the data presented in this chapter, it is unlikely that the diminished numbers of circulating or accumulating *in situ* *i*NKT cells observed in RA are the primary problem that causes disease in patients. Moreover, it is also possible that the alteration of CD4 subset contribution does not change the joint milieu sufficiently to influence the pattern of the following adaptive immune response. However, while the change in prevalence is not significant, functional deficiencies could be still substantial. This possibility is investigated in the following chapters.

Chapter 4: Proliferative responses of T cells expressing Vα24 TCR chain in rheumatoid arthritis

Various animal models of autoimmune diseases were tested for numerical changes in *i*NKT cell population as mentioned in the Introduction and previous chapter. However, publications describing differences in *i*NKT cell expansion profiles observed in the animals after antigenic stimulation *in vivo* are practically non-existent. Similarly, in the majority of human studies functional characterisation was based entirely on cytokine secretion profiles.

There are no studies describing the extent of proliferative defects in NKT cell population in animal models of inflammatory arthritis. However, two of the human studies made an attempt to investigate the issue of *i*NKT cell expansion in RA patients. Both reported diminished *i*NKT responsiveness to the artificial ligand, α-galactosylceramide (αGalCer) in affected individuals.

The first publication by Kojo *et al.* investigated various autoimmune diseases, namely RA (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and Sjögren's syndrome (SS) (Kojo *et al.* 2001). The work was carried out on samples collected from only 10 patients and these were exclusively samples of peripheral blood (Kojo *et al*, 2001). The concentration of αGalCer used was the one typically used in the expansion assays, i.e. 100 ng/ml. The measurement of *i*NKT cell enrichment in αGalCer-exposed cultures was conducted once, on day 10 of the experiment. Furthermore, the cultures were also stimulated with IL-2 from the first day. An arbitrary threshold for allocating patients into either responder or non-responder group was set as *i*NKT cell enrichment of at least 5-fold. The results indicate that peripheral blood *i*NKT cells of a significant group of patients, including up to 70% of all the RA sufferers fail to proliferate following αGalCer exposure.

In addition the authors carried out some experiments to explain the reason for *i*NKT cell unresponsiveness. These involved co-culturing APCs and *i*NKT cells of patients with *i*NKT cells or APCs from healthy donors. These experiments seemingly pointed to a TCR defect affecting *i*NKT cell population in the diseased individuals. However, limitations of the experimental approach make it impossible to unambiguously state the primary cause of the observed unresponsiveness. First of all, the number of samples in disease groups, including RA, was extremely small (one patient in each group). However, the main reason for the difficulty of interpretation is the possibility of alloreactive T cell proliferation in the co-cultures of cells from different donors. The increasing *i*NKT cell numbers in these cultures could potentially be observed due to the alloreactivity resulting in effecting in rising endogenous cytokine secretion being erroneously classified as a true antigen-driven proliferative response.

The αGalCer expansion experiments carried out by Linsen *et al.* (Linsen *et al.* 2005) included 13 samples of peripheral blood as well as 5 synovial fluid samples from RA patients. While these experiments were set up with the same antigen concentration as in the previously described study, the assays were complicated by including a re-stimulation step on day 7 of cultures. At this time point the cultures were also supplemented with IL-2. The assessment of αGalCer-reactivity was carried out after a week from the restimulation step (on day 14 from the start of cultures). The allocation to the responder/non-responder groups was based on the enrichment of at least 2% in the cultures. The results suggested that close to 50% of RA patients fall into the non-responders group. Surprisingly, the study also reported greater expansion in the responder group of RA patients than in the healthy donors and that responsiveness of synovial fluid *i*NKT cells was reported to be intact even in the patients whose peripheral blood cells did not respond to stimulation with αGalCer.

These reports suffer from a number of limitations. Firstly, the expansion assays described in both cases were set up with rather small numbers of donors. The

frequencies of proliferating *i*NKT cells in both of described studies were assessed only once during the culture period, at different time points. Moreover, the re-stimulation step and/or addition of exogenous cytokines unnecessarily complicate these assays. Furthermore, arbitrarily set, rather high thresholds of "responsiveness" used to characterize patients fail to give a complete picture of the true proliferative capacity of these cells.

Given the limitations of the previous human data it was decided that the αGalCer-driven proliferative capacity of NKT cells should be re-addressed. The results of this work are described in this chapter.

To assess the proliferative potential of *i*NKT cells, expansion experiments were conducted, as described in Materials and Methods. Briefly, peripheral blood mononuclear cells from 20 healthy donors (H), as well as 22 blood samples from patients (RAPB) and 12 synovial fluid samples (RASF), were isolated by gradient centrifugation. Cells were then stimulated with αGalCer or iGb3 at a concentration of 100 ng/ml and cultured for 14 days. At these circumstances various CD1d positive cells present in the culture acted as APCs.

100,000 cells were stained with anti-Vα24 PE and anti-CD4 FITC antibodies. Initially, to assess the time course of response, aliquots of cultured cells were collected daily for ten consecutive days (days 0 to 9), day 12 and day 14. After these primary experiments, the cells were harvested four times during a 14 day period and stained as before.

Flow cytometric analysis was conducted as described in Materials & Methods. Firstly, a gate ("a lymphocyte gate") was set on the FS/SSC profile. The gate was broader than described in the previous chapter as activated cells changed their physical properties (Figure 4.1 A). Next, $CD4^+$ and $CD4^-$ populations were assessed as shown in Figure 4.1 B.

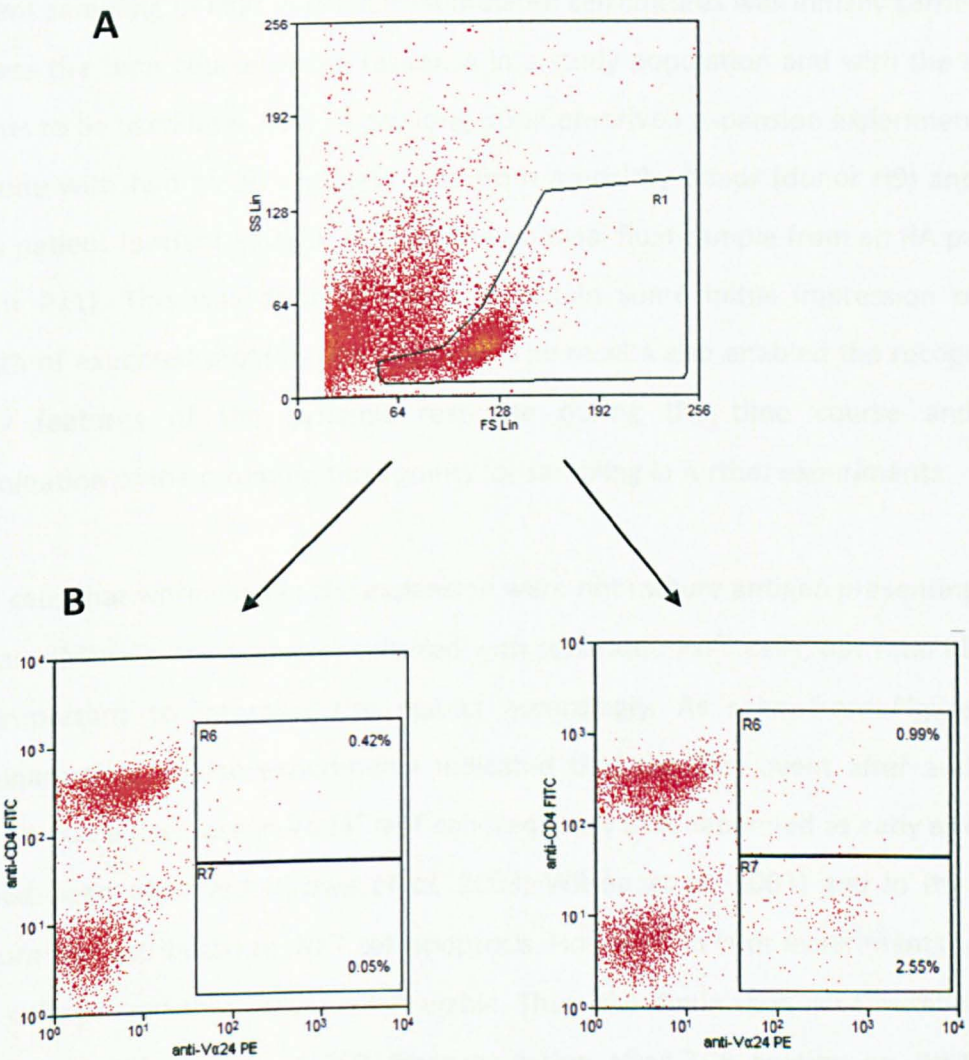


Figure 4.1 The gating used for analysis of anti-Vα24/CD4 staining during the NKT cell expansion assay (day 10, Healthy donor shown). Mononuclear cells were incubated with αGalCer for a period of two weeks. The cells were than collected on day 4, 7, 10 and 14 and stained with anti-Vα24 PE and/or CD4 FITC antibodies. A) lymphocyte gate B) $CD4^+$ $V\alpha24^+$ and $CD4^-$ $V\alpha24^+$ populations identified.

4.3. Time course of αGalCer–driven proliferation of iNKT cells

Frequent sampling of cells in αGalCer-stimulated cell cultures was initially carried out to assess the time course of the response in a study population and with the set of reagents to be used later. The 14 day long αGalCer–driven expansion experiment was first done with two blood samples: one from a healthy donor (donor H9) and one from a patient (patient P10), as well as one synovial fluid sample from an RA patient (patient P11). This was done primarily to obtain some initial impression of the strength of expected proliferative response. The results also enabled the recognition of key features of the dynamic response during the time course and the determination of the optimum time points for sampling in further experiments.

As the cells that were used in the expansion were not mature antigen presenting cells (such as mMDDCs, for example) cultured with separated *i*NKT cells, but total PBMCs, it is important to interpret the results accordingly. As seen from Figure 4.2, preliminary time course experiments indicated that the first event after antigenic exposure was a decrease in $V\alpha24^+$ NKT cell frequency that happened as early as day 1. This had been observed (Crowe *et al.* 2003; Wilson *et al.* 2003) and in the early literature was attributed to *i*NKT cell apoptosis. However, in later experiments tracing $V\alpha24^+$ cells proved that they are still viable. Thus, the diminished *i*NKT numbers are thought to be the result of TCR downregulation after TCR ligation on *i*NKT cells (Wilson *et al.* 2003).

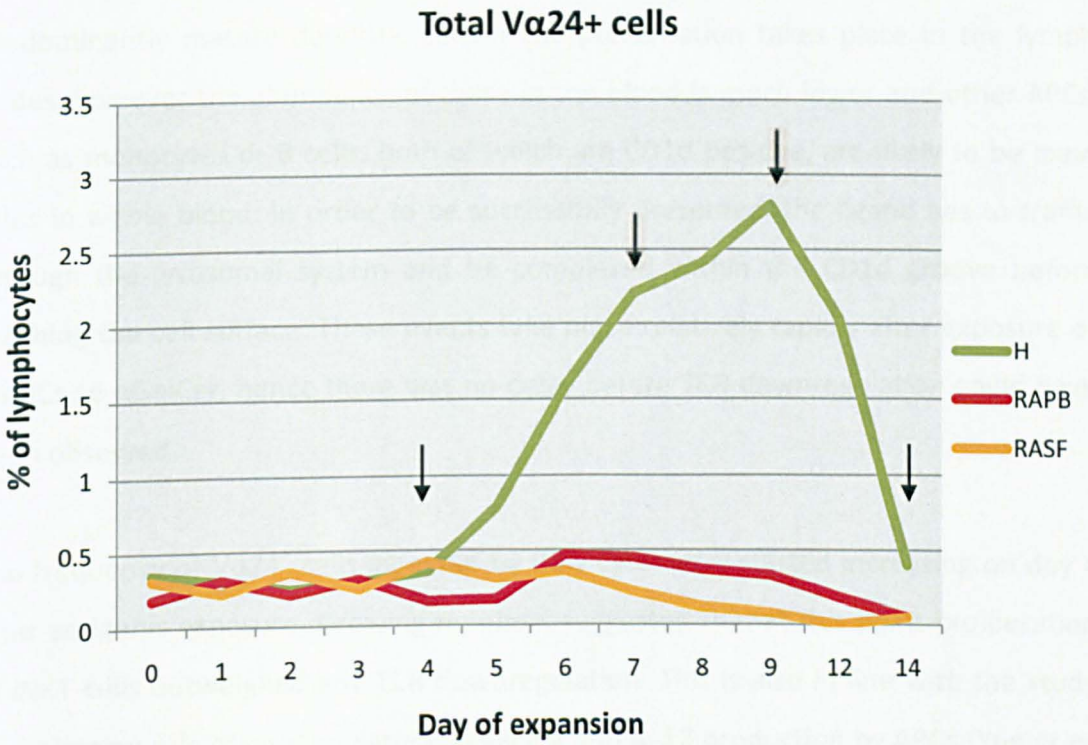


Figure 4.2 Time course of αGalCer–driven expansion of Vα24^{+} NKT cells.

PBMCs from peripheral blood of one healthy donor (H, green), one RA peripheral blood (RAPB, red) and one synovial fluid (RASF, yellow) sample were exposed to αGalCer and cultured over a period of two weeks. Aliquots were collected for ten consecutive days (day 0 to day 9), day 12 and day 14 and stained with anti–CD4 and anti–Vα24 antibodies. Samples were analyzed by flow cytometry. /NKT cell frequencies were assessed in the lymphocyte gates. Arrows indicate time points (day 4, day 7, day 10, day 14) chosen for experiments as representative of the expansion profile.

Although TCR downregulation on day 1 is the first event than could be detected using flow cytometry, it is important to remember that it is likely to be a consequence of αGalCer presentation via CD1d molecules expressed by APCs. These are predominantly mature dendritic cells if the presentation takes place in the lymph nodes, however the abundance of these in the blood is much lower and other APCs, such as monocytes or B cells, both of which are CD1d positive, are likely to be main APCs in whole blood. In order to be successfully presented, the ligand has to traffic through the lysosomal system and be complexed within the CD1d groove before reaching the cell surface. These events take place relatively rapidly after exposure of PBMCs to αGalCer, hence there was no delay before TCR downregulation could have been observed.

The frequency of $V\alpha24^+$ cells assessed by flow cytometry started increasing on day 4 after antigenic exposure. Growing numbers suggested that at this point proliferation of *i*NKT cells outweighed any TCR downregulation. This is also in line with the study investigating role of co-stimulatory signalling and IL-12 production by APCs (Yue *et al.* 2005).

The maximum responses were observed between day 7 and day 12 from the first exposure to αGalCer. On continuation of the assay a very clear decrease in $V\alpha24^+$ cell numbers was seen on day 14. Based on these observations the time points for the following experiments were chosen as day 4, day 7, day 10 and day 14 (as indicated by arrows in Figure 4.2).

Although this experiment was carried out to establish the timing for further experimental sampling from the cultures of peripheral blood and synovial fluid mononuclear cells, even this preliminary experiment showed differences in the expansion profiles of *i*NKT cells from RA patients compared to the healthy donor (Figure 4.2).

Some initial experiments included four young healthy volunteers (male aged 22, female aged 26, male aged 28 and female aged 30). The αGalCer-driven *i*NKT cell expansion in three of these donors (including two related males) was striking. Figure 4.3 shows the dot plots obtained from one of these individuals during the 14 day long culture. As the expansion seen in these younger donors far exceeded that seen in the older healthy donors, who were age matched to the RA patients, to avoid skewing the expansion data in the healthy donor group, these individuals were removed from the overall analysis.

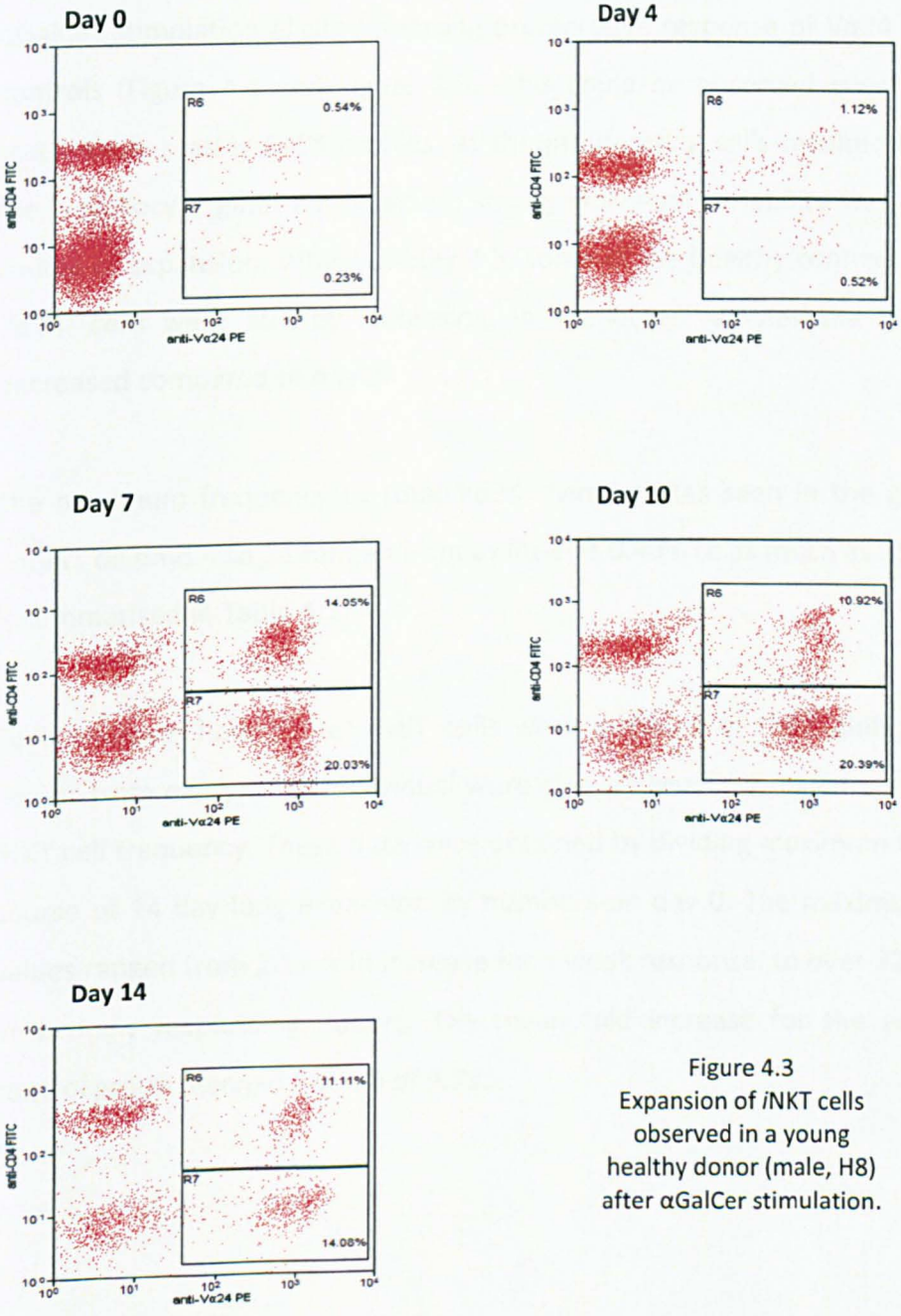


Figure 4.3
Expansion of *i*NKT cells observed in a young healthy donor (male, H8) after αGalCer stimulation.

αGalCer stimulation elicited a strong proliferative response of Vα24$^+$ cells in healthy controls (Figure 4.4 and Figure 4.5). This could be observed even on the forward scatter/side scatter FACS profiles, as the proliferating cells resulted in a widening of the lymphocyte gate. As expected, strong individual variability was observed in the course of expansion. Whilst on day 4 in some of the healthy controls the numbers of Vα24$^+$ cells were already increasing, in the other samples the frequencies were decreased compared to day 0.

The maximum frequency of total Vα24$^+$ lymphocytes seen in the group of healthy donors on days 4 to 14 ranged from as little as 0.40% to as much as 15.93%. The data is summarised in Table 4.1.

To investigate how potent *i*NKT cells were in terms of their ability to proliferate, results from every single individual were also analysed for maximum fold increase of *i*NKT cell frequency. These data were obtained by dividing maximum frequency in the course of 14 day long expansion by numbers on day 0. The maximum fold increase values ranged from 2.15-fold increase for a weak response, to over 32.5–fold increase in strongly responding donors. The mean fold increase for the expansion in the control group reached a value of 9.73.

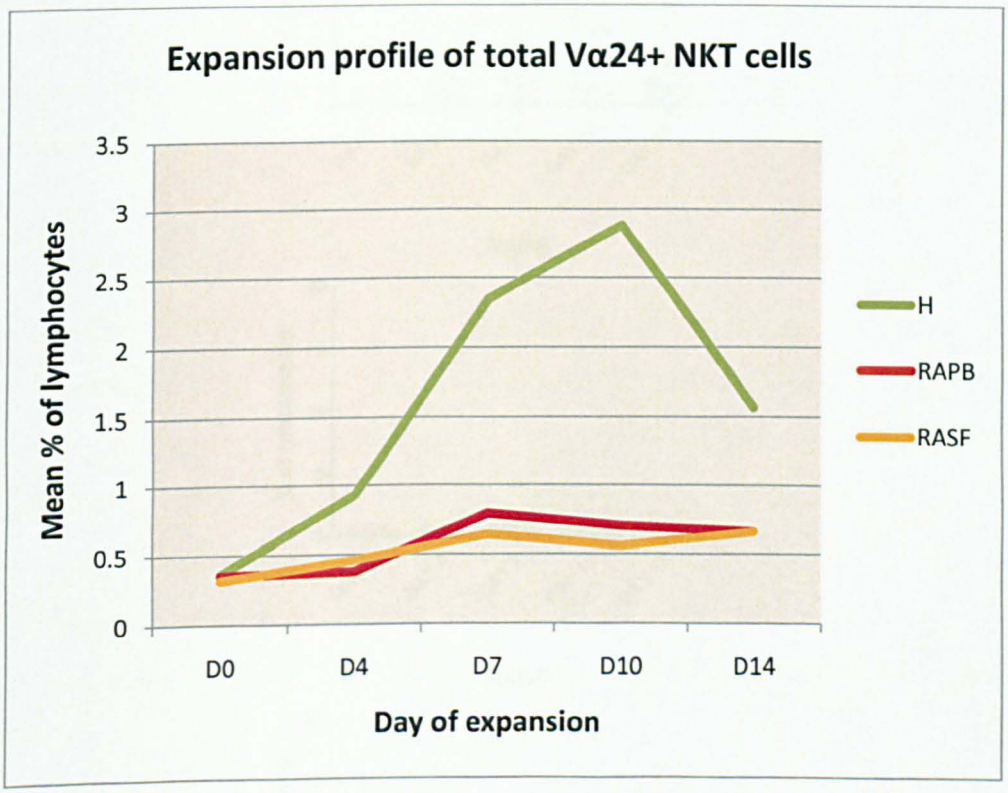


Figure 4.4 Expansion profiles of total Vα24^{+} NKT cells for H, RAPB and RASF. αGalCer-stimulated expansion of total Vα24^{+} NKT cells from the experimental groups: healthy donors (H, green, n=20), RA peripheral blood (RAPB, red, n=22) and synovial fluid (RASF, yellow, n=12) from RA patients. Mean frequencies from day 0, day 4, day 7, day 10 and day 14 are shown. The error bars were omitted for the purpose of legibility; the inter-individual variation is depicted in the next figure (Figure 4.5).

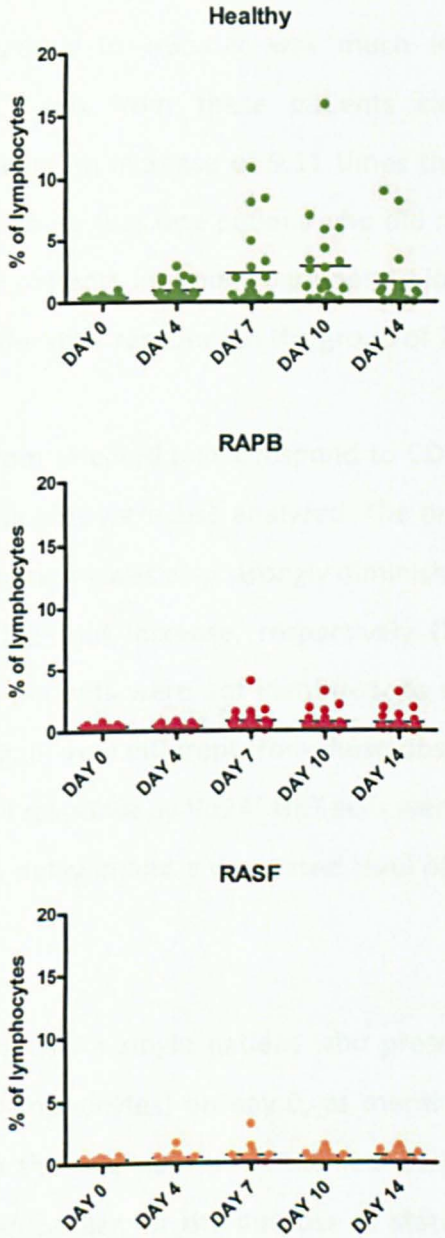


Figure 4.5 Frequencies of total *i*NKT cells in experimental cultures during the 14 day-long expansion experiment. Samples shown are: peripheral blood from donors (H, green, n=20), RA peripheral blood (RAPB, red, n=22) and synovial fluid (RASF, yellow, n=12) from RA patients. Median frequencies are shown.

In contrast to the group of healthy donors, when PBMC and SFMC from the RA patients were analyzed the response to αGalCer was much less pronounced (Table 4.1). Peripheral blood Vα24$^+$ cells from these patients clearly responded weaker to stimulation, with the maximum increase of 9.11 times the frequency of that of day 0 (Table 4.1). In this group there was one patient who did not respond to αGalCer at all, representing 4.5% of all patients in whom peripheral blood *i*NKT cell responses were analyzed. The mean proliferative response in the group of 22 patients was 4.11 fold.

To see how *i*NKT cells from affected joints respond to CD1d–mediated presentation of αGalCer, synovial fluid samples were also analyzed. The proliferative response of Vα24$^+$ cells from this group of samples was also strongly diminished, with mean and maximum responses of 3.4- and 7.2–fold increase, respectively (Table 4.1). However, in this analysis, non-responsive patients were not identified. As shown in the table, ranges of *i*NKT frequencies were again very different from those observed in the healthy controls. The profiles of the overall response by Vα24$^+$ NKT cells were very similar for both PBMCs and SFMCs, suggesting a delay in and a decreased level of proliferation (Figure 4.4 and Table 4.1).

In the RAPB group there was a single patient who presented with a very large *i*NKT population (7.5% of all lymphocytes) on day 0, as mentioned in the previous chapter (Paragraph 3.5). Due to the cell numbers obtained during the assay, this particular patient was treated as an outlier for the purpose of statistical analysis. However, it is interesting to mention that αGalCer exposure caused a proliferation with maximum enrichment of just over 18% seen and a maximum fold increase of 2.43, which was below the mean increase in the RAPB group.

Table 4.1. Expansion of total *i*NKT cells in the experimental groups.

	Day 0 frequency		Highest frequency		Fold increase			Non–responders	
	Min	Max	Min*	Max	Min	Max	Mean	Number/all samples	%
Healthy	0.16	0.93	0.40	15.93	2.15	32.51	9.73	0 / 20	0
RAPB	0.14	0.73	0.23	3.98	1.07	9.11	4.11	1 / 22	4.5%
RASF	0.08	0.61	0.27	3.1	1.9	7.2	3.41	0 / 12	0

Expansion frequencies assessed in the responding donors, non – responsive individuals excluded. Donors were assigned as non–responders if *i*NKT cell frequency on day 0 was equal or higher than a maximum *i*NKT frequency during expansion;* for responding donors.

Table 4.2 P values obtained for the expansion of total *i*NKT cells.

P value	Day 0	Day 4	Day 7	Day 10	Day 14
H vs. RAPB	> 0.05	**0.002**	**0.037**	**0.01**	> 0.05
H vs. RASF	> 0.05	> 0.05	> 0.05	**0.041**	> 0.05
RAPB vs. RASF	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Significant differences identified by Student T test ($p<0.05$) shown in red.

Following the initial assessment of proliferation experiments, additional statistical tests were carried out. This second analysis was more stringent to ensure that the most appropriate test was used to provide a description of the expansion over a period of time. Therefore a time-series analysis was performed which used two- and three- factor within subjects ANOVA. The analysis was carried out with and without post-hoc Bonferroni correction.

Although the SPSS analysis required a few samples to be removed as outliers (Figure 4.6), it confirmed previously obtained results. Total *i*NKT cells derived from the blood of healthy volunteers increased in number with time. The pairwise comparison was highly significant between the control and patient groups (repeated measures ANOVA p=0.001 and 0.003 for H vs. RAPB and H vs. RASF; confidence intervals: between 0.445 and 1.636 for the former and 0.387 and 1.795 for the latter), but was not significant between both RA patients PBMC sources (p=0.884; confidence interval: between 0.8020 and 0.9035). This was also true when Bonferroni correction was applied (respective p values: 0.003; 0.009, 1.000; confidence intervals: between 0.3060 and 1.7743; between 0.2232 and 1.9586; between -0.9035 and 0.8020, respectively).

There was marked inter-individual variability in observed response; the lack of response of *i*NKT cells from RA blood and synovial fluid reflected a general reduction in proliferative capacity of *i*NKT cells in these samples.

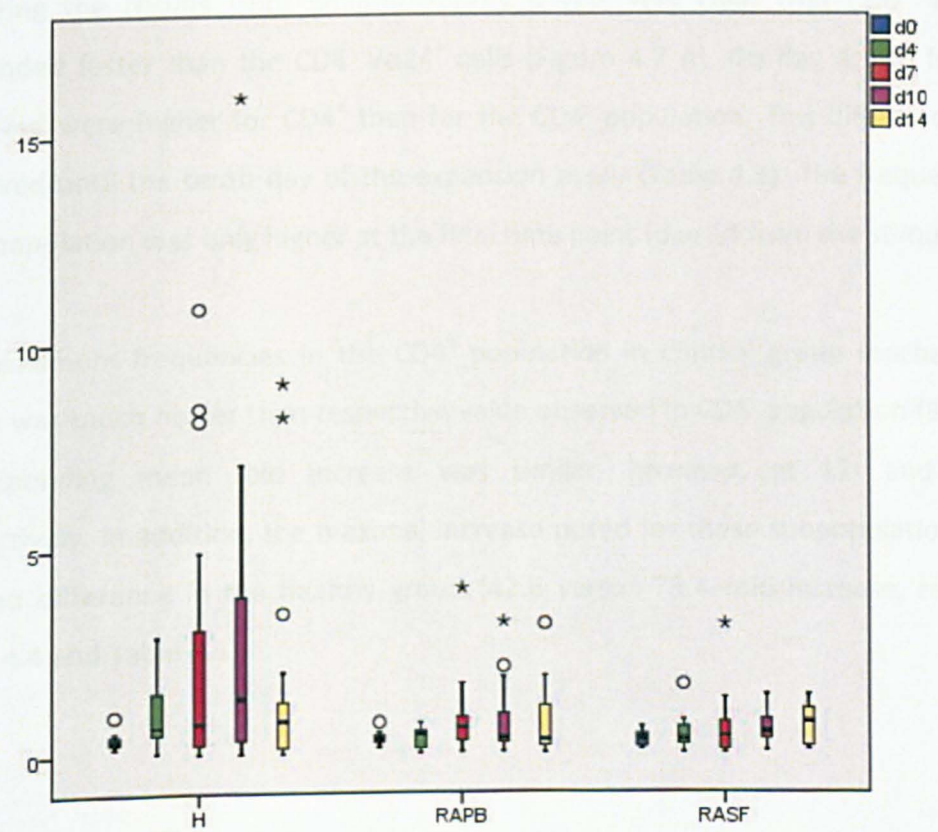


Figure 4.6 Second analysis of expansion results of total *i*NKT cells. repeated measures ANOVA p=0.001 and 0.003 for H vs RAPB and H vs RASF without Bonferroni correction; respective values with the correction applied: 0.003 and 0.009).

As mentioned in the Introduction and in Chapter 3 $CD4^+$ and $CD4^-$ *i*NKT cells show significant differences in biological behaviour. The data acquisition strategy in this study was designed to allow for the separate assessment of these NKT cell subsets. When analyzing the results from healthy donors it was very clear that $CD4^+$ $V\alpha24^+$ cells responded faster than the $CD4^-$ $V\alpha24^+$ cells (Figure 4.7 A). On day 4, the frequencies observed were higher for $CD4^+$ than for the $CD4^-$ population. This difference was still observed until the tenth day of the expansion assay (Table 4.3). The frequency of the $CD4^-$ population was only higher at the final time point (day 14 from the stimulation).

The maximum frequencies in the $CD4^+$ population in control group reached 14.48%, which was much higher than respective value observed in $CD4^-$ population (8.97%). The corresponding mean fold increase was similar, however, at 11- and 12.7-fold, respectively. In addition, the maximal increase noted for these subpopulations showed marked difference in the healthy group (42.6 versus 78.4–fold increase, respectively, Table 4.4 and Table 4.5).

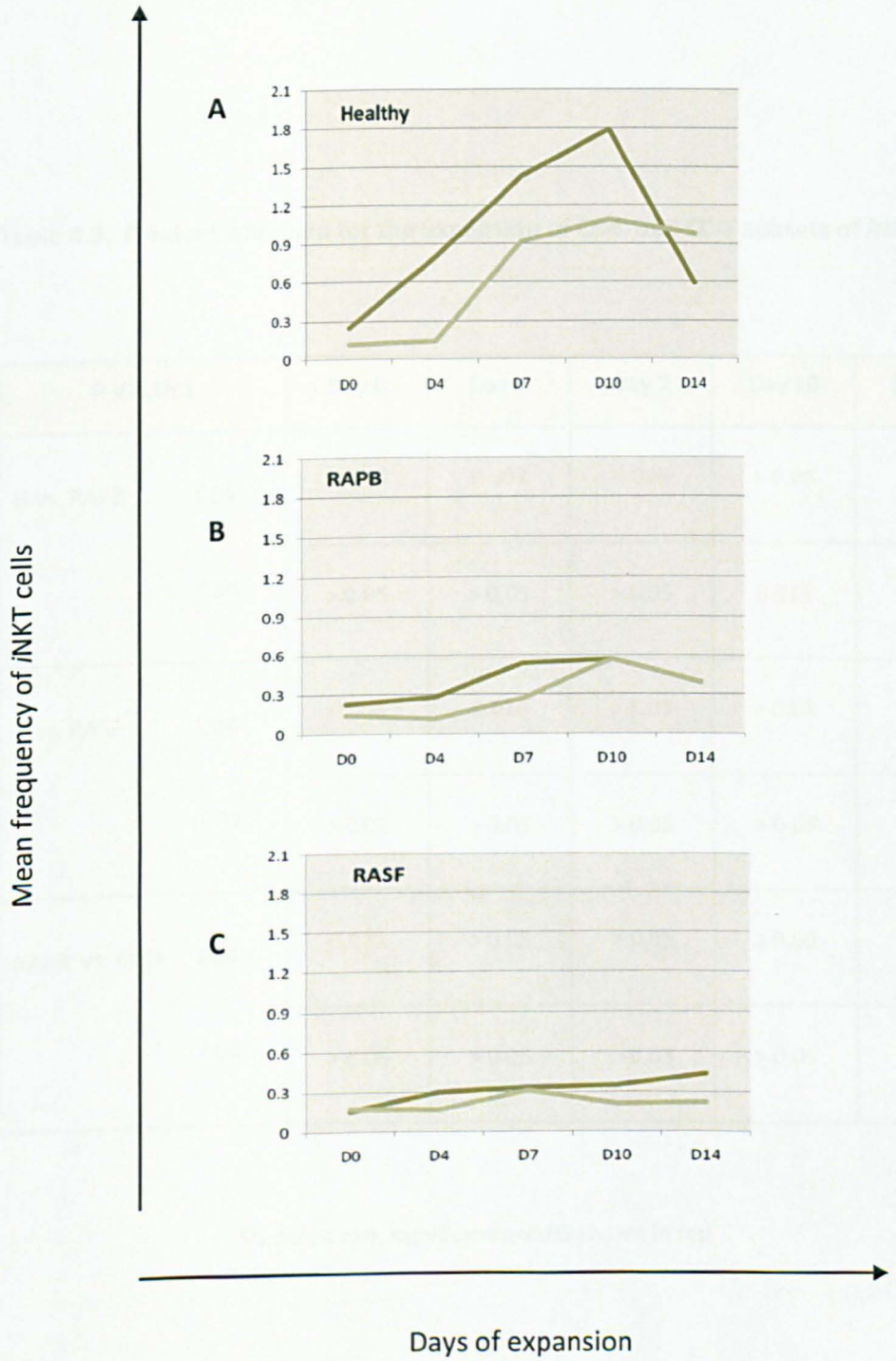


Figure 4.7. Differences in proliferative responses of *i*NKT subsets in RA patients. Expansion profiles for $CD4^+$ and $CD4^-$ subpopulations of iNKT cells in healthy controls (H, panel A), peripheral blood of RA patients (RAPB, panel B) and synovial fluid of RA patients (RASF, lower C).

Table 4.3. P values obtained for the expansion of $CD4^-$ and $CD4^-$ subsets of *i*NKT cells

P VALUES		Day 0	Day 4	Day 7	Day 10	Day 14
H vs. RAPB	$CD4^+$	> 0.05	**0.002**	**0.035**	> 0.05	> 0.05
	$CD4^-$	> 0.05	> 0.05	> 0.05	**0.012**	> 0.05
H vs. RASF	$CD4^+$	**0.003**	**0.026**	**> 0.05**	> 0.05	> 0.05
	$CD4^-$	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
RAPB vs. RASF	$CD4^+$	**0.033**	> 0.05	> 0.05	> 0.05	> 0.05
	$CD4^-$	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Student t test, significant $p<0.05$ shown in red

Data comparing PBMCs and SFMCs from the patients with RA did not show much difference. The profiles of expansion differed significantly from those for the healthy control group as shown (Figure 4.7, Figure 4.8 and Table 4.3) both in terms of the time scale and potency of the response. It was unclear whether the maximum values obtained during the 14 day long experiment were actual peak values for the proliferating PBMCs and SFMCs in the patients, as the graphs seemed to fluctuate rather than give an obvious peak.

The most noticeable differences were in the fold increase values in these cultures. The maximum response in the $CD4^+$ subset in patients' peripheral blood was in the range of 1.12 to 8.94-fold (mean=4.2), while in synovial fluid the range of response was narrower (between 2.18 and 6.5-fold; mean=4.14). Corresponding values for the $CD4^-$ population were 1.12-16.38 for the RAPB group and 2- to 9.44–fold for RASF (respective means: 4.2 and 3.9) as seen in Table 4.4 and table 4.5. When these values were compared with respective fold increase numbers for control donors, statistical significance was reached in differences between both $CD4^+$ (H vs. RAPB p=0.024; H vs. RASF=0.047) and $CD4^-$ (H vs. RAPB p=0.001; H vs. RASF=0.004) subpopulations. When Bonferroni correction was applied, only the comparisons between fold increase of $CD4^+$ subpopulations was still significantly changed (H vs. RAPB p=0.003; H vs. RASF=0.012).

An additional finding was related to the numbers of individuals classified as non–responders. For the $CD4^+$ subpopulation none of the healthy donors was found to be non–responsive. The situation was different in the RAPB patients, as almost 10% showed no $CD4^+$ *i*NKT cell expansion following αGalCer stimulation. However, the analysis did not reveal any non-responders in RASF group.

As for the $CD4^-$ subset, this discrepancy was more pronounced. While $CD4^-$ *i*NKT cells from all healthy donors expanded when exposed to αGalCer, around 10% of samples of

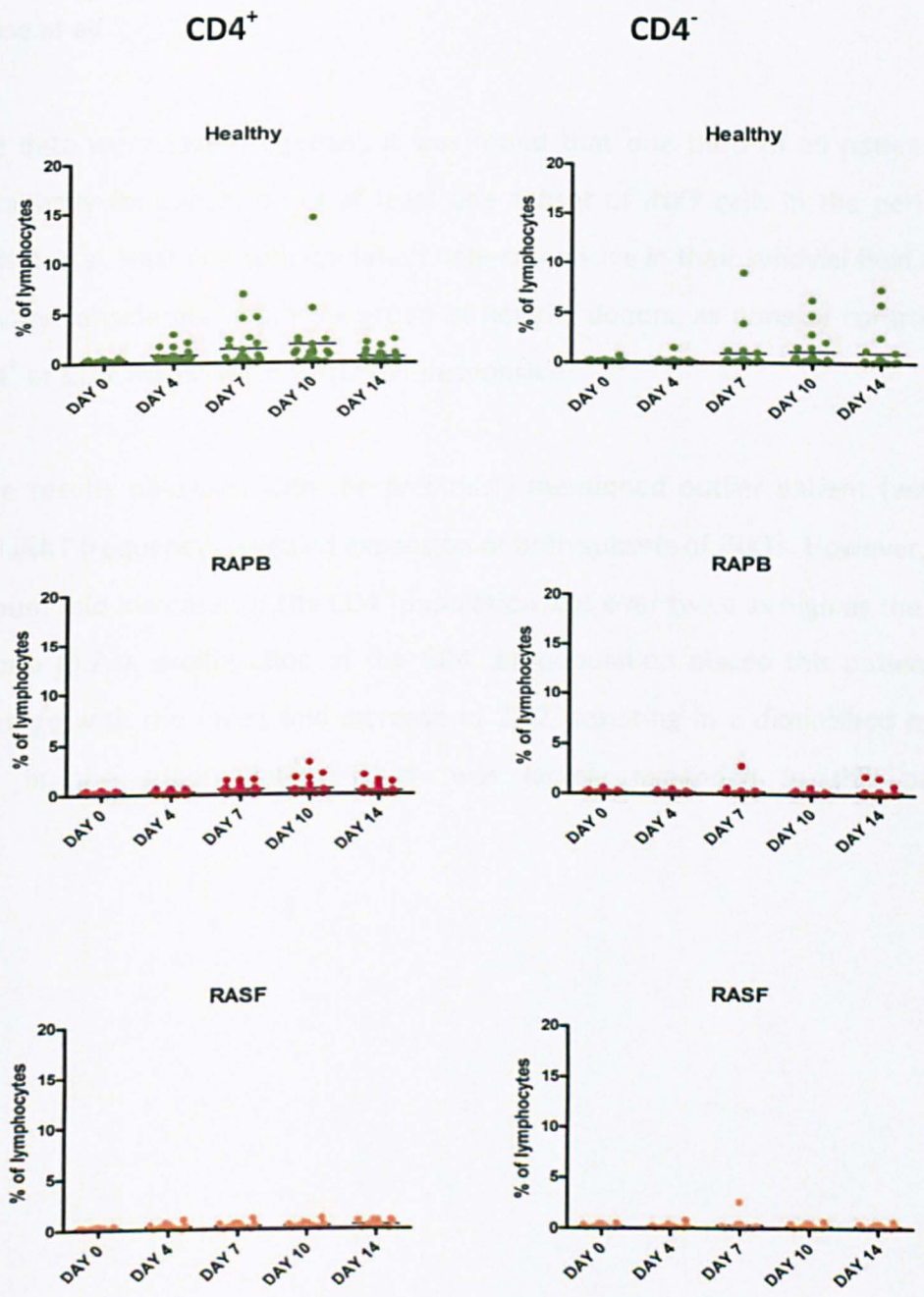


Figure 4.8. Enrichment of $CD4^+$ and $CD4^-$ *i*NKT cells in experimental cultures during expansion. Samples shown are: peripheral blood from controls (Healthy, green), peripheral blood from the patients (RAPB, red) and synovial fluid from the patients (RASF, yellow). Median frequencies are shown.

synovial fluid and 25% of peripheral blood samples obtained from RA sufferers showed no response at all.

When the data were taken together, it was found that one third of all patients had reduced capacity for expansion of at least one subset of *i*NKT cells in the peripheral blood. 10% had at least one subpopulation non–responsive in their synovial fluid. These numbers vary considerably from the group of healthy donors, as none of controls had either $CD4^+$ or $CD4^-$ *i*NKTs which were non–responsive.

Finally, the results observed with the previously mentioned outlier patient (with the high initial *i*NKT frequency) revealed expansion of both subsets of *i*NKTs. However, while the maximum fold increase for the $CD4^+$ population was over twice as high as the mean in this group (9.23), proliferation of the $CD4^-$ subpopulation placed this patient well below average with the mean fold increase of 2.02, resulting in a diminished relative expansion in the subpopulation which was largely expanded in this patient.

Table 4.4. Expansion of $CD4^+$ subset of iNKT cells in the experimental groups.

	Day 0 frequency		Highest frequency		Fold increase			Non–responders	
	Min	Max	Min	Max	Min*	Max	Mean	Number / all samples	%
Healthy	0.12	0.44	0.29	14.48	2.42	42.58	11.03	0 / 20	0
RAPB	0	0.42	0	3.14	1.12	8.94	4.21	2 / 22	9
RASF	0.05	0.25	0.24	0.99	2.18	6.5	4.14	0 / 12	0

Expansion frequencies assessed in the responding donors, non – responsive individuals excluded. Donors were assigned as non – responders if iNKT cell frequency on day 0 was equal or higher than a maximum *i*NKT frequency during expansion; * for the responding donors

Table 4.5. Expansion of $CD4^-$ subset of iNKT cells in the experimental groups.

	Day 0 frequency		Highest frequency		Fold increase			Non–responders	
	Min	Max	Min	Max	Min	Max	Mean	Number / all samples	%
Healthy	0.02	0.62	0.08	8.97	1.4	78.38	12.68	0 / 20	0
RAPB	0.02	0.51	0.04	2.78	1.12	16.36	4.22	5 / 22	23
RASF	0.01	0.41	0.06	2.55	2	9.44	3.91	1 / 12	8

Expansion frequencies assessed in the responding donors, non–responsive individuals excluded. Donors were assigned as non–responders if iNKT cell frequency on day 0 was equal to or higher than a maximum *i*NKT frequency during expansion.

ANOVA analysis was performed to compare the proliferative responses of $CD4^+$ and $CD4^-$ subsets of *i*NKT separately. Again, eve that SPSS removed some outliers (Figure 4.9), there was significantly greater $V\alpha24^+CD4^+$ cell enrichment in cultures of mononuclear cells from healthy donors over time as compared to both RA sources (pairwise comparisons p=0.003 for healthy vs. RA blood, p=0.004 for healthy vs. RA synovial fluid; confidence intervals: 0.204 to 0,946 and 0.218 to 1.095). These results are presented in Figure 4.9. However, no difference was observed between cells from RA blood and synovial fluid (p=0.705, confidence interval: -0.513 to 0.349). When Bonferroni correction was applied, the results were still highly significant for the first two comparisons (p=0.009; and 0.012, respectively; confidence intervals: 0.1172 to 1.0321 and 0.1157 to 1.1971) and non-significant for the comparison between RAPB and RASF sources (p=1; confidence interval between -0.6131 and 0.4496).

$CD4^-V\alpha24^+$ cells also showed decreased proliferation pattern, however it was less significant (the pairwise comparisons between healthy donors and RAPB and RASF groups: p=0.33, confidence interval: 0.04 to 0.891, H for the former and p=0.89, confidence interval between -0.068 to 0.937). Bonferroni correction reduced these effects even more and there was no statistically significant change observed between any of these groups (p=0.098; 0.226, respectively). The difference between RAPB and RASF sources was never statistically significant (p=0.9 and 1.000 for the analysis with and without Bonferroni correction, respectively).

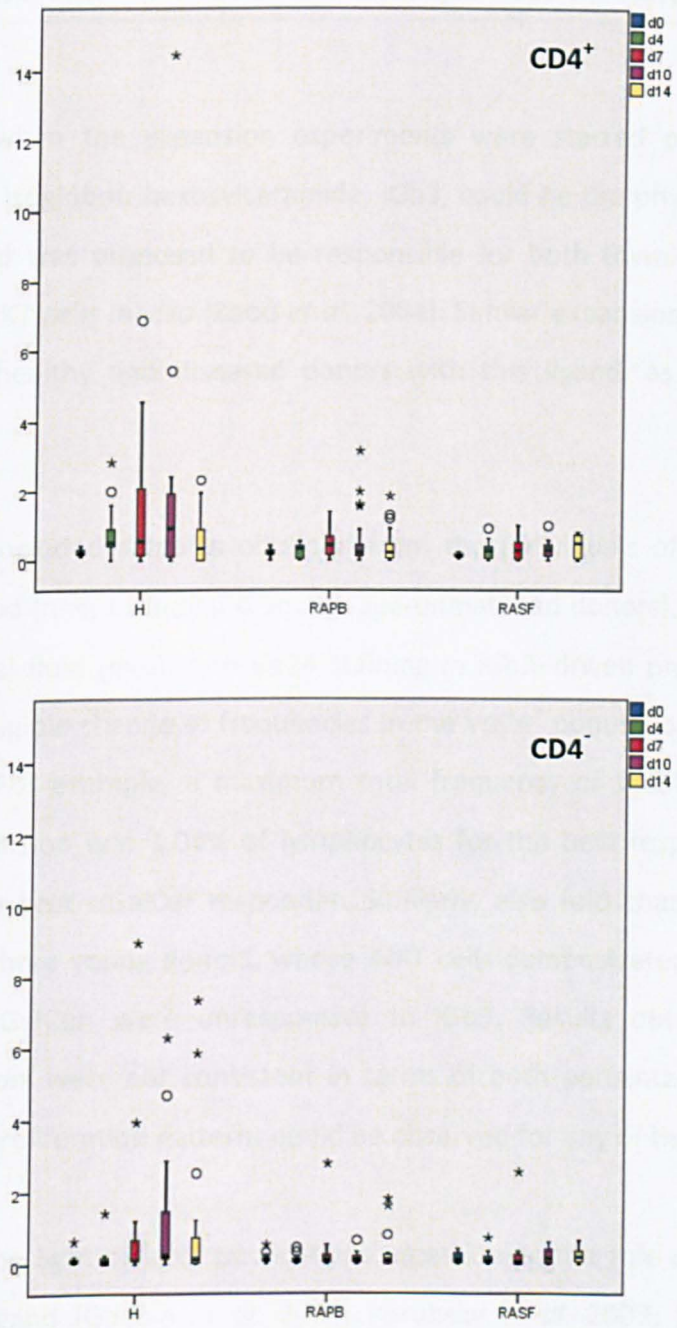


Figure 4.9. Second analysis of expansion results of the CD4 subsets of *i*NKT cells. Pairwise comparisons p=0.003 for healthy (H) vs RA blood (RAPB), p=0.004 for healthy vs. RA synovial fluid (RASF), but no difference was observed between cells from both RA sources (p=0.75). The respective numbers were 0.009, 0.012 and 1.00 when Bonferroni correction was applied.

4.7. Proliferative responses of Vα24$^+$ NKT cells after iGb3 exposure

At the time when the expansion experiments were started publications appeared that proposed that isoglobotrihexosylceramide, iGb3, could be the physiological self CD1d ligand. This compound was proposed to be responsible for both thymic selection and peripheral activation of NKT cells *in vivo* (Zhou *et al.* 2004). Similar expansion assays were therefore set up for some healthy and diseased donors with this ligand, as described in Materials & Methods.

These assays included samples obtained from the individuals of all three groups, healthy peripheral blood (n=6, including 4 young, age-unmatched donors), RA peripheral blood (n=6) and RA synovial fluid (n=6). Anti-Vα24 staining in iGb3–driven proliferation assays revealed practically negligible change of frequencies in the Vα24$^+$ population in healthy donors' group (Figure 4.10). For example, a maximum total frequency of Vα24$^+$ cells observed over the course of expansion was 1.04% of lymphocytes for the best responder, as compared with 34.08% for the best αGalCer responder. Similarly, also fold change was low (mean 1.31). Moreover, all three young donors, whose *i*NKT cells demonstrated an extraordinarily strong response to αGalCer, were unresponsive to iGb3. Results obtained with cells from all remaining donors were not consistent in terms of both percentage and time scale and no clear trend or proliferation patterns could be observed for any of the three groups.

Therefore, in the light of later publications, questioning the role of iGb3 as an endogenous physiological ligand (Gadola *et al.* 2006; Porubsky *et al.* 2007; Speak *et al.* 2007), these experiments were abandoned at an early stage.

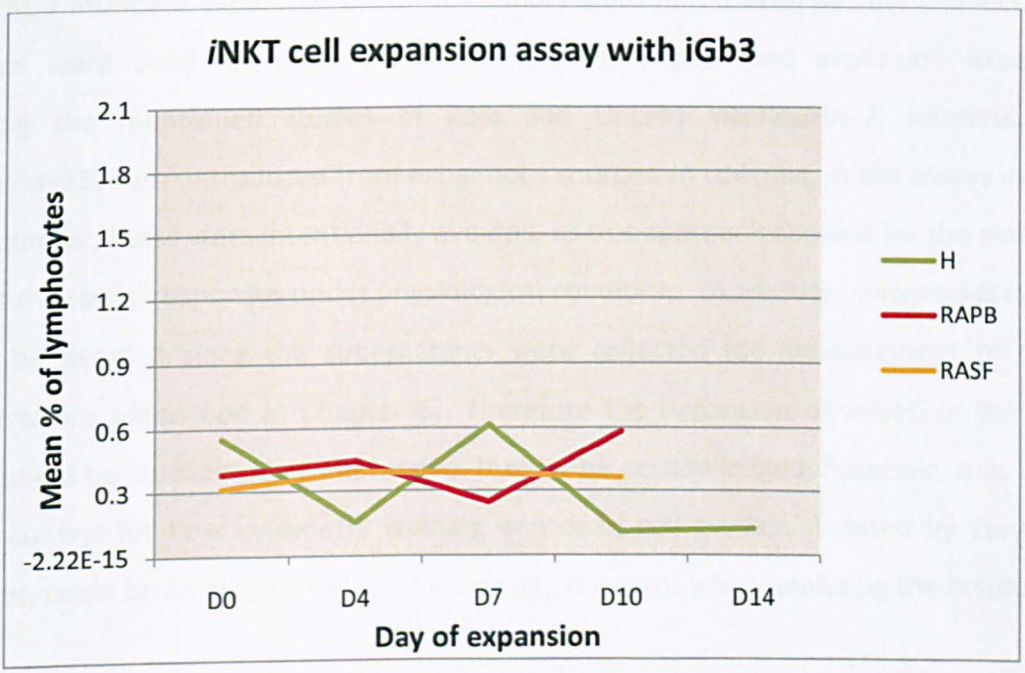


Figure 4.10 Expansion profiles in assays where iGb3 was used as an antigen.

iGb3-stimulated expansion of total Vα24^{+} NKT cells from the experimental groups: healthy donors (H, green, n=6, including 4 young donors), RA peripheral blood (RAPB, red, n=6) and synovial fluid (RASF, yellow, n=6) from RA patients. Mean frequencies from day 0, day 4, day 7, day 10 are shown for all three groups, day 14 frequency is shown only for the control group. The error bars were avoided for the purpose of legibility.

The data obtained in the experiments described in the present chapter generally confirmed previously described observations. The differences in proliferative response between groups as well as a intensive expansion in control donors were noted even despite the fact that no cytokines were used for these assays. In previously published expansion experiments (including the mentioned studies of Kojo and Linsen) interleukin–2, interleukin–7 or interleukin–15, were introduced from exogenous sources. In contrast, in the assays described in this chapter, these were intentionally avoided, as this approach allowed for the assessment of CD1d-mediated responses under physiological conditions. In addition, exogenous cytokines had to be avoided since the supernatants were collected for measurement of cytokine concentrations (described in Chapter 6). Therefore the expansion observed in the healthy group should be considered as impressive. It must be acknowledged, however, that a lack of isotype control for flow cytometry staining and dead cell marker, dictated by very limited resources, could be an additional factor increasing the error when analysing the results.

The reduction, or complete lack, of *i*NKT cell responsiveness both in the blood and in the synovial fluid of RA patients is not a surprising finding. This was described previously in the studies mentioned already (Kojo *et al.* 2001; Linsen *et al.* 2005), where it was found that RA patients belong to one of two groups: either αGalCer responders or αGalCer non-responders. However, synovial fluid samples were not tested in that study. Moreover, according to the blood results presented by Kojo *et al.*, 70% of the patients were recognised as non-responsive. This varied strikingly from the results obtained as only a small fraction of patients could have been termed as "non–responders" based on the lack of proliferation of peripheral blood total *i*NKT cells. This is probably due to the fact that all the patients with less than 5–fold increase in the initial frequency of *i*NKTs were not counted as responders in the previous study, even though their cells proliferated to some extent after the stimulation. In addition, analysis of FACS plot could not fully exclude the dead cells which could skew these results.

Also, in previously reported experiments the frequency of $V\alpha24^+$ cells was assessed just once after two weeks of culture. According to the findings presented here (Paragraph 4.2), the

frequency of *i*NKT cells on day 14 was already decreasing in the healthy controls, so it was easy to miss some of the patients, whose *i*NKTs did proliferate, but their pool contracted after the initial peak of enrichment in the culture. These patients could have been erroneously classified as "non–responders". Finally, the differences in the results obtained by the other groups could also be explained by the number of samples analysed and even by the sensitivity of flow cytometry equipment or the biological effectiveness of a given batch of αGalCer. It is important to acknowledge, however, that the assay was carried out only once for every individual. While available resources did not allow for the repeated measurements, it would have been a priority provided the lack of this limitation. Moreover, an additional assay, such as tracking of dividing populations with CFSE, could have been employed for assessment of antigen-driven proliferation in RA.

According to the study published by Linsen *et al.*, all synovial fluid *i*NKT cells, even those isolated from patients whose blood *i*NKTs were unreactive, responded to stimulation with α-galactosylceramide (Linsen *et al.* 2005). In the set of experiments presented here it was found that none of the twelve patients was a non–responder in terms of total *i*NKT cell expansion. However, although all patients showed some proliferation, the overall expansion of *i*NKT cells in the synovial fluid was clearly compromised in this group.

The differences observed in the expansion profiles for $CD4^+$ and $CD4^-$ $V\alpha24^+$ populations in the healthy donors were unexpected. The initial lower frequencies of $CD4^-$ lymphocytes could be explained by the process of TCR downregulation after antigen exposure. However, this could raise the question why $CD4^-$ cells respond slower to a given stimulus and then start expanding rapidly after day 4. Recent papers suggest that the CD4 molecule acts as co-receptor not only for MHC class II, but also CD1d–driven activation (Chen *et al.* 2007; Thedrez *et al.* 2007). This could explain why the proliferative response of $CD4^-$ cells in healthy individuals starts later than in the $CD4^+$ population.

Given the functional differences between the two subsets, it would be worth exploring what is so unique in $CD4^-$ $V\alpha24^+$ cells that enables them to react after an initial delay. The possibility remains that they use some other unknown molecule to modulate the outcome of the stimulation events they encounter.

Several molecules, including ICOS, CD40/CD40L and GITR, were shown to act as co–stimulatory molecules in the CD1–mediated pathways of lipid antigen presentation. However, subtle differences in their surface expression by various Vα24 cell subsets are unknown. The potential that one or more of these pathways could be involved in human autoimmunity remains to be explored.

The reason for the lack of *i*NKT responsiveness in RA, lupus, Sjögren's syndrome and systemic sclerosis was addressed by Kojo *et al.* in experiments with sorted cells (Kojo *et al.* 2001). The authors suggested that these cells are non–functional in patients with autoimmune diseases. However, these experiments were carried out on a very limited number of samples, i.e. only one sample for each disease was used. More importantly, these assays employed APCs from different allogeneic donors. Consequently, some of the responses observed could be attributed to the alloresponsive reaction rather than true *i*NKT proliferation after antigenic stimulation. In addition, there is no a clear cut threshold to identify the responders and non-responders in the experiments, making the interpretation of the results even more difficult.

At this point it is important to stress that, although there is a lack of a particularly good responder in both patient groups, there were some patients whose *i*NKT cells did react and expand relatively well after CD1d–mediated stimulation. This could suggest a progressive loss of proliferative function, which was surprisingly overlooked by the authors of these early studies, who focused on the non-responders and did not compare the strength of the response between patients. It would be interesting to determine if such a gradual loss of function really occurs in the disease, and if so, what the underlying mechanisms are.

In addition, it is interesting to note that the number of individuals in whom at least one subset of the *i*NKT cells is somehow "defective", and does not expand after stimulation, is quite large. Almost a third of the patients who donated a sample of peripheral blood and around 10% of the ones whose synovial fluid was obtained, had at least one *i*NKT cell subset that was not proliferating. There are numerous reasons for the reduced responsiveness of the *i*NKT cells in RA, including the possibilities described in the previous chapter (in conjunction with reduced frequencies of *i*NKT cells in RA patients). The proliferation defect could be caused by extensive cell divisions connected with telomeric erosion, as suggested by Goronzy and

Weyand (Goronzy *et al.* 2006). Such a diminishing proliferative function could lead to a total loss of capability to react.

As mentioned in Chapter 3, lymphocytes express telomerase whose activity is crucial for maintaining their immunological function. Recent findings suggest that antigenic challenge in T cells results in transient telomerase upregulation followed by a decrease in enzyme activity (reviewed in Hodes *et al.* 2002). Although the study published by Tarhan suggests an increased telomerase levels in RA patients (Tarhan *et al.* 2008), according to Thewissen, activation-induced telomerase activity in PBMCs is impaired in early arthritis patients (Thewissen *et al.* 2005). While in the former study the human telomerase reverse transcriptase (hTERT) levels were evaluated without any form of stimulation, the latter study included stimulation of total PBMCs with anti-CD3 antibodies, so these data are more relevant for the purpose of this research. Assuming that all T cell populations undergo the same processes, this insufficient telomerase activity in the early phase of RA could result in shrinking *i*NKT cell populations and diminished proliferative responses of these cells.

In addition, it could also be argued that *i*NKT cells in patients are already activated *in vivo* and therefore more sensitive to AICD. Consequently, various concentrations of αGalCer could have different effects on the cells in the cultures. The experiments described here were carried out with an addition of standard amount of the compound (100ng/ml), as recommended by the manufacturer and used by many research groups. This relatively high concentration of the ligand could have resulted in the lack of detectable expansion in the assay. Therefore, the experiments with various titrations of this, and possibly also other ligands, could provide additional information regarding the cause of observed unresponsiveness.

In summary it appears that the proliferative capacity of *i*NKT cells in RA patients is compromised in both the peripheral blood and synovial fluid. Even patients who show relatively preserved levels of expansion of these cells still end up with lower total frequency than most of the tested, age-matched healthy controls. Whether this is due to defective CD1d-mediated antigen presentation (the assays used autologous APCs), replicative senescence due to telomere erosion or a signalling defect affecting a co-stimulatory molecule

or the TCR itself remains to be seen. The experiments which including the use of a standardized presenting cell line, such as CD1d transfectant devoid of class I/class II expression, would have provided some insight into the function of APCs in patients. These experiments had been started, however the timing issue did not allow for carrying out these assays.

However, from the data presented here it is clear that previous studies, due to their limitations in experimental design and data interpretation strategy, probably underestimated the impairment of *i*NKT proliferative capacity in RA patients.

Chapter 5:

CD1d-restricted T cells in rheumatoid arthritis

Since the discovery of this lymphocyte population around 1987 (reviewed in Godfrey *et al.* 2004), numerous studies have been published which investigated functional details and the role of NKT cells in both human physiology and pathology. The majority of these publications presented experimental work employing anti–Vα24 antibodies as a tool to identify lymphocytes expressing the *semi-invariant* T Cell Receptor. Therefore, our understanding was based on the *i*NKT cell population. These studies exposed several important facts related to the nature of these cells.

With the construction of CD1d tetramers in recent years (see Paragraph 1.5.5) it has become clear however, that not all the NKT cells can be detected by staining with available anti-TCR antibodies. The staining done with this new tool revealed another subset sharing the ability to recognise lipid–derived ligands, but utilising a wider TCR selection. This suggested that NKT cell populations should be described based on the functional properties, i.e. their common ability to respond to CD1d presented antigens rather than on the phenotypical characterization (e.g. the co-expression of T and NK cell markers or usage of Vα24 TCR chain). Therefore a new name has been now proposed: "CD1d–restricted NKT cells", "CD1d–responsive NKT cells" or "CD1d–restricted T cells"

Most importantly, the availability of CD1d tetramers enabled researchers to obtain the answers to more questions on NKT cell biology. The identification of the novel NKT subset (non-*i*NKTs) revealed the dissimilarities in their phenotypical and functional profiles compared to classical *i*NKT cells (Gadola *et al.* 2002, Gumperz *et al.* 2002).

In fact, as mentioned in the Introduction, a study which investigated the responses of the two subsets of NKT cells to experimentally introduced lymphoma identified important differences between the variant and invariant cell populations. Experiments with CD1d- and Jα18-knockout mice found that *i*NKT cells protected from the tumour, while non-invariant cells suppressed the immunity against it (Renukaradhya *et al.* 2008).

Previous chapters reviewed studies on *i*NKT cell populations, both in humans and animal models of autoimmunity. The vast majority of these studies involved the detection of $V\alpha24^+$ or $V\alpha14^+$ cells by staining with antibodies against invariant TCR chain. In contrast, CD1d tetramers have not been extensively used to investigate the population of CD1d-restricted cells either in human autoimmunity or in animal models.

The first animal study which employed CD1d tetramers was the work on a MOG_{35-55} peptide-induced model of experimental autoimmune encephalomyelitis published by Jahng *et al.* in 2004 (Jahng *et al.* 2004). This study identified a sulfatide-reactive population of CD1d-restricted T cells abundant in central nervous system (CNS) in animals which developed EAE. Surprisingly however, this population did not express the Vα14 chain or recognise αGalCer. In addition, treatment of mice with sulfatide alleviated the symptoms. This was related to the inhibition of the MOG_{35-55}-reactive T cell population.

Recently, Halder *et al.* (Halder *et al.* 2007) investigated the role of non-*i*NKT cells in a concanavalin A-induced (ConA) model of hepatitis, where *i*NKT cells participate in mediating liver damage. This group also used sulfatide and sulfatide-loaded tetramers to identify non-*i*NKT cells. In the experiments sulfatide protected animals from the disease. The mechanism which was proposed by the authors was that the compound activated sulfatide-reactive non-*i*NKT cells which interacted with plasmacytoid dendritic cells (pDCs). Following this, APCs secreted IL-12 and MIP-2 which recruited invariant cells to the liver. These incoming *i*NKT cells immediately became anergic, which was mediated by pDC-derived IL-12.

The only human study which used tetramers to investigate autoimmune disease was the work of Lee *et al.* (Lee *et al.* 2002). The authors used the reagent with the aim of detecting the population of $V\alpha24^+$ CD1d-restricted cells in type 1 diabetes. This work demonstrated that the population is preserved in the disease, which contradicts the majority of animal studies in NOD mice, as well as original study in discordant twins conducted earlier by Wilson *et al.* (Wilson *et al. 1998)*. However, this publication focused on the invariant subset of CD1d-restricted cells and non-*i*NKT cells were not quantified, despite the fact that the results presented in the publication clearly indicated the existence of a $V\alpha24^-$ population identified by CD1d-αGalCer tetramer staining (Lee *et al.* 2002).

Importantly, there are no human studies which would characterize non-*i*NKT cell populations in any other autoimmune disease. However, as the invariant Vα24^{+} cells seem to have a role in conferring protection from inflammation in patients, it was assumed that non-*i*NKT cell populations would also be involved in controlling autoimmunity.

Similarly, data on CD1d-restricted populations in arthritis, both in mice and humans are lacking. Therefore, after carrying out experiments which investigated the prevalence and proliferatory response of invariant cells, it seemed logical to measure the pool of CD1d-restricted cells as a whole.

The analysis of the CD1d-restricted cell prevalence in peripheral blood and synovial fluid of RA patients was hoped to provide additional indication on the role of CD1d-restricted cells in the disease. In addition, the relative contributions of invariant and non-invariant NKT cells were hoped to be informative, as they could help to gain some insight into the role of the non-invariant cells. The results of this investigation, which employed CD1d tetramers, are presented in this chapter.

To assess the frequencies of CD1d–restricted cells in synovial fluid and peripheral blood of RA patients and compare these with the blood of healthy donors, empty APC–conjugated CD1d tetramers were first loaded with α–galactosylceramide. After initial optimisation flow cytometry staining was performed on thirty samples in three groups (H, n=10; RAPB, n=10 and RASF, n=10).

Briefly: 500,000 cells were triple stained with APC conjugated CD1d tetramers-αGalCer, anti–CD4 FITC and anti–CD19 PE antibodies. Loaded tetramers were employed at the same time as antibodies and staining was conducted for 30 min on ice as described in Materials and Methods. The anti-CD19 antibody was added to eliminate any non-specific staining of B cells. Consequently, the analysis was conducted by assessing CD1dtetramer-αGalCer staining on the $CD4^{+/-}$ $CD19^{-}$ cells, as shown in Figure 5.1 B

The CD1d tetramer-αGalCer gating was set as shown in Figure 5.1 C. The positive gate was drawn low, just bordering the negative gate to ensure that all CD1d-restricted cells specific to αGalCer were included. Similar gating has been successfully applied before by various research groups (Brigl *et al.* 2003; Fischer *et al.* 2004; Zhou *et al.* 2004; Ikarashi *et al.* 2005).

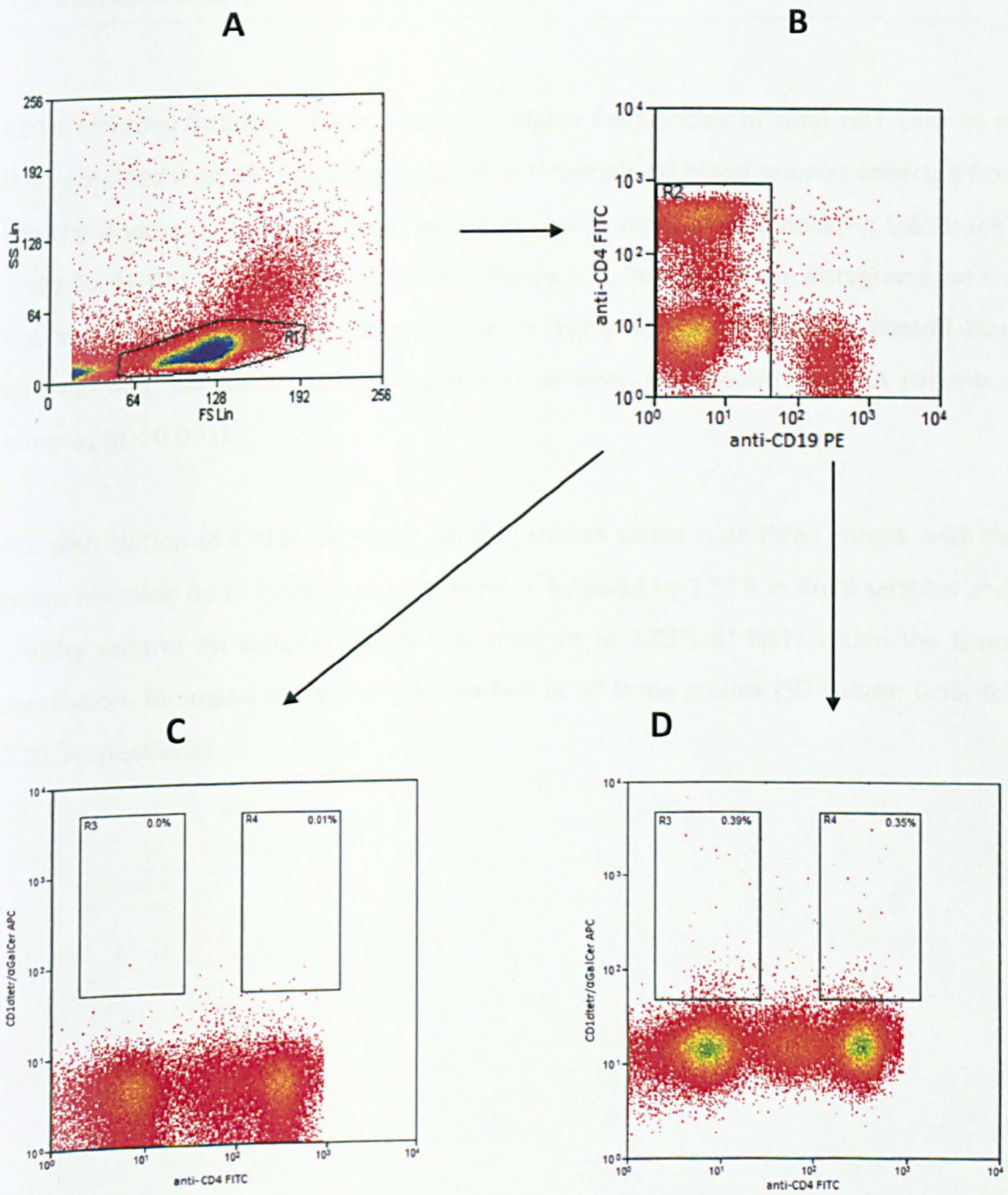


Figure 5.1. The gating used for analysis of anti-CD4 CD1d tetramer/αGalCer staining (healthy donor) The mononuclear cells were isolated from the donors and triple stained for 1 hour on ice. A) lymphocyte gate B) exclusion of $CD19^{+}$ cells to remove the positive signal from unspecific binding to B cells as recommended by the manufacturer of the reagent C) the negative control and D) the tetramer staining identifying CD4 and $CD4^{-}$ populations of CD1d-restricted cells.

CD1d tetramer/αGalCer staining revealed higher frequencies of total NKT cells in synovial fluid obtained from RA patients compared with peripheral blood samples collected from both healthy donors or patients (mean values for percentages of lymphocytes: 0.655; 0.672 and 1.591 for H, RAPB and RASF respectively; Figure 5.3). Representative histograms are shown in Figure 5.2. Statistically the differences were highly significant between control blood and synovial fluid samples ($p < 0.001$) and also between PB samples from RA patients and SF samples ($p < 0.001$).

The distribution of CD1d–restricted cell frequencies varied in all three groups, with the RASF group reaching up to 2.4% of all lymphocytes, followed by 1.39% in RAPB samples and finally healthy control PB samples which contained up to 1.05% of NKTs within the lymphocyte population. Biological variability was marked in all three groups (SD values: 0.55, 0.29 and 0.20, respectively).

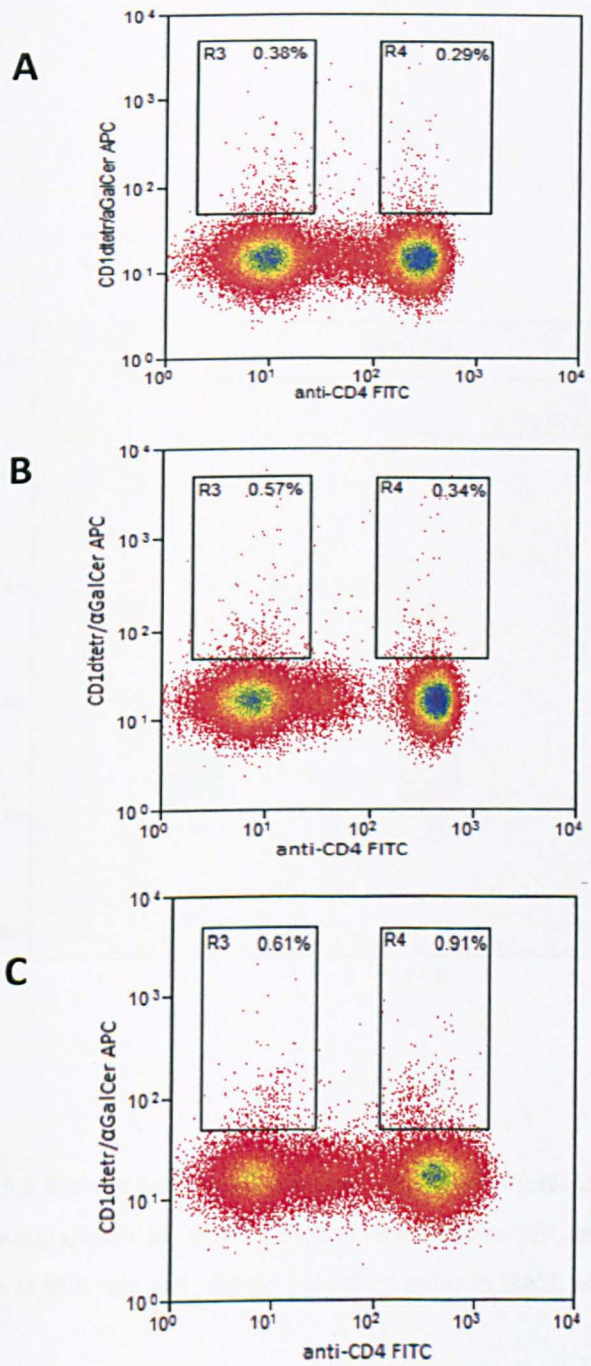


Figure 5.2. Double staining with anti-CD4 antibodies and αGalCer–loaded CD1d tetramers. Representative plots are shown for a healthy donor (A), peripheral blood sample from RA patient (B) and synovial fluid sample from RA patient (C).

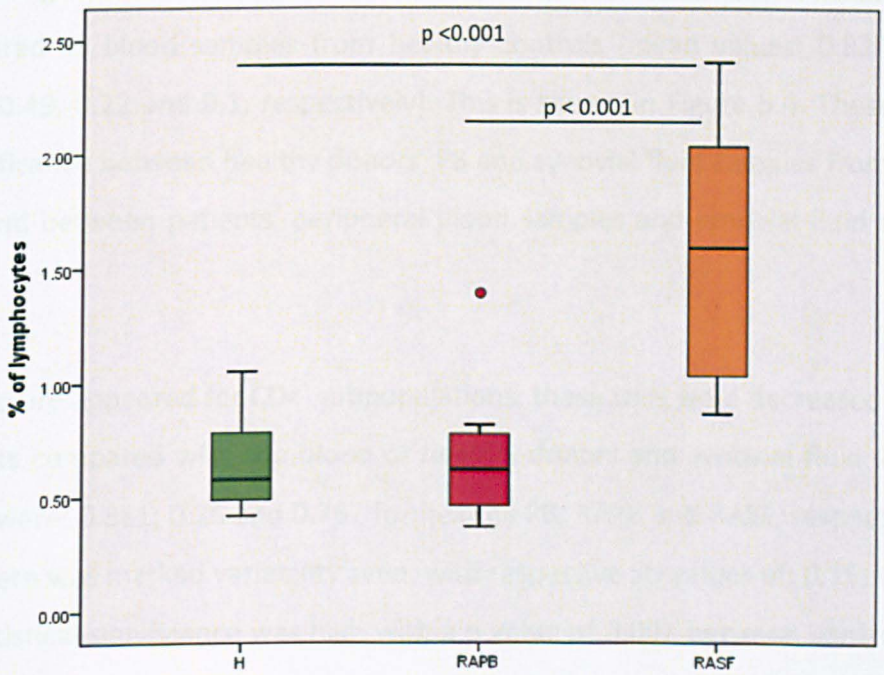


Figure 5.3 Box and whisker plots of the frequencies of total CD1d–restricted NKT cells in experimental groups; peripheral blood of healthy donors (H, green), peripheral blood of RA patients (RAPB, red) and synovial fluid of RA patients (RASF, yellow). Medians are shown.

As anti-CD4 staining was conducted along with the tetramer staining, it was possible to compare the subpopulations of CD1d–restricted T cells within the total NKT pool, based on their positivity for this marker. The results indicated that $CD4^+$ CD1d tetramer/αGalCer$^+$ cell frequency among all the lymphocytes was increased for both RA synovial fluid and peripheral blood compared to blood samples from healthy controls (mean values: 0.829; 0.412 and 0.274, SD = 0.49, 0.22 and 0.1, respectively). This is shown in Figure 5.4. These differences reached significance between healthy donors' PB and synovial fluid samples from RA patients ($p = 0.001$) and between patients' peripheral blood samples and synovial fluid samples ($p < 0.01$).

A different picture appeared for $CD4^-$ subpopulations: these cells were decreased in the blood of the patients compared with the blood of healthy donors and synovial fluid samples. The mean values were: 0.381; 0.26 and 0.762 for healthy PB, RAPB and RASF, respectively (Figure 5.4). Again there was marked variability seen, with respective SD values of: 0.15; 0.1 and 0.37. However, statistical significance was high with a p value of 0.001 between control blood and SF samples and $p < 0.001$ between samples of blood and synovial fluid obtained from the RA patients. The abundance of $CD4^-$ CD1d–restricted NKT cells observed in the synovial fluid of patients was on average almost 3 times higher than in the blood of patients.

It is noteworthy that the relative frequency of $CD4^+$ and $CD4^-$ NKT cell subsets changes in patients compared to healthy donors. $CD4^+$ cells constitute about 40% of CD1d-restricted NKTs in the peripheral blood of the latter. However, they form around 60% of all the CD1d-restricted cells in the blood of the patients and 50% in the synovial fluid (Figure 5.5). Although these differences may seem small, this result was found to be statistically significant between the peripheral blood samples from the control and the RAPB group.

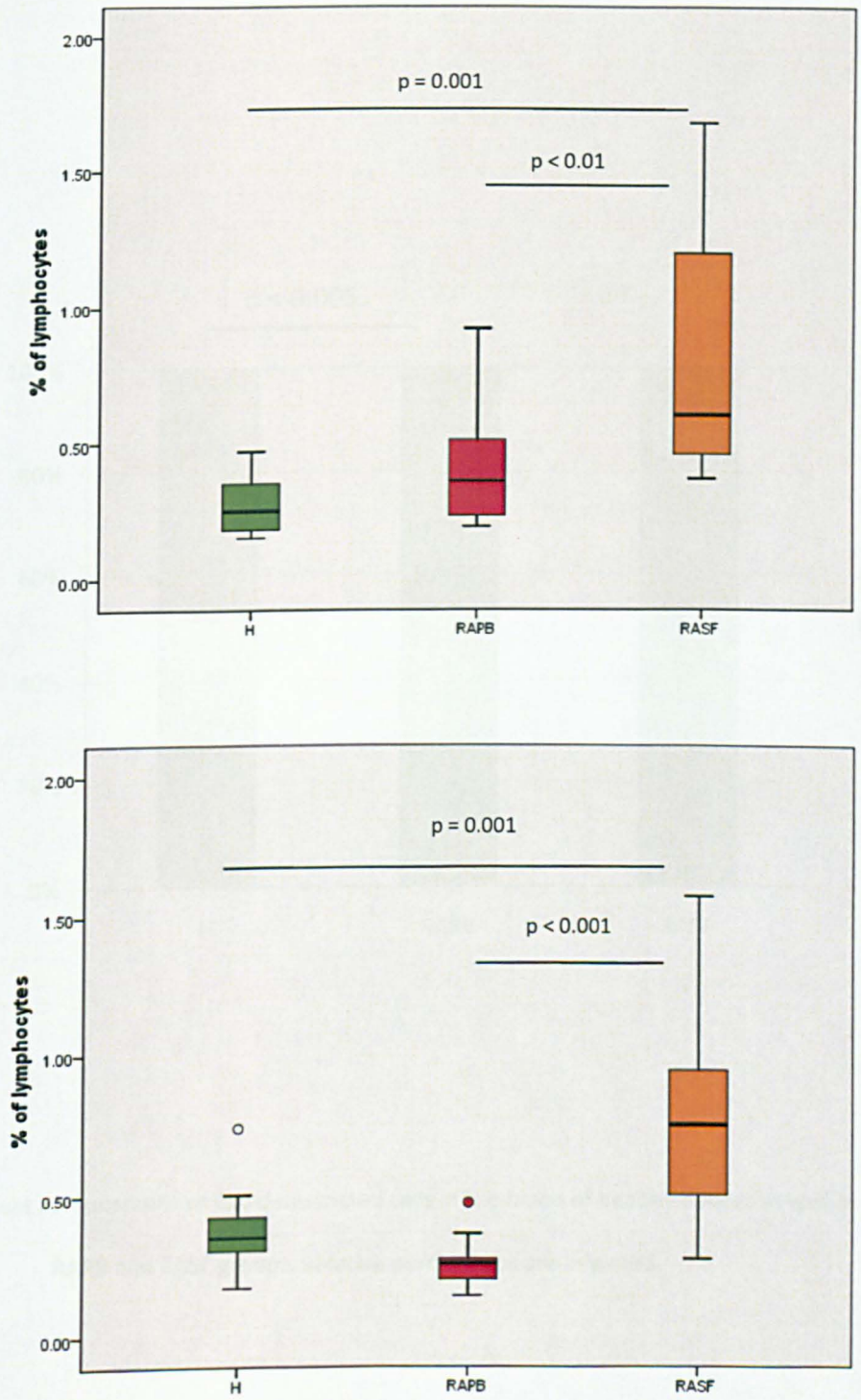


Figure 5.4 Frequencies of CD4 subpopulations of CD1d–restricted NKT cells in experimental groups; peripheral blood of healthy donors (H, green), peripheral blood of RA patients (RAPB, red) and synovial fluid of RA patients (RASF, yellow). $CD4^+$ is shown in the upper panel, $CD4^-$ in the lower. Medians are shown.

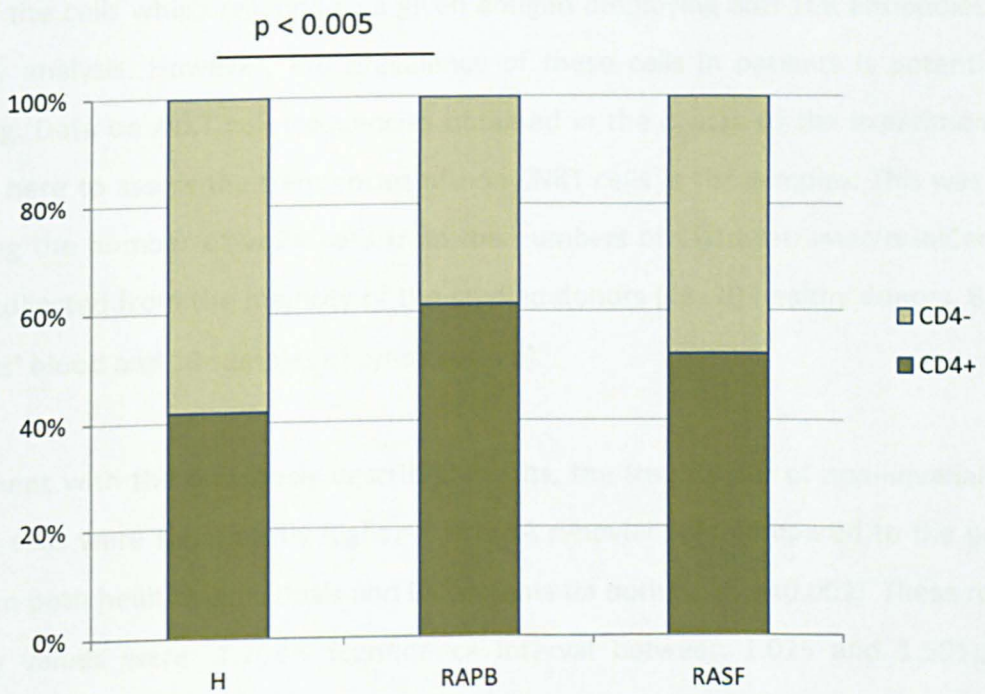


Figure 5.5 Subset compositions of CD1d–restricted cells in the blood of healthy donors as well as RAPB and RASF groups. Relative percentages are depicted.

As explained in the Introduction, the non-invariant subset of CD1d-restricted cells represents a collection of NKT cells characterised by variable TCRs. Therefore it is virtually impossible to detect all the cells which respond to a given antigen employing anti-TCR antibodies for flow cytometry analysis. However, the prevalence of these cells in patients is potentially very interesting. Data on *i*NKT cell frequencies obtained in the course of the experimental work was used here to assess the frequencies of non–*i*NKT cells in the samples. This was done by subtracting the number of Vα24$^+$cells from the numbers of CD1d tetramer/αGalCer$^+$ cells in samples collected from the majority of the studied donors (i.e. 10 healthy donors, 8 samples of patients' blood and 10 samples of synovial fluid).

In agreement with the previously described results, the frequencies of non–invariant CD1d–restricted cells were significantly higher in the RA synovial fluid compared to the peripheral blood from both healthy individuals and RA patients (in both cases $p<0.001$). These respective frequency values were: 1.263% (confidence interval between 1.025 and 1.501), 0.317% (confidence interval between 0.079 and 0.555) and 0.269% (confidence interval between 0.003 and 0.535) of all the cells found in the lymphocyte gate (Figure 5.6) meaning a 4– or 4.7- fold increase in the disease–affected joints compared with the peripheral blood of health and diseased donors, respectively.

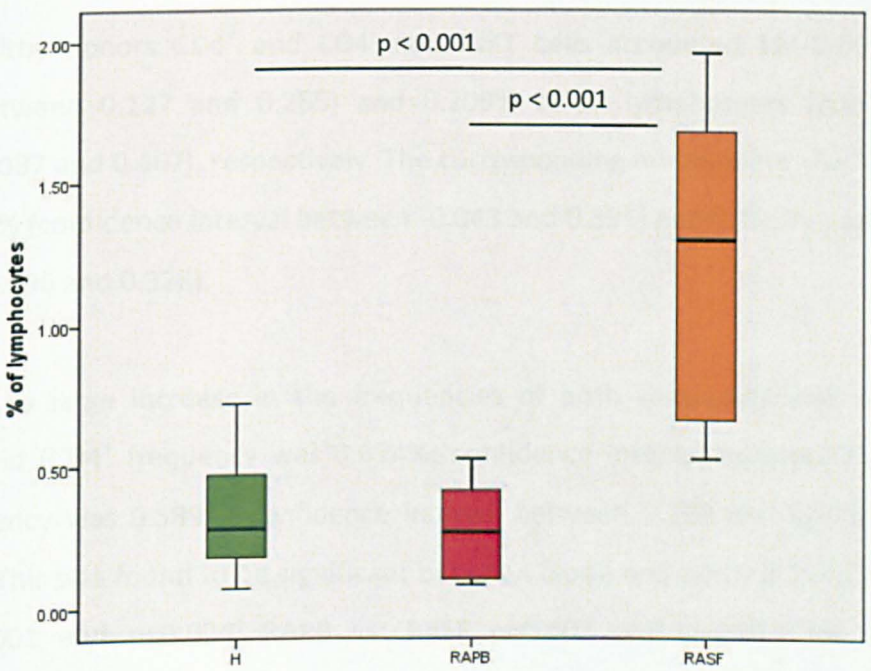


Figure 5.6. Frequencies of non–invariant NKT cells within the CD1d–restricted populations in all experimental groups. The frequencies of non-invariant CD1d-restricted cells were calculated based on the subtraction of invariant cell counts from the total CD1d-restricted cell frequency obtained for a given donor.

While there are still some aspects to be clarified regarding the role of non-*i*NKT cells, it is possible that $CD4^+$ and $CD4^-$ subsets differ as in the case of *i*NKTs. For this reason CD4 expression within the total CD1d-reactive T cell pool was analysed.

In the healthy donors $CD4^+$ and $CD4^-$ non–*i*NKT cells accounted for 0.069% (confidence interval between 0.127 and 0.265) and 0.209% of all lymphocytes (confidence interval between 0.087 and 0.467), respectively. The corresponding numbers for the blood of patients were 0.176% (confidence interval between -0.043 and 0.395) and 0.116% (confidence interval between -0.096 and 0.328).

In contrast, a large increase in the frequencies of both subpopulations was seen in the synovial fluid ($CD4^+$ frequency was 0.674%; confidence interval between 0.478 and 0.870, $CD4^-$ frequency was 0.589%; confidence interval between 0.399 and 0.779) as depicted in Figure 5.7. This was found to be significant between blood and synovial fluid groups (i.e. H vs. RASF $p<0.001$ and $p=0.024$; RAPB vs. RASF $p=0.002$ and $p=0.002$ for $CD4^+$ and $CD4^-$ populations, respectively), indicating accumulation of the non-invariant subset in the joints.

When the relative contributions of these $CD4^+$ and $CD4^-$ subsets were analysed it appeared that while in healthy controls the $CD4^+$ subset is much less abundant, accounting for only a quarter of all non–*i*NKT cells, in both the peripheral blood and synovial fluid of the patients, this was the predominant cell subpopulation. The differences were statistically significant between both patients blood ($p=0.033$) and synovial fluid samples (0.004) and healthy group (Figure 5.8).

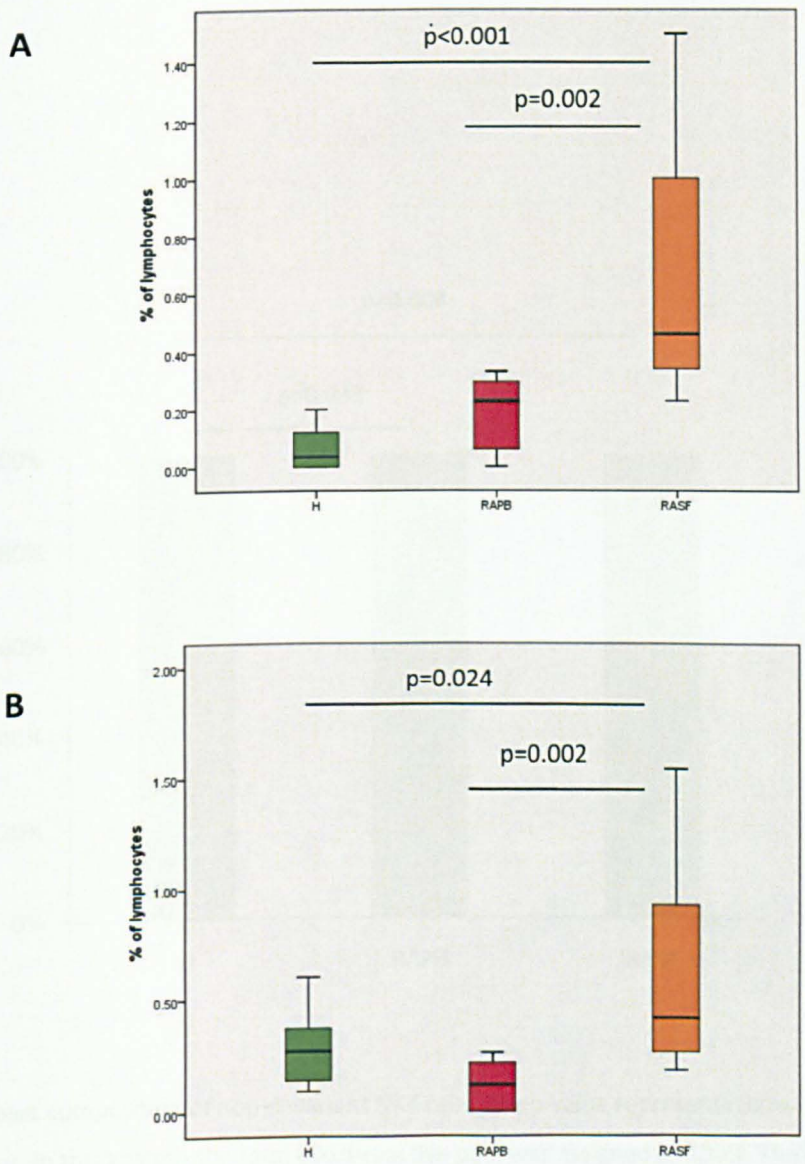


Figure 5.7. Frequencies of CD4 subsets of non–invariant NKT cells within the CD1d–restricted population. The frequencies of non-invariant CD1d-restricted cells were calculated based on the subtraction of invariant cell counts from the total CD1d-restricted cell frequency obtained for a given donor. A) and B) represent $CD4^+$ and $CD4^-$ subsets, respectively.

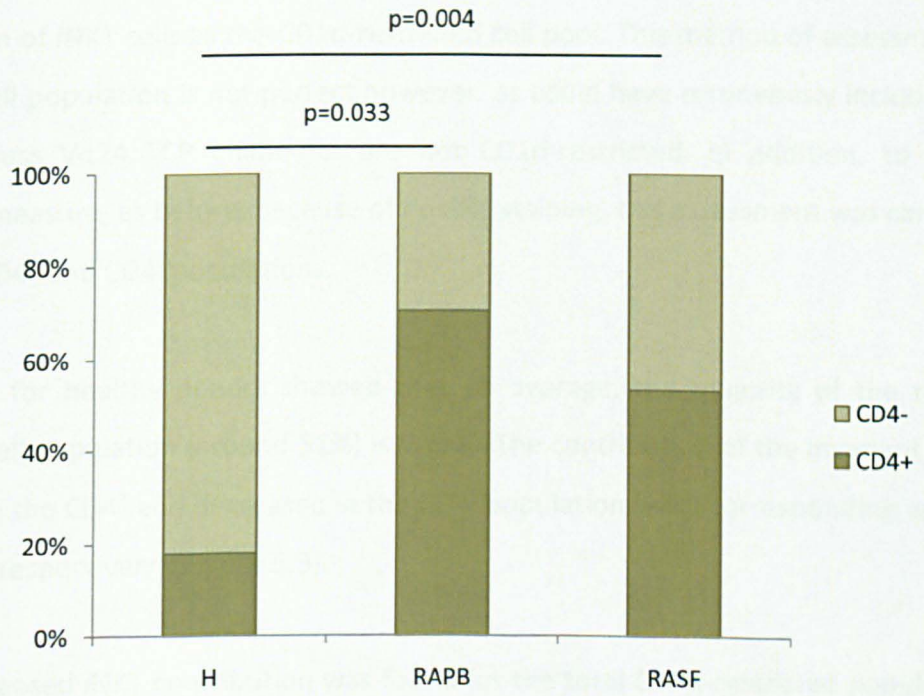


Figure 5.8. Subset composition of non–invariant NKT cells. The p value represents differences between H and RA groups. In this analysis the total CD1d-reactive pool was assigned as 100%. The contribution of the $CD4^+$ and $CD4^-$ tetramer reactive subsets within this pool is illustrated in this figure.

Further data analysis enabled results for Vα24$^+$ cells to be obtained as a fraction of CD1d tetramer/αGalCer–stained NKTs for individual samples. This was assessed by subtracting the *i*NKT cell frequency obtained for a given donor as presented in Paragraphs 5.5 and 5.6. from the total CD1d-restricted cell pool and then subsequent calculation of the percentage contribution of *i*NKT cells to the CD1d-restricted cell pool. This method of assessment of non-invariant cell population is not perfect however, as could have erroneously included the cells which express Vα24 TCR chain but are not CD1d-restricted. In addition, to the overall frequency measure, as before, because of double staining, this assessment was carried out for the total, CD4$^+$ and CD4$^-$ populations.

The results for healthy donors showed that on average, the majority of the total CD1d–restricted cell population (around 51%) is Vα24$^+$. The contribution of the invariant subset was increased in the CD4$^+$ and decreased in the CD4$^-$ population (with corresponding values 84.5% and 27.3%, respectively) (Figure 5.9).

Slightly increased *i*NKT contribution was found for the total CD1d-restricted population in the peripheral blood from patients (58.9%), this proportion was decreased in the CD4$^+$ and increased in CD4$^-$ subset (RAPB CD4$^+$: 62.8%; RAPB CD4$^-$: 69.3%). However, these changes were not statistically significant.

In sharp contrast, a striking difference was observed for synovial fluid samples. The contribution of Vα24$^+$ cells was largely reduced in both total cell pool and the subsets (total RASF: 22.86%, CD4$^+$: 22.76% and CD4$^-$: 29.42%; Figure 5.9). The changes were statistically significant between total (H vs. RASF $p<0.001$ and RAPB vs. RASF $p<0.001$), CD4+ (H vs. RASF $p<0.001$ and RAPB vs. RASF $p=0.02$) and CD4- (H vs. RASF $p=0.027$ and RAPB vs. RASF $p=0.002$) subpopulations.

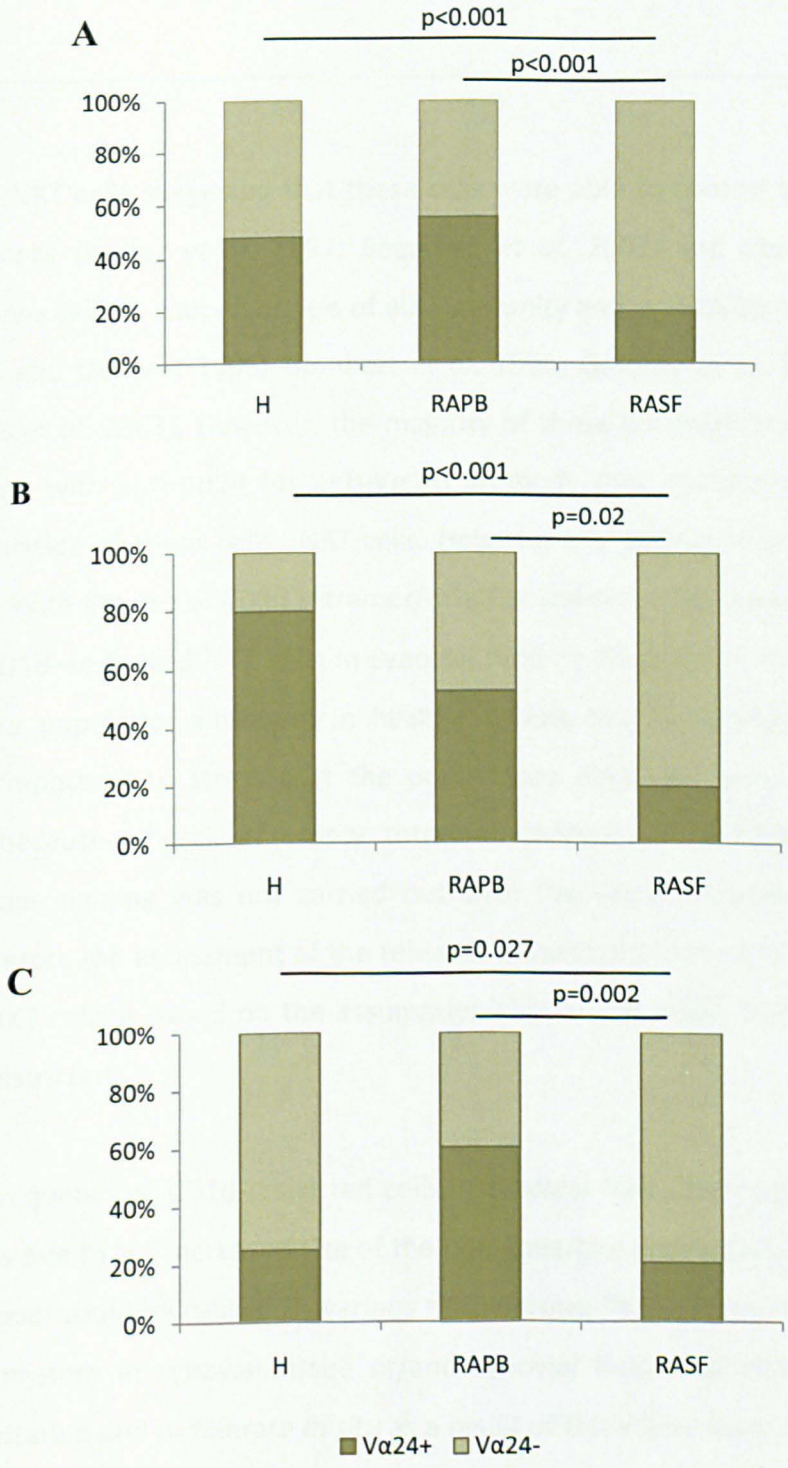


Figure 5.9 Relative contribution of invariant and non–invariant NKT cells within CD1d–restricted NKT cell populations. Data shown for the total CD1d – restricted cells (A) and for the $CD4^+$ and $CD4^-$ subpopulations (shown as B) and C), respectively).

Early studies on NKT cells suggested that these cells were able to control the emergence of autoreactive T cells (Baxter *et al.* 1997; Beaudoin *et al.* 2002) and observed a reduced population of these cells in animal models of autoimmunity and individuals with autoimmune disease (Takeda and Dennert 1993; Gombert *et al.* 1996; Godfrey *et al.* 1997; Mars *et al.* 2002; Demoulins *et al.* 2003). However, the majority of these observations were exclusively based on staining with anti-Vα24 (or anti-Vα14) antibody that enables tracking one very specific subpopulation of these cells, *i*NKT cells. However this approach failed to detect the entire NKT pool. With the use of CD1d tetramer/αGalCer staining it has become clear that the population of CD1d–restricted NKT cells in synovial fluid of RA patients is over-represented compared to the population observed in healthy donors and the blood of the patients. However, it is important to stress that the populations observed here could have been overestimated because of lack of empty tetramer controls. In addition, as mentioned previously, double staining was not carried out with the CD1d tetramers and anti-Vα24 antibodies. Therefore the assessment of the relevant subset contribution of the invariant and non-invariant *i*NKT cells is based on the assumption that all the Vα24 TCR chain-expressing cells are CD1d-restricted.

The increased frequency of CD1d-restricted cells in synovial fluid observed in the described experiments was due to the increased size of the non–invariant population. This enlargement of the NKT cell pool could be caused by various mechanisms. The first possible explanation is that NKT cells present in synovial tissue or/and synovial fluid encounter specific, CD1d-mediated presentation and proliferate *in situ* as a result of this stimulation. Based on the data presented here it seems possible that, as a consequence of the activation, these multiplying non–*i*NKT cells could have a primary role in the immunological response leading to the autoimmune process that boosts the progression of joint destruction.

The ability of non-*i*NKT cells to undergo pronounced antigen–driven expansion in vitro supports this possibility (Gadola *et al.* 2002). Despite the fact that naturally existing self–ligand(s) for the CD1d molecule are presently unknown, it is conceivable that both central

(thymic) and peripheral presentation of autologous CD1d ligands occurs physiologically and/or in pathological conditions. Therefore it could be speculated that there is a CD1d–bound antigen presented in the joints affected by RA.

The second possibility is that the primary stimulation of NKT cells in RA patients occurs in the peripheral blood or lymphoid tissue, leading to an antigen–driven expansion. Unfortunately, no paired blood samples were collected to see any correlation between the peripheral blood and synovial fluid in individuals. If the activation in fact takes place prior to entry to the joints, these stimulated cells could subsequently selectively accumulate at the site affected by the disease, possibly due to the changes in their migration profile i.e. presence of adhesion molecules and chemokine receptors. It would therefore be very informative to screen both peripheral blood and synovial fluid CD1d–restricted cell populations for the surface expression of these markers.

Both of the above possibilities assume an antigen driven NKT cell response either within or outside the affected joints. Investigating these possibilities further is hampered by our limited understanding of potential CD1d ligands. While there is clear experimental evidence supporting the existence of CD1d-bound self ligands that activate NKT cells *in vitro* and in *vivo*, as discussed in the Introduction, the chemical nature of these remains to be elucidated. The majority of currently identified CD1d ligands are glycolipids, mostly of glycosidic structure, but also derived from other families of ceramides (Silk *et al.* 2008). Ceramides are second messengers in signalling pathways and have been previously shown to be important in apoptosis pathways in chondrocytes (Sabatini *et al.* 2000) and synoviocytes (Mizushima *et al.* 1998; Migita *et al.* 2000), as well as in conjunction with cartilage destruction (Sabatini *et al.* 2000).

In addition, in line with the possibility of CD1d–mediated presentation *in situ*, analysis of synovial fluid compounds found an abundance of ceramides in RA synovial fluid (Ciurtin *et al.* 2006) that were present at a much lower concentration in osteoarthris. One or more of these compounds could potentially be presented to CD1d–restricted cells in the joint, causing the expansion of this cell population. Thus, stimulating NKT cells with fractionated ceramides derived from RA synovial fluid could be very interesting. In the experiments presented here

αGalCer was used to expand NKT cells. However, this compound is not found physiologically in eukaryotes. CD1d tetramer-based analysis is influenced by the bound antigenic ligand and this fact contributes to the specificity of the detection of NKT cells. Under these circumstances using αGalCer as an artificial ligand may over or underestimate the biologically relevant NKT cell population in a given site.

Apart from antigen–driven expansion, NKT cells might be able to respond to non-specific stimulation. In fact, it was shown that murine *i*NKTs ($V\alpha14^+$ cells) can undergo activation through FcγIII receptors in antibody-induced arthritis (Kim *et al.* 2006). It is easy to envisage a situation where immunoglobulin complexes accumulating *in situ* could have a similar effect in patients. Rheumatoid factor (RF) and anti-citrullinated cyclic peptide antibodies could both have an influence via this pathway.

In addition, other non–specific mechanisms could also be implicated. NKT cells can be activated by the action of certain cytokines without the involvement of a TCR–related signal. For example, the combination of IL-12 and IL-18, was described to activate NKT cells *in vitro* (Nagarajan and Kronenberg 2007). Consequently, it is possible that a changed cytokine milieu in the peripheral blood or joint could affect CD1d–restricted cells in the patients. Spectratyping the TCR repertoire of non–invariant CD1d–restricted cells from the RA patients could potentially discriminate between a specific ligand-driven clonal response and a non-specific (cytokine or FcγIII-mediated) proliferation.

The results on the relative contribution of the subsets also indicate that there is a significant predominance of $CD4^+$ cells over $CD4^-$ cells in the patients in both the total CD1d–restricted population and the non–invariant subset regardless of the sample source (i.e. this predominance is observed in both synovial fluid and the peripheral blood). These results contrast significantly with the data obtained from healthy individuals, where $CD4^+$ cells comprise the minority of the NKT pool.

Previous chapters showed a reduction in the frequency and functional unresponsiveness of *i*NKT cell subset in RA. The proliferation and accumulation of non-*i*NKT cells, bearing variable TCRs, is therefore curious. As the immunological role of these cells remains to be fully

elucidated it is possible that these cells do not have the same protective role as their invariant counterparts. They could even have a detrimental impact on the disease, perhaps starting the sequence of events leading to autoimmunity.

Based on the results presented in this chapter the hypothesis was formed that CD1d–restricted T cells could undergo some sort of a secretory shift in RA. To identify the differences in these functional abilities of NKT cells from the patients compared to healthy volunteers, profiles of cytokine production were determined. These experiments are described in the next chapter and are based on the measurement of the cytokine concentrations in supernatant samples after stimulation of CD1d–restricted NKT cells from RA patients with αGalCer. The main aim of these assays was to reveal whether the secretory abilities of NKT cells are just generally diminished quantitatively or whether maybe an overall pattern of cytokine response is also altered in RA.

Given the observations in this chapter, demonstrating an expanded non-*i*NKT population in the joints it was important to investigate cytokine secretion by the total identifiable NKT cell pool in RA.

Chapter 6: Secretory responses of CD1d-restricted T cells in rheumatoid arthritis

The ability to secrete copious amounts of IL-4 very rapidly following TCR ligation was one of the first detected functional features of NKT cells. As discussed in the Introduction the cytokines can be of both Th1 and Th2 type and the secretion of Th17 cytokines has been reported (Yoshiga *et al.* 2008) and it is widely accepted that the cytokines secreted by these cells are central to their immunoregulatory role.

In the literature numerous publications report the effects of NKT cell stimulation in autoimmune disease models in rodents. These animal studies indicate the alteration of clinical outcome in mice in which NKT cell function was modulated b external CD1d ligands. Most of these studies also identify the crucial role of cytokines, especially IL-4, in executing NKT cell function in autoimmunity. Likewise, murine models of inflammatory arthritis have also been tested for differences in NKT cell-related secretory responses.

The administration of OCH, a αGalCer analogue with a truncated sphingosine chain has a substantial protective effect in collagen-induced arthritis (CIA), induced in mice of both C57BL/6 and SJL backgrounds. This was not observed in *i*NKT cell-deficient mice (Chiba *et al.* 2004). This protective function of OCH was interpreted to be an effect of selective Th2 responses elicited by the compound, which were in contrast to Th0 cytokine profiles observed when αGalCer was used. Also partial or complete abrogation of the protection could be seen following the administration of a blocking anti-IL-4 or anti-IL-10 antibody in these experiments. In this study, αGalCer administration conferred some or no protection in the C57BL/6 and SJL strains, respectively.

CD1d-mediated antigen stimulation also protected the DBA/1 mouse strain from CIA. This was indicated by decreased clinical and histological scores in animals treated with αGalCer, even though serum levels of IL-4 and IFN-γ were reduced compared with C57BL/6 mice (Miellot *et al.* 2005). This study proposed a crucial role of IL-10 in protection against the collagen-induced arthritis as an antibody against IL-10 receptor was able to abrogate the protective effect of αGalCer stimulation.

Moreover, when αGalCer and its analogue, α-C-GalCer, was tested in this mouse strain by a different group (Coppieters *et al.* 2007), both of these antigens also conferred protection from CIA, decreasing levels of mRNA encoding proimflammatory cytokines (IL1-β and IL-6) in the knee joints. This study also showed that early treatment with CD1d ligands is beneficial and results in a Th2 deviation, especially following αGalCer administration. This protection was dependent on the timing of the αGalCer administration, as animals exposed to the same antigens later in the course of the disease showed no benefit. In addition, a dual role of IFN-γ, protective in the early stages and detrimental in the later phase of the disease, was also identified by the authors.

In contrast to these results, another publication which described CIA in C57BL/6 mice suggested that both invariant and non-invariant NKT cell populations are responsible for alleviation of the disease in this murine model (Ohnishi *et al.* 2005). $CD1d^{-/-}$ and $J\alpha18^{-/-}$ animals were reported to have significantly decreased clinical scores and lower levels of antibodies against type II collagen compared to WT mice. However, this study failed to observe early differences in cytokine secretion between $J\alpha18^{-/-}$ and wild type mice. The discrepancies were only observed after a booster immunisation; animals which were devoid of *i*NKT cells had lower IL-4 and higher IFN-γ mRNA expression in the spleen, and had lower IL-1β mRNA levels during the development phase of CIA.

Early activation of NKT cells with another αGalCer analogue, SGL-S23, mediated protection in another murine model, antibody-induced arthritis (AbIA), where the disease was provoked by a transfer of serum (containing pathogenic anti–glucose-6-phosphate isomerase antibodies and TNF-α) from K/BxN mice to an animal of C57BL/6 background (Kaieda *et al.* 2007). In this study IFN-γ conferred protection and antibodies against the cytokine abolished the effects of this early NKT cell stimulation.

Surprisingly however, in another study on AbIA in C57BL/6 mice both *i*NKT cells and CD1d-restricted cells seemed to promote arthritis (Kim *et al.* 2005). This was demonstrated by a resistance of CD1d- or Jα18-deficient animals to the disease, while wild type mice developed symptoms after αGalCer administration. The protection was found to be TGF-β-mediated. Moreover, it was shown that IL-4 and IFN-γ suppress TGF-β levels.

In 2006 Kim *et al.* investigated the role of Fcγ receptors in non-specific activation of Vα14^{+} NKT cells in antibody-induced arthritis and concluded that the accumulation of antibody complexes observed in the joints during the disease contributed to the activation of NKT cells in this animal model (Kim *et al.* 2006). This resulted in a secretory response, characterised by increased IL-4, IL-10, IL-13 and IFN-γ levels *in vivo*. However, the stimulation mediated by these antibody complexes did not appear essential to the disease process as NKT cells from FcγR$^{-/-}$ animals responded to αGalCer with a cytokine profile comparable to that of the WT.
Finally, when a *Chlamydia trachomatis*-induced arthritis model (CtIA) was investigated in BALB/c mice, it appeared that the severity of the disease in CD1d-deficient animals was significantly increased compared with WT animals (Bharhani *et al.* 2009). This was associated with a decrease in IL-4, IL-10 and IFN-γ levels and increased MIP-2 (macrophage inflammatory protein-2; CXCL1) and IP-10 (IFN-γ-inducible protein-10; CXCL10) concentrations in the synovium. Treatment of WT mice with αGalCer ameliorated the disease at both early and late stages of arthritis, by reversing the cytokine pattern. Improvement of clinical scores was also associated with an increased clearance of the pathogen.

The studies presented above suggest that while CD1d-mediated antigen presentation and the resulting cytokine secretion seem to be involved in the pathogenesis and course of arthritis in animals, the outcome depends on the model used, mouse strain and time course of the stimulation, making the interpretation of these murine studies difficult. Most of these studies, and those in the NOD diabetes model, support a role for IL-4 in protecting the host against autoimmunity. However, at least one publication seems to contradict this notion. In 1998 Wang *et al.* backcrossed IL-4 knockout mice onto the NOD background and failed to observe increased histological features (insulitis) or incidence in these animals compared with IL-4$^{+/+}$NOD mice, regardless of whether the diabetogenic TCR (BDC2.5) was also introduced or not (Wang *et al.* 1998). The results of that study implied that IL-4 is not required in controlling autoimmunity. On the contrary, however, recently published work by Mi *et al.* which involved NOD.IL-4$^{-/-}$ mice suggests that this cytokine is necessary for αGalCer-mediated protection (Mi *et al.* 2004).
In contrast to the ample amounts of animal data, human studies on RA are scarce. Available data related to the NKT cell cytokine secretory function in RA come from the publications described previously (Chapter 3 and 4). In the paper published by Kojo *et al.* (Kojo *et al.* 2001)

the expression of IL-4 and IFN-γ was assessed by RT–PCR. As a result, in healthy controls three types of response after αGalCer stimulation were identified: combined IFN-γ and IL–4 production, exclusive IFN-γ production or complete lack of secretion of both cytokines. In this group of patients, samples from 9 donors with various autoimmune diseases were analysed. For the whole of this group it was found that some patients can be high IL–4 producers. Unfortunately, only 2 peripheral blood samples from RA patients, both αGalCer–responders, were analysed and the outcome was inconclusive as secretion patterns differed (one patient was found to be a non-secretor, the other secreted both IL-4 and IFN-γ). Moreover, the cells which were sorted for these experiments were peripheral blood lymphocytes of $CD3^+$ $CD4^-$ $CD8^-$ phenotype, providing information on DN NKT cells alone.

The second study that investigated this issue provided more insight into the ability of NKT cells to release cytokines. Linsen found that significantly fewer cells produced both IFN-γ and IL–4 in RA patients compared with healthy donors (Linsen *et al.* 2005). The assessment was carried out using an ELISPOT assay with peripheral blood MCs stimulated with αGalCer. When IL–4/IFN-γ ratios were calculated it became clear that these were reduced in patients, owing to the severely reduced number of IL–4 producing cells. This skewed the cytokine profile towards a Th1 response.

A second set of experiments conducted by these authors was based on the intracellular staining of αGalCer–stimulated $V\alpha24^+$ cells cultured for 14 days. The staining profiles suggested that *i*NKT cells from the blood of RA sufferers produce significantly more IFN-γ and also less IL–4 compared to healthy controls. The cells from synovial fluid samples, however, presented a very similar pattern of cytokine expression to what was observed in healthy controls, maintaining a similar Th0 type secretion pattern. The important distinction between *i*NKT cells from synovial fluid and healthy control peripheral blood *i*NKTs was related to the decreased overall cytokine production of the latter. However, while expansion experiment identified a group of responders and non-responders, all the patients were able to secrete cytokines after stimulation. The authors concluded that the secretory capacity of *i*NKT cells is preserved even when their ability to proliferate is reduced. However, recent data, revealing the existence of a non-*i*NKT cell population now suggests that this assumption could have been wrong.

Intracellular staining data were also presented for CD4 subsets. The results were comparable to what would be expected in healthy people, i.e. $CD4^+$ cells were responsible for IL–4 secretion and $CD4^-$ cells were mainly IFN-γ producers. However, both *i*NKT subsets in the patient group produced relatively more IFN-γ cytokine production skewing towards a Th1 pattern. There was a noticeable reduction in the number of cells exclusively secreting IL-4. Unfortunately, no data were presented for synovial fluid cells.

Given the limitations of the previously published cytokine secretion data it was decided to re-evaluate cytokine production patterns in RA patients following αGalCer stimulation *in vitro*. In addition, to investigate the possibility that NKT cells produce a wider range of cytokines, a multi bead assay was used as a detection platform.

6.2. Experimental set up

The experiments presented in this chapter were aimed at the analysis of cytokine secretion in the supernatant samples from cultured mononuclear cells (both PBMCs and SFMCs). These supernatants were harvested on day 4 after αGalCer exposure, to gain an insight into the profile of secretory events elicited by NKT cell activation.

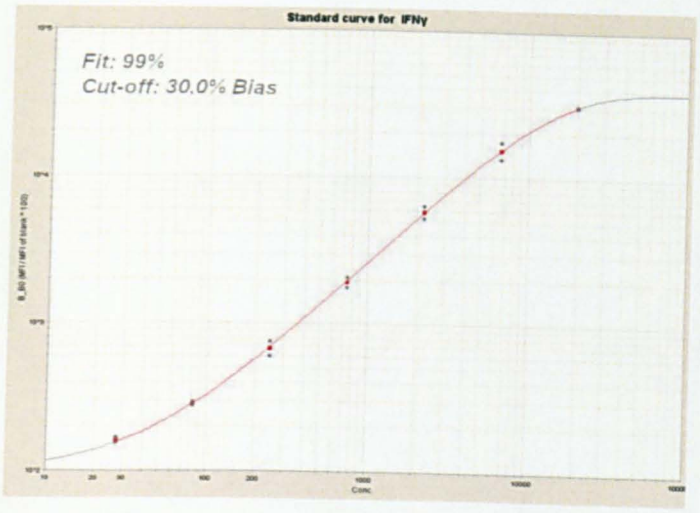
As shown in previous chapters, day 4 was the earliest time point when NKT cell proliferation could be observed in healthy control individuals. While it is widely documented that NKT cells secrete IL-4 and IFN-γ within less than an hour from stimulation (Stetson *et al.* 2003) the experiments were designed to detect additional cytokines, which may not be secreted so rapidly. Thus, a very early time point may not have detected these. On the other hand, sampling at a later time point could have resulted in losing some of the cytokine molecules due to proteolysis and/or absorption by the cells present in the cultures. Ideally, the collection of supernatants and cytokine concentration assessment would have been conducted both at earlier (day 1 on even before) or later (day 7, 10 and 14) time points. However, this was not possible due to the volume of supernatant in the cultures and the financial constraints of conducting multi bead assays.

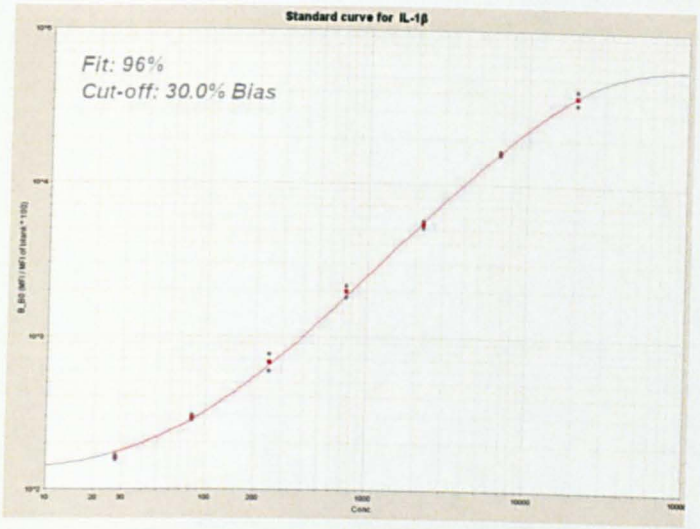
By day 4 some of the non-NKT cells (APCs and conventional lymphocytes) may have contributed to the cytokine production. However, differences in these αGalCer-initiated responses would be potentially interesting. Thus it was felt that sampling the tissue culture supernatants on day 4 after the initial αGalCer exposure represented a good compromise for the experiments described here.

The multi bead assay used detected the concentration of 11 different cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α and TNF-β. The samples were then acquired on the flow cytometer and the analysis was performed as described in Materials and Methods. The major advantage of the technique is that cytokines could be measured in a relatively small volume of supernatants, allowing the use of relatively few cells for these experiments.

The cells which were targeted by the stimulation in the assays were all the CD1d–restricted NKT cells which express TCR with a structure complementary to CD1d–bound αGalCer, i.e. both invariant and non–invariant cells. The use of αGalCer, the most extensively tested NKT cell activator allowed the comparison of the data with previously published results.

To measure the concentrations of the required cytokines in the supernatants, a series of standard dilutions was prepared and acquired in the same way as actual experimental samples. The concentrations were assessed based on the standard curves drawn by the FlowPlex software according to the signal detection in those standard samples. These curves are illustrated in Figures 6.1 to 6.4. The fit of the curves ranged from 91% to 100%, with results equal to or exceeding 95% for 8 of the screened cytokines. This ensured that the technical abilities were sufficient for the accurate detection of the measured analytes.





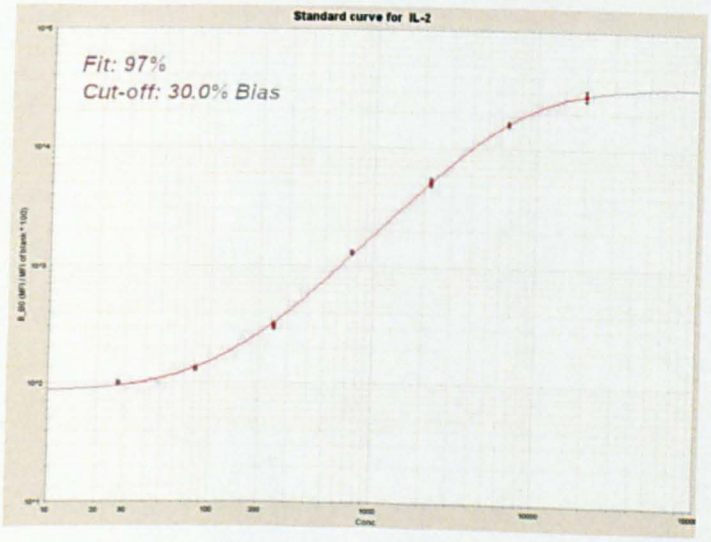
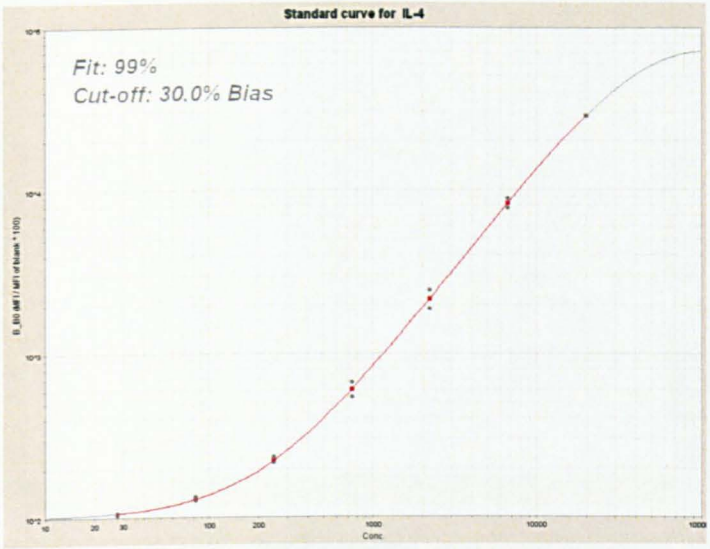
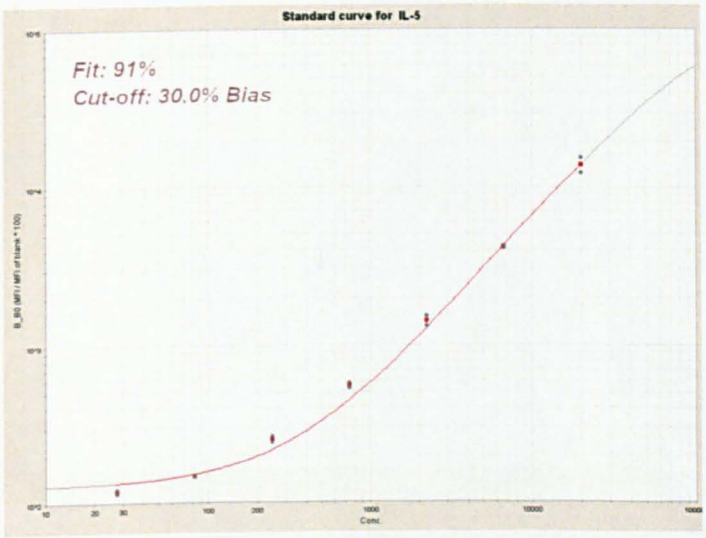


Figure 6.1 Standard curves for concentration quantitation of: IFN-γ, IL-1β and IL- 2 as generated by UK Bio software.





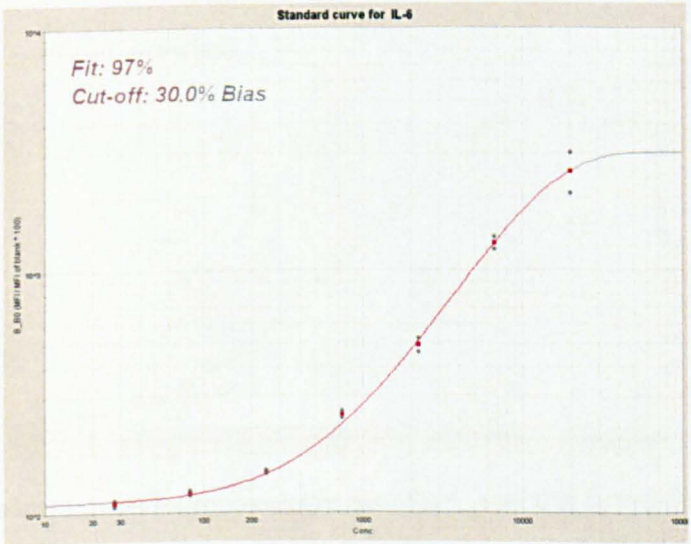
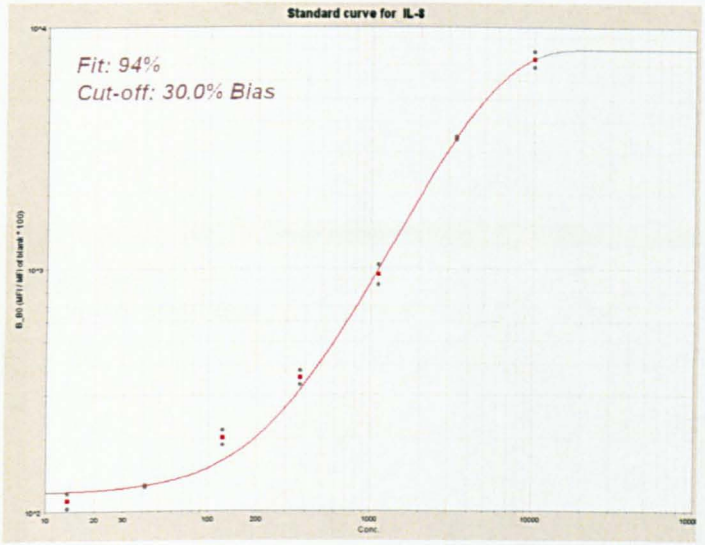
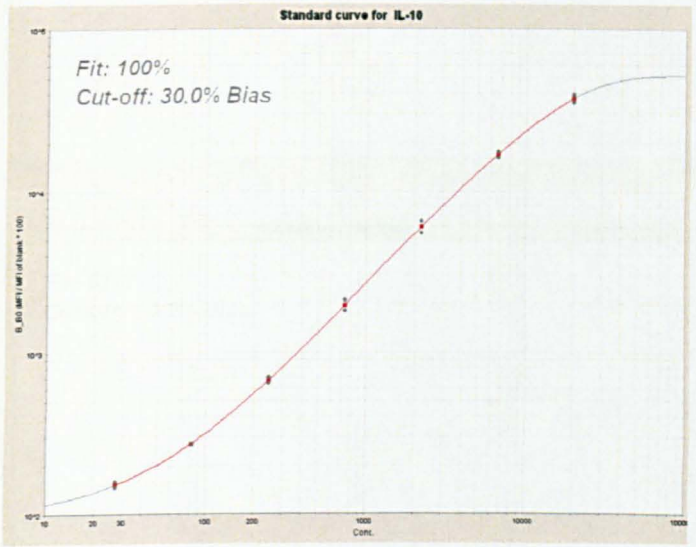


Figure 6.2 Standard curves for concentration quantitation of: IL-4, IL-5 and IL-6 as generated by UK Bio software.





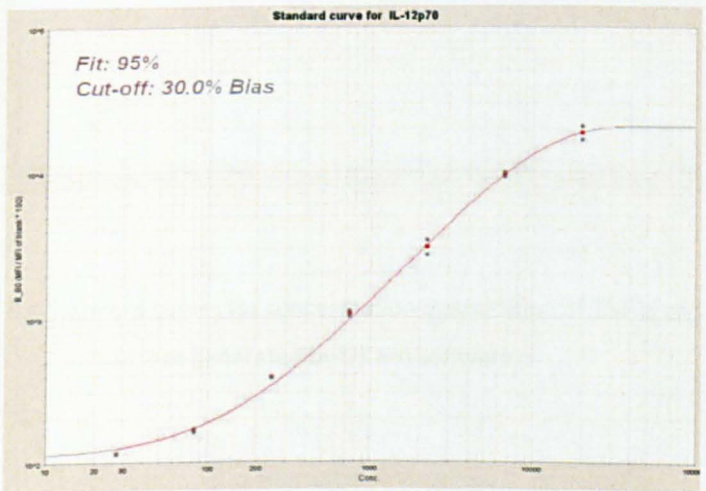


Figure 6.3 Standard curves for concentration quantitation of: IL-8, IL-10 and IL-12p70 as generated by UK Bio software.

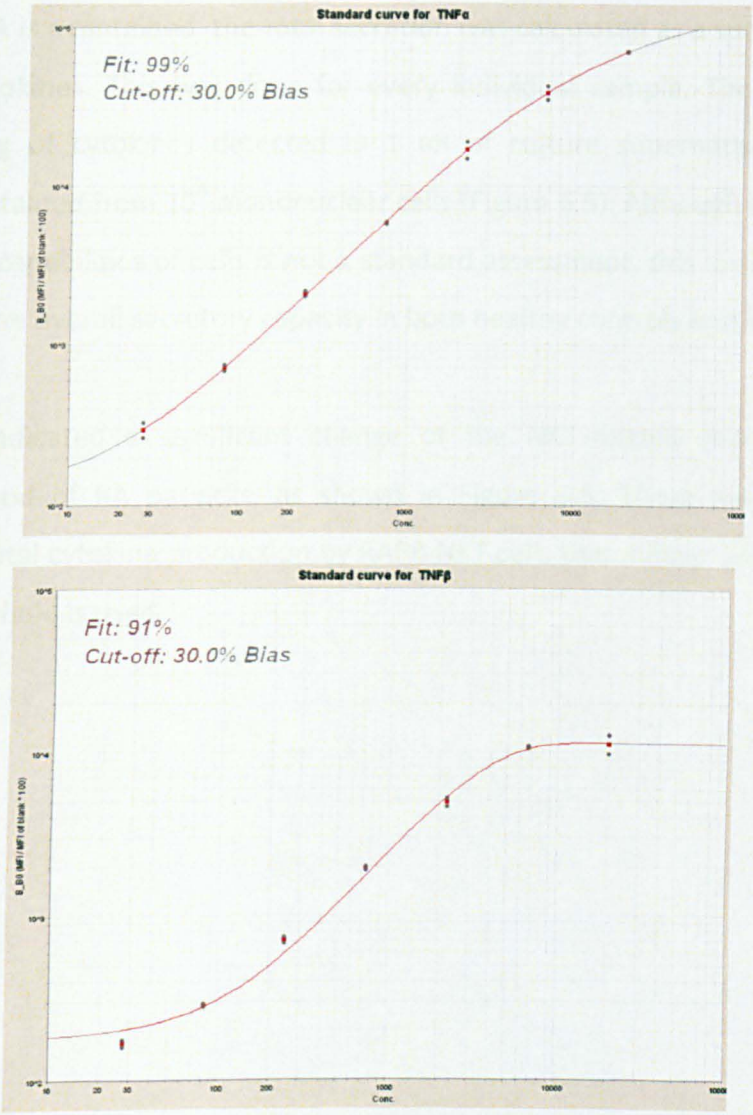


Figure 6.4 Standard curves for concentration quantitation of TNF-α and TNF-β as generated by UK Bio software.

To distinguish whether the overall production of cytokines elicited by specific stimulation of NKT cells in RA is maintained, the total secretion was calculated as a sum of concentrations of all eleven cytokines. This was done for every individual sample. The result calculates the number of pg of cytokines detected in 1 ml of culture supernatant, which equals the production obtained from 10^6 mononuclear cells (Figure 6.5). Although this way of estimating the secretory capabilities of cells is not a standard assessment, this analysis would give some indication of the overall secretory capacity in both healthy controls and RA patients.

The results indicated a significant change of the NKT-related secretory output in the peripheral blood of RA patients, as shown in Figure 6.5. These results point towards a reduction of total cytokine production by RAPB NKT cells that almost returns to normal levels when synovial fluid is used.

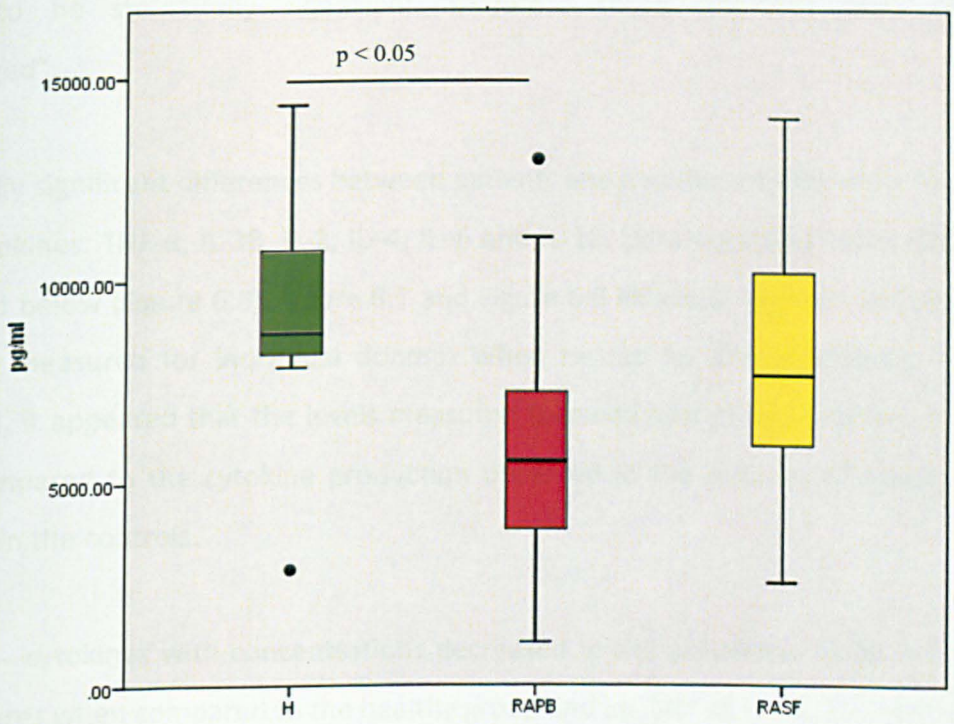


Figure 6.5 Total cytokine concentration detected in 1 ml of supernatant samples. Mononuclear cells were stimulated with αGalCer cultured. Supernatant samples were collected on day 4 from the antigen exposure and the concentrations of 11 different Th1 and Th2 cytokines was quantified by multibead assay. The overall cytokine production was calculated for 1 ml of supernatant by addition of concentrations of all analytes present in the samples. Medians are shown.

The spectrum of Th1 and Th2 cytokines measured in culture supernatants on the fourth day after exposure of mononuclear cells to αGalCer showed that no single cytokine secreted in the cultures of patients' cells mirrored the secretion seen in the healthy donors exactly. However, the changes in IFN-γ, IL-5 and IL–8 levels detected were only minor and did not appear to be statistically significant, therefore these cytokines were classified as "unchanged".

Statistically significant differences between patients and healthy subjects were found for a set of six cytokines: TNF-α, IL-1β, IL-2, IL–4, IL–6 and IL-10. Data regarding these differences are presented below (Figure 6.6). Figure 6.7 and Figure 6.8 illustrate the concentrations of these cytokines measured for individual donors. When results for these cytokines were closely examined, it appeared that the levels measured followed one of two patterns of expression when compared to the cytokine production observed in the cultures of mononuclear cells obtained in the controls.

Pattern I - cytokines with concentrations decreased in the peripheral blood or/and synovial fluid cultures when compared to the healthy group and i.e. TNF-α, IL-1β, IL–2 and IL–4;

Pattern II – cytokines for which concentrations in the peripheral blood were comparable to healthy blood cultures, but were increased in the synovial fluid, i.e. IL–6 and IL–10

Mean concentrations of these cytokines are shown in Figure 6.6. It was not possible to measure the concentrations of TNF–β, due to the lack of any signal in all the samples including controls.

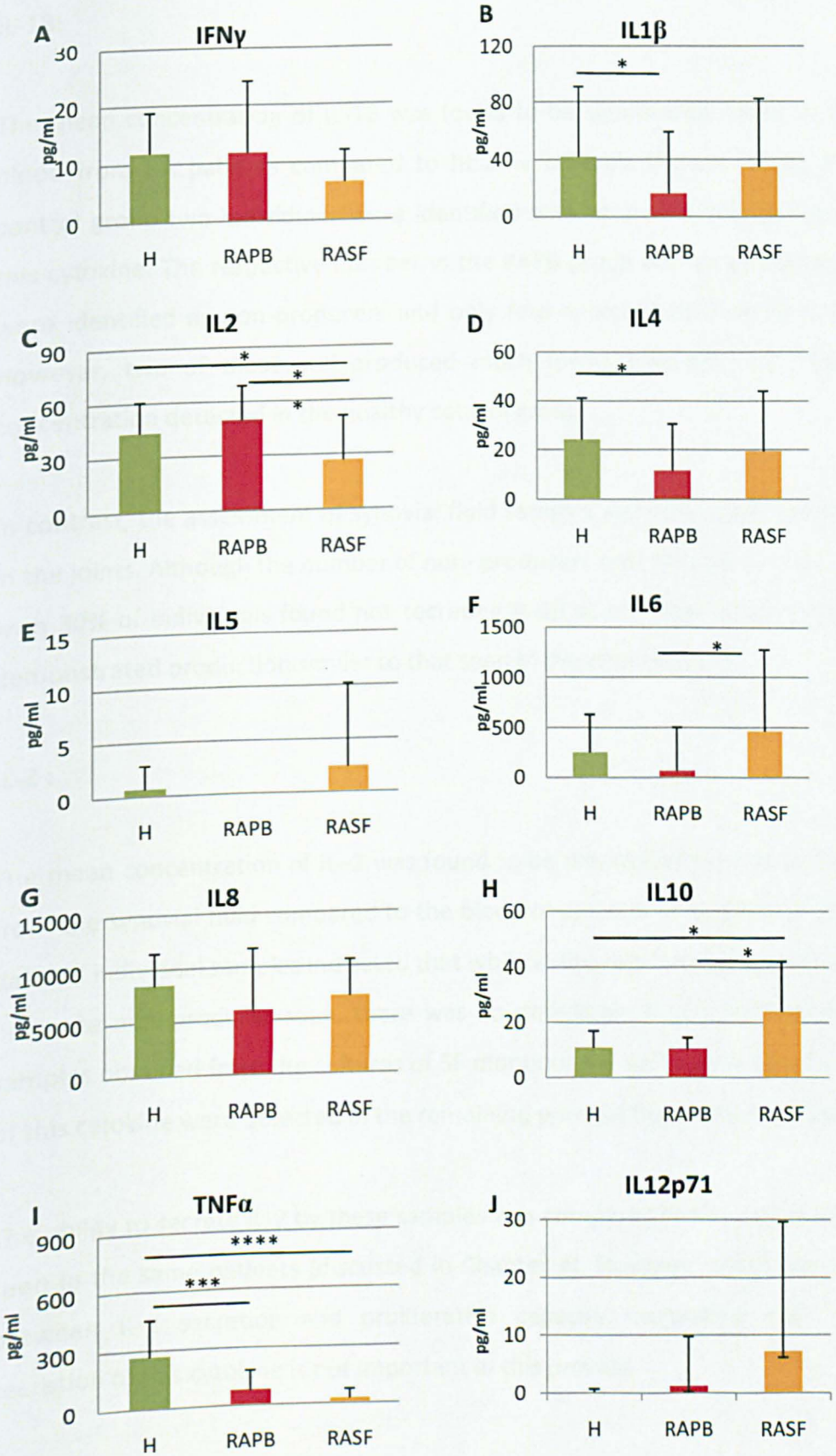


Figure 6.6 Mean cytokine secretion following αGalCer exposure. Cytokines were measured in the samples of supernatant collected from cultures of mononuclear cells on day 4 post stimulation.
*p<0,05, **p<0.02, ***p=0.005, ****p=0.0001

IL-1β:

The mean concentration of IL-1β was found to be significantly lower in the peripheral blood from RA patients compared to healthy controls (Figure 6.6 B). In the healthy control group two individuals were identified who produced undetectable amounts of this cytokine. The respective number in the RAPB group was much higher; 6 individuals were identified as non-producers and only four secreted this cytokine (Figure 6.7 A). However, two of those still produced much lower amounts, less than the lowest concentration detected in the healthy control group.

In contrast, the assessment of synovial fluid samples showed higher production of IL-1β in the joints. Although the number of non–producers was still higher in the synovial fluid, with 30% of individuals found not secreting IL-1β at all, most of the remaining donors demonstrated production similar to that seen in the control group.

IL-2 :

The mean concentration of IL–2 was found to be diminished in cultures containing MCs from the synovial fluid compared to the blood of patients or controls (Figure 6.6 C). The data on individual samples indicated that while in the two latter groups there was always only one non–producer seen, there was no detectable IL–2 in 40% of all supernatant samples obtained from the cultures of SF mononuclear cells, while near to normal levels of this cytokine were detected in the remaining synovial fluid samples (Figure 6.7 B).

The ability to secrete IL-2 by these samples was compared to the proliferative responses seen in the same patients (discussed in Chapter 4). However, there was no correlation between IL-2 secretion and proliferative capacity, suggesting that the autocrine secretion of this cytokine is not important in this process.

IL–4:

The pattern of cytokine secretion in the patients revealed a significant decrease in IL–4 production in the peripheral blood samples from RA sufferers (Figure 6.6 D). While only one healthy individual was identified whose NKT cells did not secrete IL–4 upon stimulation, there were two non-secretors in the blood from the patients and four in the synovial fluid cultures (Figure 6.7 C). Surprisingly however, the mean concentration of this cytokine was only slightly decreased in the cultures of mononuclear cells derived from synovial fluid and this change was not significant. This was mainly due to the fact that there was a single individual in the synovial fluid group who produced unusually high levels of IL–4.

IL–6:

IL–6 was not secreted in particularly large amounts by αGalCer–stimulated cells from control donors. Moreover, in two healthy individuals the secreted amounts fell below the detection threshold (Figure 6.8 A). The number of non–producers detected in the peripheral blood and synovial fluid from RA patients was higher, especially in the former (50% and 20%, respectively). Overall, the mean concentration of IL–6 secreted by the synovial fluid mononuclear cells was increased compared with the results from healthy donors and blood of patients, which was statistically significant for the latter comparison (RAPB vs. RASF) as depicted in Figure 6.6 F. Individual secretion pattern seemed to follow a bimodal distribution, with some patients secreting negligible amounts while others producing significant levels of IL–6.

The data on RA peripheral blood showed a decrease of IL–6 production compared to healthy individuals, but this difference was not statistically significant. To the contrary, a significant change was seen in the patients between peripheral blood and synovial fluid cultures with the latter containing more IL–6. Similarly to IL-4 this was due to one individual secreting relatively high concentrations of this mediator, skewing the overall picture.

IL–10:

IL–10 production by the cells isolated from RA peripheral blood showed no differences from the control samples and the mean concentrations were very similar (Figure 6.6 H). The results obtained by the measurements of IL–10 in the supernatants, however, revealed a significant increase in this cytokine in synovial fluid compared with peripheral blood in both healthy donors and the patient group. This result was obtained regardless the presence of a single non–producer in both control and synovial fluid group (Figure 6.8 B). Moreover, the maximum concentration observed in this group was three–fold higher than the maximum secretion seen in the control samples.

TNF–α:

The results on TNF–α concentration in the collected supernatant samples showed a pattern of progressive decrease in RA (Figure 6.6 I). When analysing mean values, this cytokine was found to be significantly decreased in the peripheral blood of patients. There were also 20% of non–producers in this group (Figure 6.8 C). The synovial fluid group showed an even more pronounced reduction of the mean TNF–α concentration. This was significantly lower when compared to mononuclear cell cultures derived from healthy individuals. The number of non-producers was similar to the RAPB group with two donors producing no detectable amounts of TNF–α.

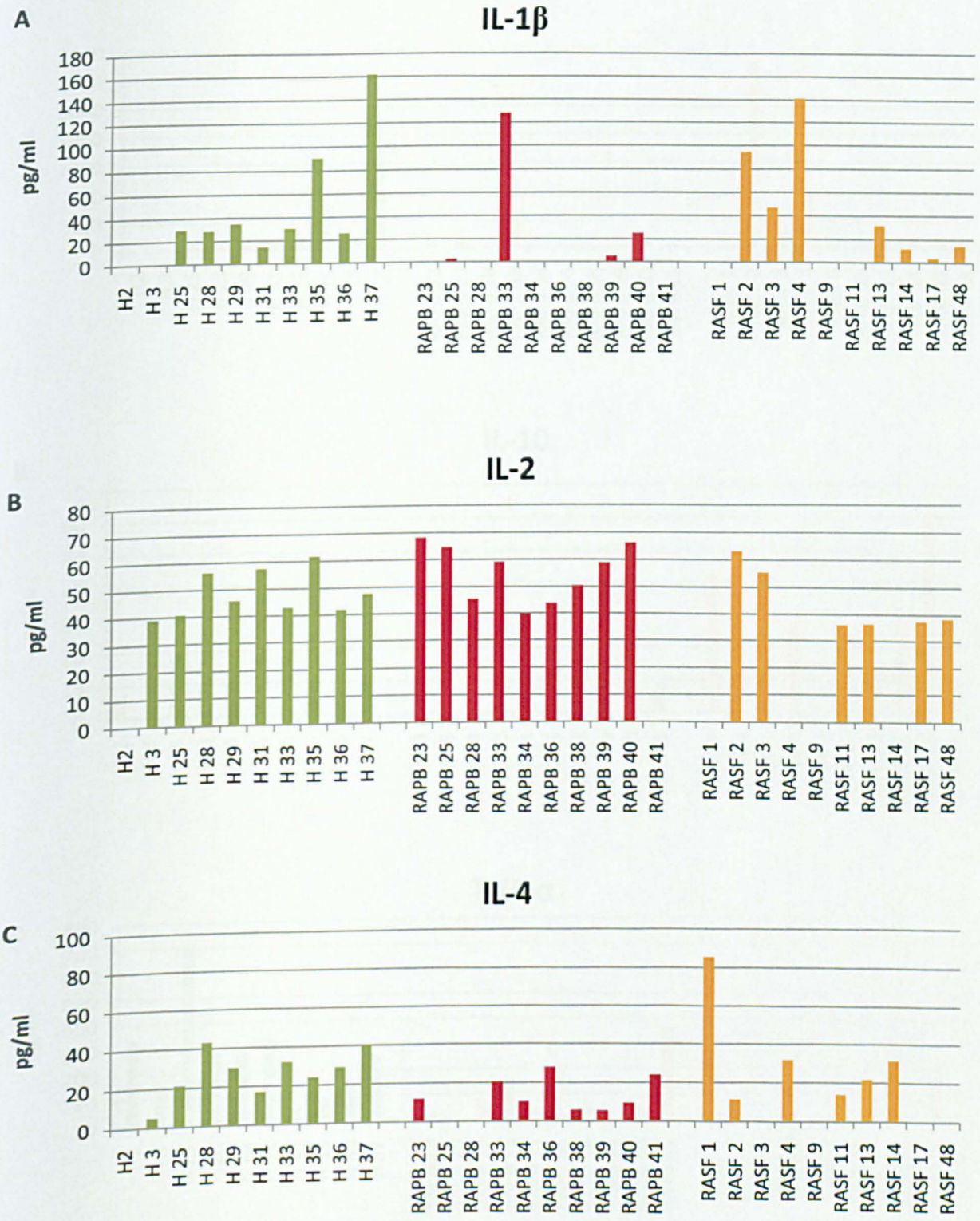


Figure 6.7 Production of IL1β, IL2 and IL4 in the individual samples. Cytokines were measured in the samples of supernatant collected from cultures of mononuclear cells on day 4 post stimulation. The quantified cytokines are indicated above each figure.

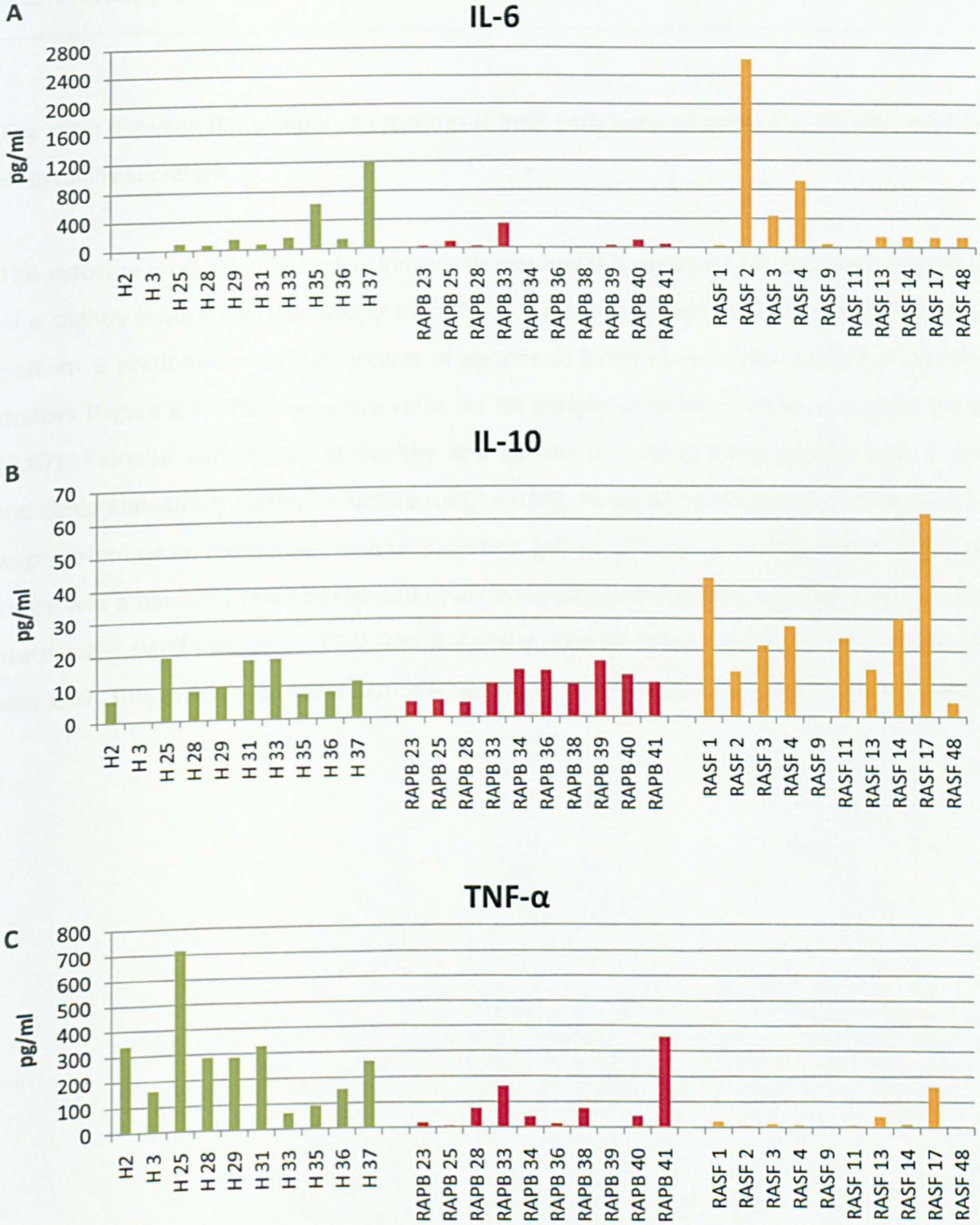


Figure 6.8 Production of IL-6, IL-10 and TNF-α in individual samples. Cytokines were measured in the samples of supernatant collected from cultures of mononuclear cells on day 4 post stimulation. The quantified cytokines are indicated above each figure.

6.6. IL–4/IFN-γ ratio

The ratio between IFN-γ and IL–4 cytokines is frequently used to assess the Th1/Th2 polarity of cytokine secretion.

The cytokine production in control individuals resulted in a ratio of 2.17. As IL–4 is a cytokine of a slightly lower molecular weight than IFN-γ, this result demonstrates that a Th2 cytokine pattern is predominant in the cultures of peripheral blood mononuclear cells from healthy donors (Figure 6.9). The respective value for RA peripheral blood samples was much lower (0.87). Pairwise comparison of healthy and patient peripheral blood groups with T test indicated statistically significant difference ($p < 0.05$). However, ANOVA analysis that is more appropriate when comparing three independent groups of samples indicated that, although there was a tendency towards reduced ratios in the blood of patients, the difference was not statistically significant ($p = 0.056$). The IL-4/IFN-γ ratio calculated for synovial fluid cultures was 1.87. This change was not statistically significant, irrespective of the statistical test used.

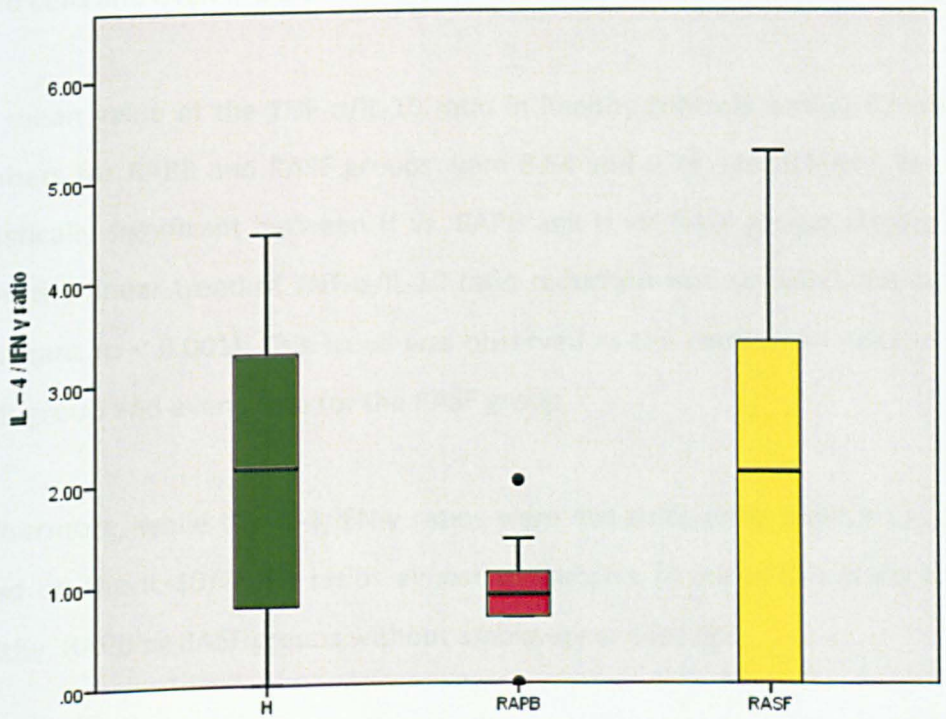


Figure 6.9 IL–4/IFN-γ ratio calculated for the supernatant samples from αGalCer–stimulated cell cultures as detected by the multibead assay(11 plex). The ratios were calculated for every single sample. Median is shown.

6.7. TNF-α/IL-10 ratio

When comparing the groups it become apparent that two cytokines, namely IL-10 and TNF-α demonstrated patterns distinguishing between the sample sources. IL-10 was increased in synovial fluid MC cultures, while TNF-α levels decreased in cultures of patients' peripheral blood cells and even more in SFMC cultures.

The mean value of the TNF-α/IL-10 ratio in healthy controls was 22.62 while corresponding numbers for RAPB and RASF groups were 8.64 and 0.78, respectively. The differences were statistically significant between H vs. RAPB and H vs. RASF groups (Figure 6.10). In addition when the linear trend of TNF-α/IL-10 ratio reduction was analyzed, the outcome was highly significant ($p < 0.001$). This trend was observed as the confidence interval narrowed for the RAPB group and even more for the RASF group.

Furthermore, while the IL-4/IFN-γ ratios were not sufficiently distinct to group the samples, based on the IL-10/TNF-α ratios almost all samples (8 out of 10) could be allocated to the healthy, RAPB or RASF groups without ambiguity or overlap.

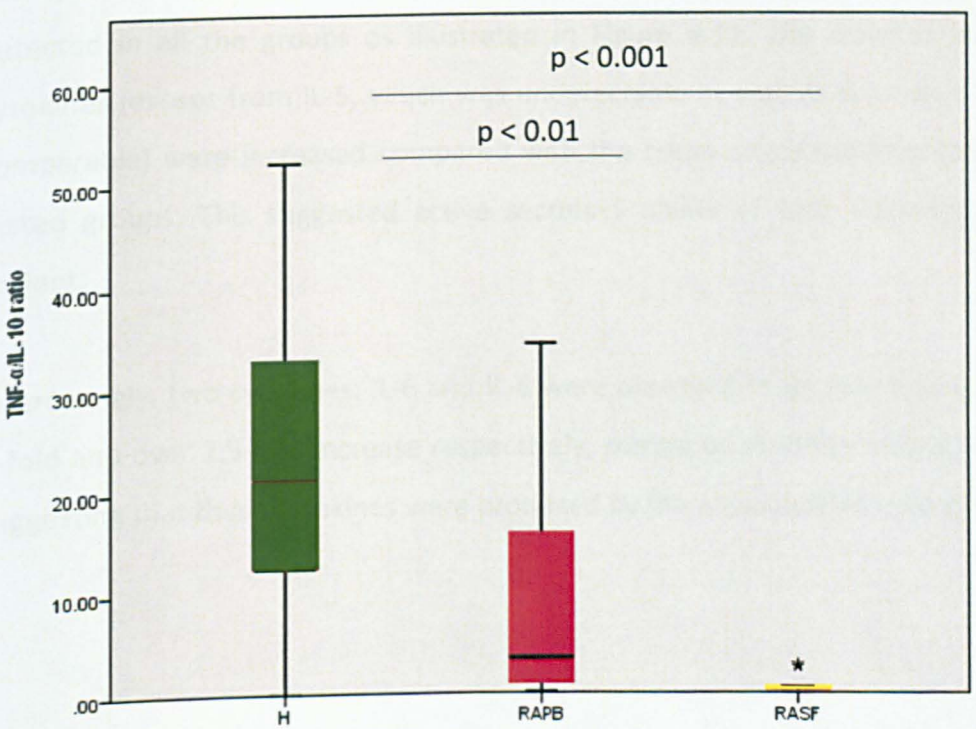


Figure 6.10 TNF-α/IL-10 ratio in supernatant samples from αGalCer–stimulated cell cultures as detected by the multibead assay(11 plex). The ratios were calculated for every single sample. Median is shown.

Although the patient who presented with an expanded *i*NKT cell population (*i*NKT frequency of 7.5% versus the mean for RAPB population of 0.36%, Figure 3.6) was excluded from the analysis of cytokine concentrations in culture supernatants, the sample from this particular individual was still collected and assessed for cytokine concentrations. To assess differences in the cytokine pattern observed in P5, the data was compared to the mean concentrations detected in all the groups as illustrated in Figure 6.11. The concentrations of almost all cytokines (except from IL-5, which was undetectable as well as IL-10 and TNF-α which were comparable) were increased compared with the mean concentrations observed in all three tested groups. This suggested active secretory ability of cells observed in this particular patient.

Interestingly, two cytokines: IL-6 and IL-8 were observed to be very high in Patient 5, almost 6-fold and over 2.5-fold increase respectively, compared with the means for healthy donors, suggesting that these cytokines were produced by the expanded NKT cell population.

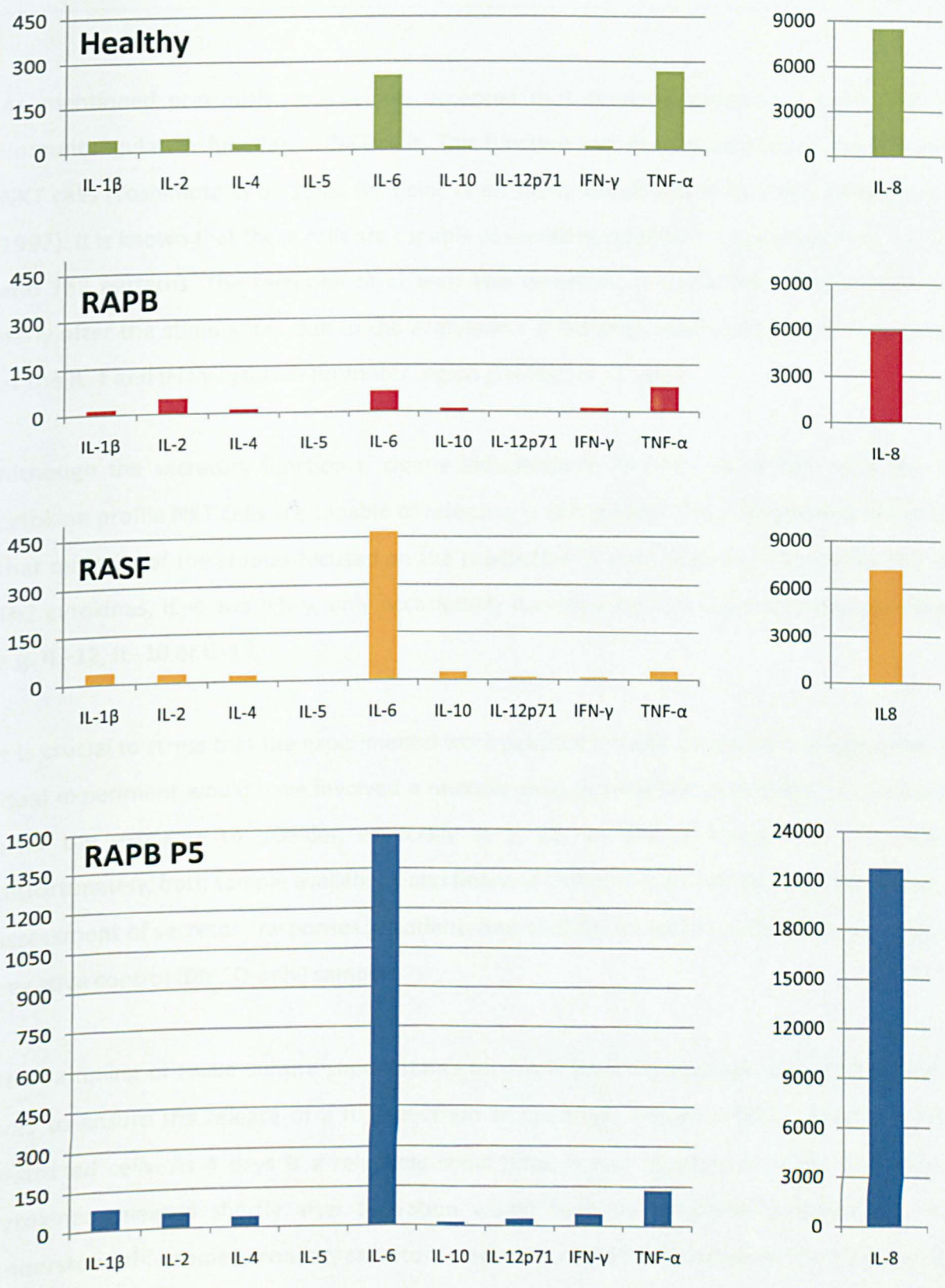


Figure 6.11 Cytokine concentrations measured by multibead assay in SN sample from culture of PBMCs derived from the patient 5. Mean concentrations observed in all study group are provided for comparison.

As mentioned previously, it is widely accepted that cytokine secretion is central to the immunoregulatory function of NKT cells. This function was discovered early in the history of NKT cells (Yoshimoto *et al.* 1995; Bendelac *et al.* 1996; Mendiratta *et al.* 1997; Santiago *et al.* 1997). It is known that these cells are capable of secreting cytokines of opposing roles, i.e. Th1 and Th2 patterns. The secretion of at least two cytokines, IL-4 and IFN-γ, takes place very early after the stimulation, due to the acetylation of histones associated with the chromatin at the IL-4 and IFN-γ cytokine promoter region (Stetson *et al.* 2003).

Although the secretory function is clearly indispensable for the role of NKT cells, the full cytokine profile NKT cells are capable of releasing is still lacking. This is mainly due to the fact that majority of the studies focused on the production of only single representative Th1 and Th2 cytokines, IL–4 and IFN-γ, only occasionally was data also provided for other cytokines, e.g. IL–12, IL–10 or IL-13.

It is crucial to stress that the experimental work described in this chapter is a compromise. An ideal experiment would have involved a multiple sample collection during the 14 day period after the exposure to αGalCer, especially at 1, 12, 24 and 48 hours after stimulation. Unfortunately, both sample availability and financial limitations did not allow for the repeated assessment of secretory responses in patients and controls as well as performing the assay on negative control (DMSO-only) samples.

The sampling of tissue culture supernatants on day 4 from the exposure of cells to αGalCer was to ensure the release of a full spectrum of cytokines following TCR ligation on CD1d-restricted cells. As 4 days is a relatively short time, it was assumed that the NKT-derived cytokines released shortly after activation would have not degraded substantially. It is understood that some secondary secretory responses could have started by this time, as cells were not sorted in these experiments and other mononuclear populations were present. However, the observation of a bystander-derived wave of cytokines was hoped to give some clues on the course of overall immune response after a single exposure of cells to the CD1d

ligand. However, this way of carrying out the experiment had hindered the possibility of identifying the exact source of the cytokines observed in the supernatant samples. Therefore future studies should include assessment of cytokine secretion in the assays with sorted NKT cell subsets.

The cytokine production illustrated here represents the secretory response of all CD1d–restricted cells capable of recognising αGalCer irrespective of TCR expression (i.e. both invariant and non–invariant subsets). However it is conceivable that not all CD1d–restricted cells were stimulated by αGalCer.

The concentrations measured in culture supernatant may not exactly mirror NKT cell–derived cytokine production in the peripheral blood or in the joints affected by the autoimmune process in the patients under stimulation by physiological or pathological self-ligands. However, the results obtained provide some indication regarding the secretory capabilities of NKT cells in RA patients.

The multi bead assay system employed here enabled the testing of a more comprehensive Th1/Th2 secretory profile of NKT cells after exposure to αGalCer, from a relatively small volume of tissue culture supernatant. Conventional ELISA assays would have required a much larger sample volume, which would have been impossible given the number of cells isolated from the samples available for the experiment. Furthermore, the cost of such an experiment would have been prohibitive.

Reports published to date in RA, and other autoimmune diseases, suggest a reduced NKT cell–derived cytokine secretion and a skewed Th1 profile of this response (Wilson *et al.* 1998; Singh *et al.* 2001; Linsen *et al.* 2005). The results presented in this chapter indicated a decrease in cytokine secretion by peripheral blood NKT cells of RA patients. However, such changes were not observed in synovial fluid. It is important to point out that NKT cell numbers were not quantified in the cultures of mononuclear cells. Thus, an increase in this population in synovial fluid samples (as shown in the previous chapter) could potentially compensate for diminished cytokine secretion capabilities of these cells.

When the IL–4/IFN-γ ratio was calculated for peripheral blood NKT cells in RA in the study by Linsen *et al.*, a shift of the immunological response towards a presumably deleterious Th1 pattern was seen (Linsen *et al.* 2005). Unfortunately, ELISPOT assays were only conducted for the mononuclear cells from blood samples; hence the ratio was not calculated for the cells obtained from the site of the inflammation. The assumption of a lack of Th1 bias in the joints was made based on the fact that the differences in intracellular IL-4/IFN-γ staining of cells cultured in the presence of αGalCer for 14 days were not statistically significant compared to the healthy group.

Linsen's findings on Th1 bias of the IL-4/IFN-γ ratio in the peripheral blood were not reproduced in the current project, although a tendency towards reduced IL-4/IFN-γ ratios was present. In addition, no change in the ratio was observed in synovial fluid, which suggests that CD1d–restricted cells at the inflammation site maintain the Th1/Th2 balance seen in the healthy donors. This corresponds with the assumption made by Linsen.

The second observation made in this study was that although the production of another Th2 cytokine, IL–10, is slightly (but not statistically significantly) diminished in the peripheral blood of the patients, the concentration of this cytokine is increased in the synovial fluid. This outcome is quite unexpected. A decrease in Th2 responses has been attributed to autoimmune diseases and emergence of autoaggressive T cells (Beaudoin *et al.* 2002) and explained by the lack of their recognised suppressive role. The function of this cytokine in the process of cartilage reconstruction was also recognised (van Roon *et al.* 1995). In particular, the secretion of IL–10 by stimulated NKT cells proved to be effective in the protection against collagen–induced arthritis (Chiba *et al.* 2004; Miellot *et al.* 2005). Moreover, IL–10 was identified as a cytokine with the ability of increasing proteoglycan (PG) synthesis in RA, especially in conjunction with IL–4 (van Roon *et al.* 1996), reversing the process of cartilage degradation. The increased secretion at the inflammation site identified here should therefore be beneficial.

The third interesting finding was the fact that almost all the patients could be characterised by a significant decrease of TNF-α levels produced in peripheral blood cell cultures. An even more striking decrease was observed in the cultures of SFMCs. In addition, an increased

number of non–producers were identified amongst RA patients than in the healthy individuals. These differences were highly statistically significant. When the data was corrected for the differences in those patients who were undergoing anti–TNF therapy, the significance was even greater, proving that although TNF–α blockage could potentially slightly change the ability of CD1d–restricted cells to secrete this cytokine, TNF–α blockage alone was insufficient to explain the differences presented here. Given the success of anti-TNF therapy in RA, the role of the cytokine in the disease is significant. Therefore the influence of NKT cell activation on TNF-α levels *in vivo* could be also of great importance.

Higher IL–6 concentrations in the supernatant samples from synovial fluid–derived cell cultures were also observed. The abundance of IL–6 in synovial fluid samples from RA patients has been amply documented in the past (Houssiau *et al.* 1988; Lettesjo *et al.* 1998; Steiner *et al.* 1999). Histological characteristics of a chronic synovitis were dependent on local concentrations of IL–6 *in situ* (Sack *et al.* 1993). Correlation was found between IL–6 and sIL–6R levels in synovial fluid and radiological changes, with a suggestion that the IL–6 axis influences osteoclastogenesis (Kotake *et al.* 1996). Various cell types can produce IL–6, however, it is conceivable that CD1d–restricted cells could be the major source of this detrimental cytokine due to their potent secretory function. Data on IL-6 secretion by the patient with an expanded *i*NKT cell population also seem to support this notion. As IL–6 seems to have an influence on promoting naive T cells to switch Th17 cell phenotype (reviewed in Oukka 2008) the production of this cytokine by NKT cells may be relevant to the activation of this pathway in RA.

Although IL-8 secretion was not statistically significantly changed, the concentrations of IL–8 elicited by αGalCer were very high. If the majority of IL-8 was released from CD1d–restricted cells, an assumption of NKT cells as an IL-8 source is supported by the results obtained in Patient 5, whose cell cultures contained 2.5 times more of this cytokine than the culture of cells from an average healthy donor, NKT cells may also play a role in the influx of neutrophils to the inflammatory site, where they are responsible for the degradation of the cartilage components collagen and proteoglycans (Chatham *et al.* 1993; De Clerck *et al.* 1995).

A few studies demonstrated that IL-2 is indispensable for the suppressive function of $CD4^+CD25^+$ T cells (Thornton *et al.* 2004; Thornton 2006). In addition, a malfunction of regulatory T cells at the site of inflammation has been proposed as one of the main factors causing a breakdown of immunotolerance in autoimmunity, including RA (*reviewed in* Anderson and Isaacs 2008). Importantly, these regulatory T cells are present in synovial fluid in abundance and appear functional when separated and cultured in vitro (van Amelsfort *et al.* 2004), which suggests the important role of external mechanisms in controlling the T_{reg} population. A study on *i*NKT cells showed that there was an interaction between these cells and the T_{reg} population as αGalCer-primed *i*NKT cells demonstrated a substantial secretion of IL–2 (Jiang *et al.* 2005) and were able to promote proliferation of fully functional $CD4^+CD25^+$ T_{regs}. Therefore a decrease of IL-2 in the joint *milieu* could facilitate the escape of autoreactive T cells from this control mechanism.

A lack of a strong detection signal for IL–12p70 in the majority of samples was a surprising result. The importance of this cytokine in NKT cell function in antitumor immunity in both primary (Cui *et al.* 1997; Kitamura *et al.* 1999) and metastatic tumours was demonstrated (Shin *et al.* 2001). Moreover, it was also suggested that CD1d–mediated ligand presentation to NKT cells results in CD40/CD40L signalling between the NKT cell and APC (Yue *et al.* 2005). The activation of the NF-κB pathway in the latter results in IL-12 secretion. This positive IL-12 feedback loop accelerates the initiation of the immune response. The lack of this cytokine in the supernatants would therefore advocate that the cytokines measured in the assay are mostly derived from CD1d–restricted cells but not from the other cell sources, as the stimulation of IL-12 secretion by APCs interacting with CD1d-resticted cells is an early event. Alternatively, the lack of the signal in the experiment could be a result of IL-12 degradation by day 4.

Taken together, *in vitro* stimulation with αGalCer seems to elicit a cytokine response that should, in theory, suppress T cell activation. This finding is compatible with the beneficial effect of NKT cell activation in experimental autoimmune disease models. Given that NKT cells present in the synovial fluid and able to produce this potentially beneficial cytokine mix raises the question: why do these cells fail to control the disease *in vivo*?

There are at least three likely explanations here. First of all, it is possible that the presentation of NKT cell antigens at the inflammation site may be inadequate, resulting in a failure to release cytokines. This could happen in the absence of CD1d molecules or co–stimulatory signal expression on antigen presenting cells in the joints. The former is probably unlikely, as preliminary staining of RA joint tissue revealed widespread CD1d expression on synoviocytes (see final Discussion). In addition, the expression of CD1d molecules on professional antigen presenting cells in synovial fluid can be expected and new data showed that some NKT cells can also express this molecule (Hameg *et al.* 2000). On the other hand, co-stimulatory signals have never been investigated and there is a possibility that there are deficiencies in their expression in RA joints.

Secondly, it is also conceivable that CD1d–restricted cells become activated and secrete cytokines in the joints. The lack of improvement could be the result of non–responsiveness of target cells to these cytokines. However, it is hard to envisage that this insufficiency in cytokine responsiveness would not result in any other obvious manifestations in the phenotype of the RA sufferers.

Finally, cytokine profiles described in this chapter represent responses to a pharmacological ligand. A secretory response after exposure to self–ligand(s) *in vivo* is not known and may be distinct from the patterns detected in the experiments here. All the studies conducted to date were based on the assumption that αGalCer is similar to the as yet unidentified self–ligand present in mammals. However, this assumption may be completely wrong as naturally occurring self–antigen could significantly vary from the well-studied synthetic counterpart. These dissimilarities could affect the outcome of NKT cell activation in physiology and disease.

In fact, the diversity of cytokine secretion patterns as a consequence of stimulation with various agonists is a well recognised phenomenon in NKT cell biology. As discussed in the Introduction, relatively few studies were conducted with other naturally–derived or synthetic antigens (Chiba *et al.* 2004; Oki *et al.* 2004; Xia *et al.* 2007) and these measured the secretion of IL–4, which, based on the results presented here, may be insufficient to fully elucidate the complete immunological consequences of NKT cell activation.

In addition, in animal models every CD1d–restricted compound discovered so far seems to elicit a unique cytokine fingerprint (Chiba *et al.* 2004; Coppieters *et al.* 2007). This secretory outcome depends on the length of a sphingosine chain buried within the F' pocket of the CD1d molecule and the relationship of the carbohydrate moiety to the (in)variant TCR expressed by the NKT cell recognising it. The former feature influences the duration of ligand presentation, while the sugar head determines the specificity of TCR recognition (Oki *et al.* 2004). This association of a chemical structure with the secretory outcome was termed "structure–activity–relationship" (SAR). In the case of antigen presentation to NKT cells it was shown that ligands with shorter sphingosine chains, which are easily released from the CD1d molecule, induce more pronounced Th2 responses (Oki *et al.* 2004). To the contrary, longer–chained antigens, sitting deeper in CD1d groove, preferably induce Th1 responses. Interestingly, similar cytokine bias was found a few years ago for peptide presentation by MHC class II molecules (Kumar *et al.* 1995).

Overall, the pattern of cytokine secretion after αGalCer exposure seems to be beneficial. Especially, the decrease of TNF-α levels, accompanied by an increase of IL-10 levels in the cultures of synovial fluid mononuclear cells, suggests that the compound could potentially become a successful drug. It is possible that the CD1d–mediated presentation of a self–ligand occurs physiologically in the joint in healthy people to prevent potential self-reactivity at this location. After all, maintaining mobility is essential for the survival of the individual. A disturbance of the synthesis of such a ligand or, alternatively, an increased abundance of a competitive compound with a different cytokine secretory profile could lead to the initiation of the disease.

While this is an interesting proposition two additional facts need to be considered. Firstly, a study by McCarthy *et al.* failed to reproduce the SAR effect of various CD1d ligands using human NKT cells (McCarthy *et al.* 2007). The other consideration suggests that a change in CD1d ligand expression is not the only way to alter NKT cell secretion profiles. Other alterations in APCs, costimulatory molecules or the presence of cytokines could all affect the secretory activity of NKT cells.

Nonetheless, the possibility that the affected joints in RA may contain alternative CD1d self ligand(s) represents a potentially rewarding experimental approach to the understanding of immunopathology.

Chapter 7: Discussion

The purpose of the work presented in this thesis was to revisit some earlier assumptions on the role of NKT cells in RA and attempt to investigate issues that were not studied in detail before. As discussed widely in various chapters the protective role of TCR Vα14$^+$ *i*NKT cells in rodents is well substantiated in experimental models of autoimmunity. However, whether the equivalent TCR Vα24$^+$ cells play cells a similar regulatory role in humans remains to be proven. Despite the overall similarity the human and the rodent immune system show many striking differences and many of these involve NKT cells. The dissimilarities include the strikingly different genetic organization of the CD1 gene cluster, with the complete lack of CD1a, b and c in mice, as well as the striking difference between the abundance of *i*NKT cells within the lymphocyte pool of the two species.

Curiously, early observations in human autoimmune disease found reduced *i*NKT cell numbers in a range of human diseases. However, by the time the work presented here was started contradictory observations in the most widely studied human disease, IDDM, were also reported. As presented earlier, the studies by Lee *et al.* (Lee et al. 2002) and Oikawa *et al.* (Oikawa *et al.* 2002) questioned the validity of the original results by Wilson *et al.* (Wilson *et al.* 1998). While the former study failed to detect any significance difference between healthy and patient group, including discordant twins, the latter have even observed an increase of the *i*NKT cell population in recent onset diabetes.

The data presented in Chapter 3 seem to indicate that, although there is a tendency to reduced *i*NKT cell numbers in RA peripheral blood and synovial fluid, these changes do not reach statistical significance in the current study. This finding varies from previous reports that documented a significant decrease in the number of DN *i*NKT cells in RA. However, the trend towards a reduction has been clearly observed. The possible reasons, which include AICD, the downregulation of T cell receptors after antigenic encounter, low thymic output or competitive overgrowth of other T cell populations, are discussed in detail in Chapter 3. Given that patient numbers in both the present work and in previous reports were relatively low, the most likely explanation is probably a statistical one, increasing the number of observations may change the statistical outcome in the present study.

An IL-7 deficiency proposed by Ponchel *et al.* could be a possible explanation for the reduced prevalence of *i*NKT cells in the periphery (Ponchel *et al.* 2005). The authors suggest that this mechanism is responsible for a defect in replenishment of the T cell pool in RA patients. This cytokine seems to be crucial in T cell homeostasis, regulating both the generation of new cells in the thymus and maintaining the expansion of circulating cells (as reviewed in Aspinall 2006). Interestingly, it has been shown that IL-7 is an important trigger of differentiation and acquisition of effector phenotype by the *i*NKT cell population, and provides essential signals during *i*NKT cell development in the thymus (de Lalla *et al.* 2008). Therefore the deficiency of this cytokine or a reduced responsiveness of this signalling pathway in RA could directly affect the *i*NKT cell output as well.

As described in Chapter 4 the proliferative responses of *i*NKT cells show a striking reduction in RA patients. This aspect of NKT cell responses did not receive much attention in prior literature. Findings presented here are the results of experiments that are more sensitive to small changes in proliferative capacity and represent more physiological conditions than work published before. Furthermore, if the regulatory function of *i*NKT cells is dependent on cell-cell contact, for example with various forms of dendritic cells, then a proliferative defect would be more functionally relevant than the reduced cytokine production observed by prior observations. In addition to this, as described in Chapter 5 at the site of the inflammation, in the synovial fluid, the total CD1d-restricted NKT cell pool appears expanded. The emerging picture, a reduced proliferative capacity of *i*NKT cells coupled with a relatively increased abundance of non-*i*NKT cells raises a number of issues.

This imbalance between the two subpopulations could be explained by the hypothesis of Goronzy and Weyand mentioned in previous chapters (Goronzy and Weyand 2007). The authors propose that a reduction in thymic output and premature cell aging in RA results in a clonal expansion of some T cell clones and contraction of others. This in turn affects the diversity of TCR repertoire, which may lead to the expansion of autoaggressive and the suppression of protective clones. It is easy to envisage that the same mechanism could also affect the population of CD1d-restricted cells, altering the balance between *i*NKT cells and cells carrying non invariant TCR.

The over abundance of non-*i*NKT cells in the joints raises the possibility that these cells expand *in situ* as a result of an antigen-driven process. Is it likely that such CD1d-driven proliferation takes place in the joints? Up to date there are no studies reporting CD1d expression or the prevalence of non-iNKT cells in healthy joints. Our own, extremely preliminary, findings on the topic suggest that CD1d is expressed in the synovial tissue. Immunofluorescent staining of a single RA synovial tissue sample, shown in Figure 7.1 demonstrates unexpectedly strong staining in the synoviocytes layer and additional, weaker signal in the cells in the deeper layers.

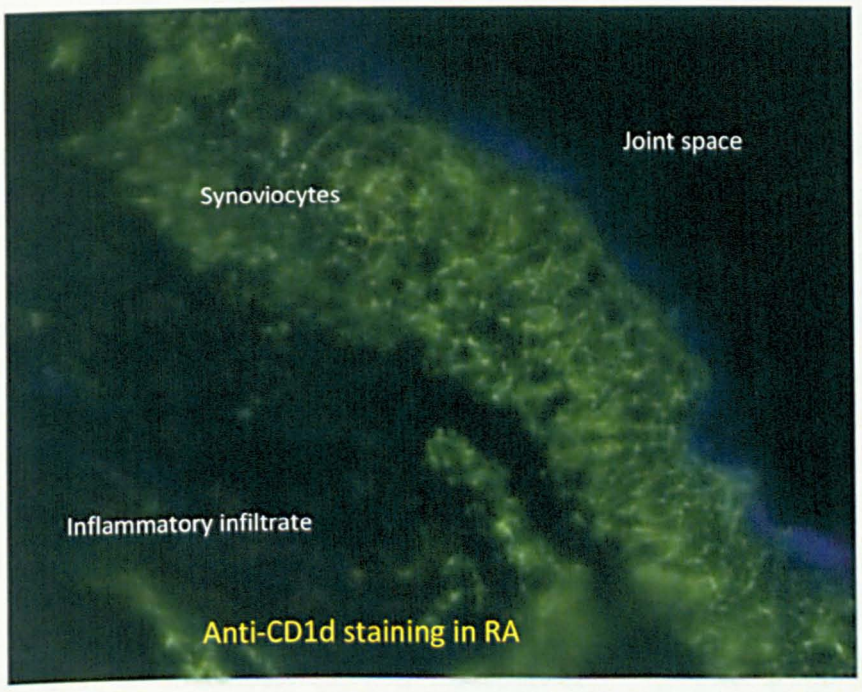


Figure 7.1 CD1d expression by synoviocytes in RA joint. CD1d expression shown in green visualizes a thick layer of expanded synoviocytes which seem to be brightly stained by anti-CD1d antibody, suggesting possibility of CD1d-mediated antigenic stimulation at the site of inflammatory damage in RA; staining carried out by Mrs Anne Phillips in Prof Martin A Birchall's laboratory.

Although it is possible that the uniformly strong signal seen on the synoviocytes is an artefact, staining in the deeper layer, which contains most of the inflammatory infiltrate, strongly

argues for the presence of CD1d in the affected joints. The presence of other elements potentially required for effective CD1d-mediated antigen presentation, such as co-stimulatory and adhesion molecules, remain to be determined. Also, if the staining of synoviocytes is real then it raises the question whether this represents a pathological situation. Alternatively, the expression of CD1d here may be physiological and could represent a pathway aimed at protecting the joint space from immunological insult that could immobilize the host, jeopardising its survival.

If the CD1d-mediated protective pathway is functionally relevant, this would also raise the issue whether an alteration in the balance between *i*NKT cells and non-*i*NKT cells at a given location has a relevance to the outcome of the immune response. It is important to reiterate that both subpopulations of CD1d-restricted NKT cells seem to represent two functionally distinct subsets. Thus, non-*i*NKT cells accumulating in affected joints could promote the inflammation rather than suppress it in the way attributed to *i*NKT cells. While there is still a lot to clarify regarding functional differences between CD1d-restricted NKT cell subsets, it has been reported that these subpopulations act differently in some animal models, such as a response to a transplanted syngeneic lymphoma in mice (De Santo *et al.* 2008). Here, *i*NKT cells seemed to protect from tumour progression, while non-invariant cells promoted it.

In an autoimmune hepatitis model these populations not only had contrasting roles, but also it appeared that the interaction between APCs and non-invariant cells in the liver resulted in the anergy of $V\alpha14^+$ cells, which are known to promote liver damage (Halder *et al.* 2007). While this negative feedback between *i*NKT cells and non-*i*NKT cells results in clinical benefits in the hepatitis model, it may be of a different functional relevance in RA. It is easy to envisage a scenario where the activation of non-invariant cells and suppression of *i*NKT cells occurs in RA, resulting in chronic inflammation. However, in this case the cross-talk between these two populations could produce detrimental effects on the disease progression and clinical scores.

The fact that NKT cells are abundant at the site of autoimmune damage does not necessarily mean that the initial stimulation takes place in the joints. Data presented in this study (Paragraph 3.4 and Paragraph 5.6) suggest that NKT cells may undergo activation before they

enter the joint, as CD4 subpopulations are already disturbed in peripheral blood of RA patients. For example, the activation could take place in the liver, where NKT cells are abundant or in the gut mucosa, where they could be exposed to a variety of ligands. Thus, accumulation of non-*i*NKT cells in synovial fluid could be a result of selective recruitment of these cells to the joint after activation.

A numerical imbalance between invariant and non-invariant subsets of CD1d–restricted cells in synovial fluid from RA patients could also be a result of differences in ligand recognition between NKT cell subsets. Assuming that the non-invariant CD1d-restricted cell subset recognizes a ligand with higher affinity than *i*NKT cells the stimulation would result in an increased proliferation by the non-invariant cells. At the same time, very low affinity towards the invariant TCR could also cause *i*NKT cell anergy. Alternatively, the possibility of completely different ligand specificities and the possibility of the expression of alternative ligands in RA cannot be ruled out. If either of these mechanisms provided a proliferative advantage to non-*i*NKT cells over their invariant counterparts during the selection and/or peripheral expansion, a numerical difference between these populations could be observed, in agreement with the *expansion of non-iNKT cells seen in the present study.*

The increased prevalence of RA in families, although not particularly strong, suggests a genetic link. In fact genome-wide association studies have identified a number of genes that may play a role in the development of the disease. One of the most comprehensive studies, which investigated single nucleotide polymorphisms (SNPs) with the aim to identify genetic predispositions to common human diseases (Wellcome Trust 2007) has identified several SNPs throughout the genome of RA patients. The most prominent of these is localized within HLA-DRB1 locus on chromosome 6, which is not surprising as this association has been long known.

It seems intuitive to assume that if NKT cells and/or the CD1d mediated antigen presentation pathways are involved in the pathological mechanism leading to the development of RA than then at least some of the linked SNPs would cluster around genes that are relevant to this pathway. However, while a strong association was detected at the 1p13 locus, this is situated far from the genes of the MHC class Ib region where CD1d itself is located. However, there are

several loci which contain SNPs bearing a moderate risk for developing RA and these contain genes with a recognized or putative function in NKT cell biology. These include the CTLA-4 molecule, the IL-2 receptor chains (CD25 and CD122), granzyme B and protein kinase C θ (PRKCQ). Protein products of these genes can potentially alter the regulation of the cell cycle and functional properties of CD1d-restricted cells. For example, protein kinase C θ is the main kinase involved in the pathway of activation through the TCR, mediating signalling from ZAP-70 towards NFκB in NKT cell precursors, while granzymes facilitate cytotoxicity and CTLA-4 provides a negative feedback control mechanism after activation. The CD25 receptor is essential in the proliferative role of IL-2 and controlling T cell activation status. Interestingly, the presence of this latter SNP form seen in RA sufferers also confers susceptibility to type 1 diabetes, which suggests that a polymorphism here can be associated with more general mechanisms leading to the induction of autoimmunity.

However, these genetic associations do not satisfactorily explain the induction of RA in all patients. Especially, the LOD scores, which calculate the strengths of the association of a given polymorphism with the risk of developing a disease in a family, are relatively low even for the HLA-DR genes (Legrand *et al.* 1984, Buckner and Nepom 2002), indicating the importance of environmental factors in the initiation of autoimmunity. In fact, according to epidemiological studies, RA incidence in the same ethnic population depends on as yet undefined factors in the environment, and is higher in an urban setting compared to a rural lifestyle (Solomon *et al.* 1975).

The effects of the environment are likely to be related to the change in the spectrum of antigens an individual is exposed to. A vast proportion of known naturally occurring CD1d ligands are derived from bacterial species. This makes it easy to envisage that the introduction of different environmental factors and modification of lifestyle could lead to an alteration in exposure to bacteria, both commensal and pathogenic. Also other pathogens, such as helminths, could be potential sources of CD1d ligands. The resulting changes in CD1d-bound antigens could, in theory, affect NKT cell responses. While this change may be permanent, theoretically even a transient exposure to a particular antigen could result in the induction of the disease, provided that the response promotes the escape of autoreactive T cells from the normal control mechanism provided by NKT cells.

This importance of environmental factors seems to fit well with both epidemiology and poor genetic linkage strengths. This interpretation also implies that the lack of protective ability of NKT cells is not necessarily due to their intrinsic defect but rather to the existence of inflammatory environmental factors.

The role of T cells in the development of RA has seen a major re-assessment recently. There are a number of factors that seem to suggest that $CD4^+$ T cells, found at the inflammatory site were "bystanders" rather than "key players" in the development of RA. These cells primarily show a memory phenotype. Such cells would be recruited to an inflammation site non-specifically, and their presence does not necessarily indicate engaging in T cell-mediated inflammation. Consistent with this concept the proliferative capacity and cytokine secretion of intra-articular T cells was consistently reported to be reduced (Davis *et al.* 2001). Obviously, if $CD4^+$ T cells play no role in the development of RA then regulatory pathways controlling their activation, such as T_{regs} and NKT cells, would have little relevance.

However, the success of an anti-CTLA treatment (Ostor 2008; Taylor 2009) led to the re-evaluation of the role of T cells in RA. The action of CTLA-4Ig blocks the costimulatory signal during the priming of naïve T cells, while the presence of this signal is not necessary for the activation of memory cells (reviewed in Salomon and Bluestone 2001). The fact that the CTLA-4Ig blockade of CD28-mediated co-stimulation shows beneficial effects even in established RA, in the light of our current understanding, strongly argues that the activation of naive T cell is a part of the ongoing immunological mechanisms that maintain the inflammation in RA. These observations represent the strongest evidence to date that T cells play an important role in the pathological mechanism of the disease. This finding in turn implies that a regulatory mechanism, for example NKT cells, could play an important role in preventing disease development.

Importantly, a spectroscopic analysis of synovial fluid samples from RA patients revealed a significant increase in ceramide concentration compared to samples from patients with osteoarthritis (Ciurtin *et al.* 2006). This finding implies that there is an abundance of potentially relevant ligands, which could be presented in the joints to various lipid-specific T cell populations, resulting in their expansion. The finding of the accumulation of CD1d-

restricted cells at the site of inflammation presented in this study is further supported by previous work in our laboratory, which identified an increase of $CD4^+CD161^+$ T cell frequency in RA synovial fluid samples compared to the blood from both RA patients and healthy individuals (H. Kamarova, PhD thesis, University of Liverpool, 2008). While $CD4^+CD161^+$ and NKT cell populations are not directly comparable, the existence of a larger population of CD161-expressing T cells is potentially compatible with the presentation of non-peptide ligands to CD1d- or MR1-restricted, so called type III NKT cells. Thus, antigen presentation mediated by MHC class Ib molecules may be of a crucial importance for either maintaining the integrity of the joint or intensifying the pathology seen in RA.

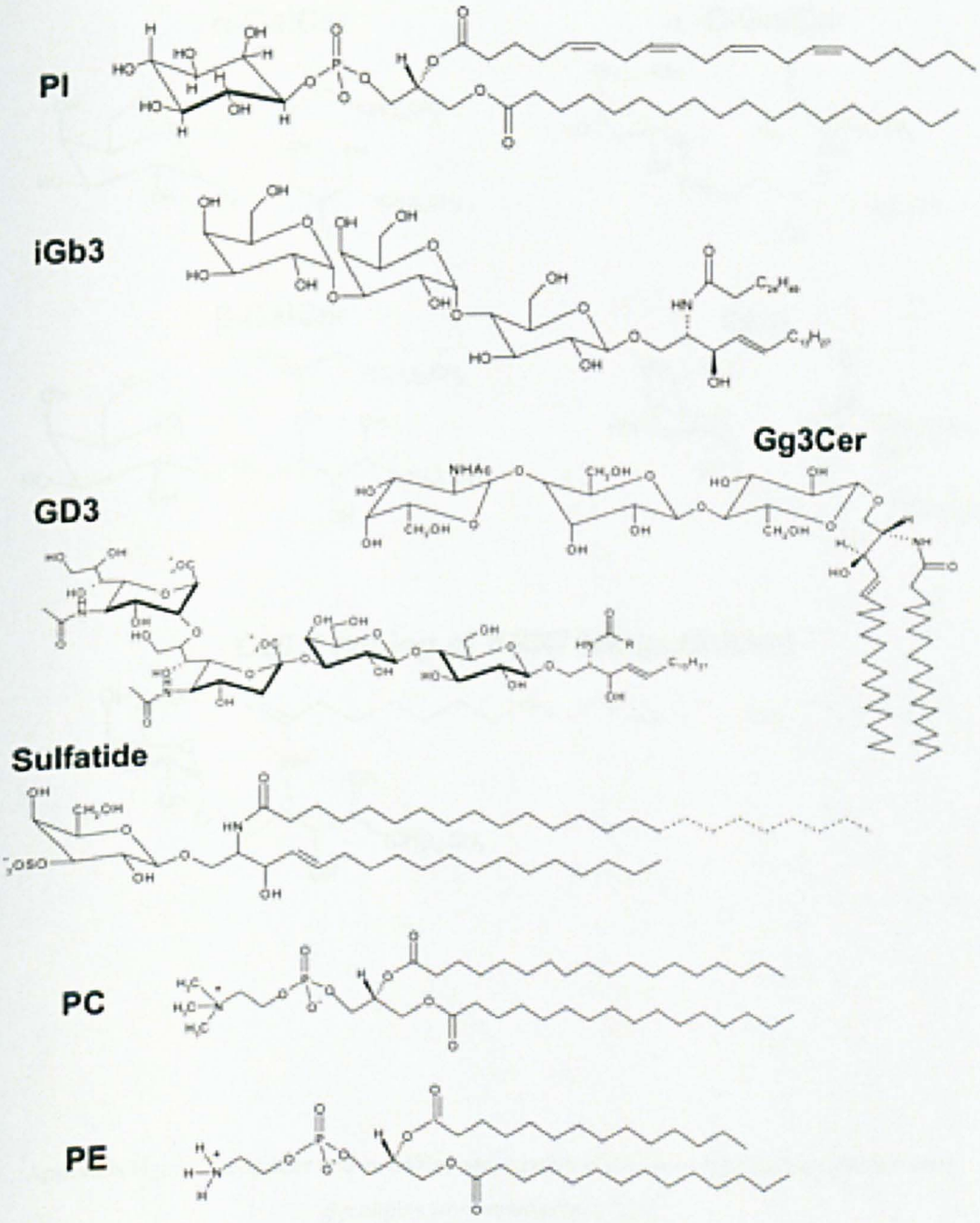
The main hypothesis on the study assumed a differential function of non-*i*NKT cells in RA and varying roles of two subpopulations of NKT cells in the pathogenesis of the disease. Therefore, the aims of the study, set out to clarify the involvement of both subsets were met and the results confirmed this initial supposition. The findings presented here suggest that there is a defect in the proliferative responses of Vα24 TCR chain-expressing NKT cells. At the same time an elevated level $V\alpha24^-$ NKT cells accumulates in the affected joints. This altered balance between the two subsets, which is unique to the synovial fluid and is not seen the peripheral blood of the patients, may result from CD1d-mediated antigen presentation in situ. As the results of the cytokine expression studies show, this could lead to a unique cytokine environment, low in TNF-α and high in IL-10.

Future studies should therefore focus on the characterisation of non-*i*NKT population, its responsiveness in RA patients and the cross-talk between these cells and *i*NKT cells. Analysis of glycolipids in RA synovial fluid, especially the elution of ligands from joint-derived CD1d molecules (expressed by synoviocytes or soluble), could provide invaluable information on the role of endogenous ligand(s) in the disease and shed more light on the existence of various CD1d-restricted populations at the site of autoimmune damage in patients.

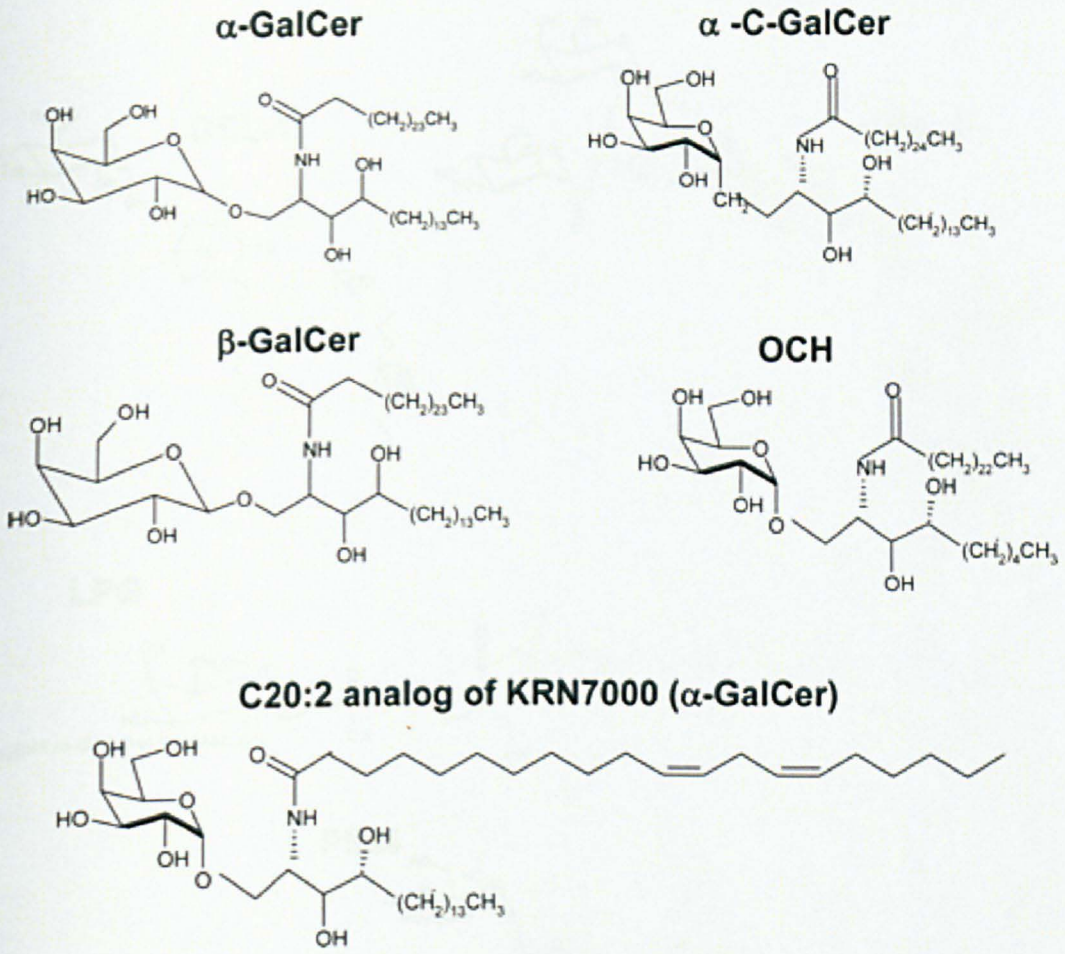
Understanding the mechanisms that underlie the reduced *i*NKT cell proliferation, the mechanisms leading to the expansion of non-invariant subset and the importance of the altered cytokine profile, may hold clues to the defective immunoregulation in the disease. Furthermore, modifying/altering these pathways may bring therapeutic benefits.

Appendix: CD1d Ligands

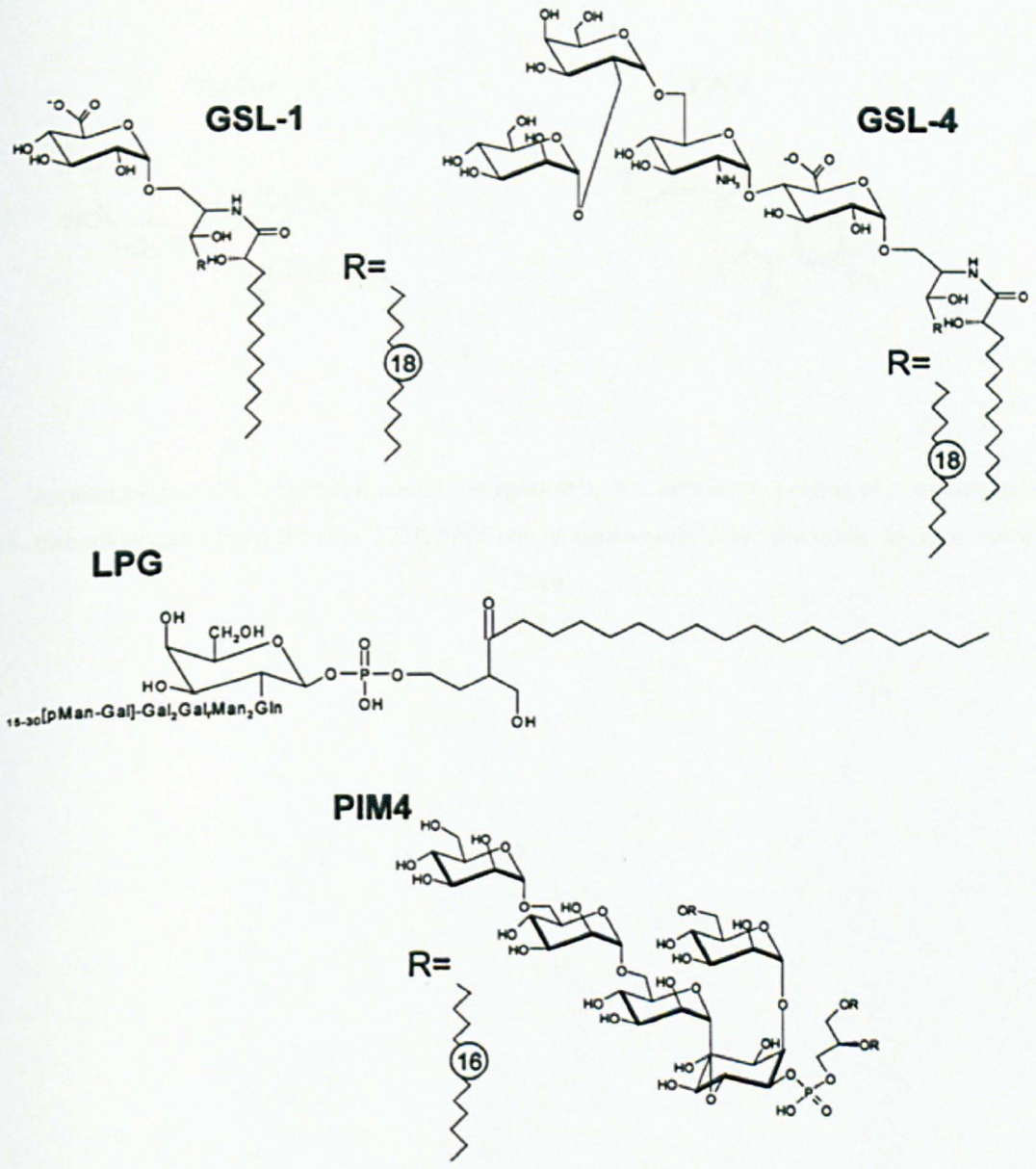
This appendix contains chemical structures of known CD1d ligands. Because of a large number of compounds now discovered, only a small selection is presented. Figures show natural ligands, αGalCer analogues, pathogen-derived antigens and synthetic compounds, recognized by CD1d-restricted cells.



Appendix Figure 1. "Natural" ligands of CD1d. A selection of lipids derived from mammalian cells. PI, phosphatidylinositol; iGb3, isoglobotrihexosylceramide; Gg3Cer, gangliotriaosylceramide; GD3, disialoganglioside; PC phosphatidylcholine; PE, phosphatidylethanolamine from Brutkiewicz 2006

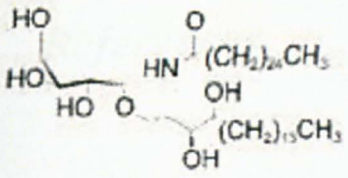


Appendix Figure 2 α-GalCer and α-GalCer analogs. Synthetic α-GalCer and α-GalCer-based glycolipids from Brutkiewicz 2006.

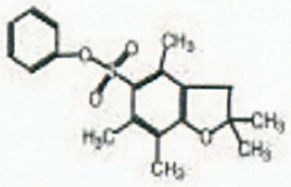


Appendix Figure 3 Pathogen-derived CD1d ligands. CD1d ligands from *Sphingomonas spp.*, *Leishmania donovani*, and *Mycobacterium tuberculosis*, LPG, lipophosphoglycan from *L. donovani*; PIM4, PIM from *M. tuberculosis*. From Brutkiewicz 2006, modified

ThrCer



PPDF



Appendix Figure 4 Synthetic compounds recognized by NKT cells in the context of CD1d molecule ThrCer, threitolceramide from Silk *et al.* 2008; PPDF phenyl pentamethyldihydrobenzofuran from Van Rhijn *et al.* 2004

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