transnationale
UNIVERSITEIT LIMBURG

School voor Levenswetenschappen

Role of Natural Killer T cells in Rheumatoid Arthritis

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Biomedische Wetenschappen,
te verdedigen door

Loes LINSEN

Promotor : Prof. Dr. P. Stinissen
Copromotor : Prof. Dr. J. Raus

2005

DOCTORAATSPROEFSCHRIFT

Universiteit Maastricht

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You will do foolish things,
but do them with enthusiasm.
Colette

Members of the jury

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Table of Contents

List of Abbreviations

α-GalCer	α-galactosylceramide
AP-1	activator protein 1
AP-3	adaptor protein complex 3
APC	antigen presenting cells
BSA	bovine serum albumine
CatL	cathepsin L
CatS	cathepsin S
CCP	cyclic citrullinated modified peptides and proteins
CD	cluster of differentiation
CDR	complementary determining region
Cpm	counts per minute
CsA	cyclosporin A
CTLA-4Ig	cytotoxic T lymphocyte-associated antigen-4 immunoglobulin
DC	dendritic cells
DMARDS	disease modifying anti-rheumatic drugs
DN	$CD4^-CD8^-$, double negative
EAE	experimental allergic/autoimmune encephalomyelitis
ER	endoplasmatic reticulum
E/T	effector to target ratio
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
HC	healthy control
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSP	heat shock protein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1Ra	IL-1 receptor antagonist
LT	lymphotoxin
LTβR	LTβ receptor
mAb	monoclonal antibody
MEC	medullary epithelial cells

MHC	major histocompatibility complex
MP	methylprednisolone
MS	multiple sclerosis
MTX	methotrexate
NBT/BCIP	nitro blue tetrazolium/5-bromo-4-chloro-3-indolyphosphate
NF	nuclear factor
NIK	NF-κB-inducing kinase
NKT	natural killer T cells
NOD	non-obese diabetic
NSAIDS	non-steroidal anti-inflammatory drugs
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PHA	phytohemagglutinin
PMA	phorbol-12-myristate-13-acetate
RA	rheumatoid arthritis
RANKL	receptor activator of NF-κB ligand
RF	rheumatoid factor
rhIL-2	recombinant human interleukin-2
RT	room temperature
SEM	standard error of mean
SF	synovial fluid
SFMC	synovial fluid mononuclear cells
SI	stimulation index
SLE	systemic lupus erythematosus
ST	synovial tissue
TCL	T cell line
TCR	T cell receptor
Th0/1/2	T helper 0/1/2
TNF	tumor necrosis factor
Tr1	regulatory T 1 cell
Treg	$CD4^+CD25^+$ regulatory T cells
TSST	Toxic-Shock-Syndrome Toxin
TT	Tetanus Toxoid
V	variable region of the TCR α/β chain

List of Figures

Chapter 1.

Chapter 3.

Chapter 4.

Chapter 5.

Nederlandse samenvatting.

Chapter 1

Introduction

Etiology of rheumatoid arthritis and

Immunoregulation of autoimmunity by Natural Killer T cells

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, inflammatory disease that predominantly affects the joints. The clinical picture varies from mild joint symptoms to severe inflammation and damage of the joints, which eventually can lead to manifestations beyond the joint. Genetic and environmental factors affect the progression, extent and pattern of the inflammatory responses and are most likely responsible for the heterogeneous clinical features. Investigations of RA at the molecular level have markedly changed our understanding of the pathogenesis although the etiology remains unresolved.

1.1.1 Clinical features and epidemiology

RA is classified according to criteria published by the American Rheumatism Association[1]. A patient is diagnosed to have RA if he/she has fulfilled at least 4 of the following 7 criteria: morning stiffness, arthritis of 3 or more joint areas, arthritis of hand joints, symmetric arthritis, rheumatoid nodules, increased serum rheumatoid factor and radiographic changes in the joint. Initially, these criteria were designed to classify the disease for research purposes, but they are used nowadays as guidelines for the diagnosis. The prominent signs of joint inflammation in RA are tenderness and swelling of the joint while local heat and reduced mobility are also often present. Structural damage in a typical patient most often starts between the first and second year of disease. Commonly, the joints of the wrists and small joints of hand and feet are involved. Involvement of the large joints (such as knee and hip) may occur later on and is associated with moderate to severe disease[2]. Affection of non-joint organs (extra-articular manifestations) occurs predominantly when specific 'rheumatoid' antibodies are present. Increased mortality rates arise largely from extra-articular organ involvement[3].

RA is distributed worldwide and involves all ethnic groups. In Flanders and The Netherlands approximately 200 000 people suffer from RA[4]. The occurrence of RA is relatively constant with a prevalence of between 0.5 and 1.0 %, a frequency that has been reported in several European and North-American populations[5]. However, higher prevalence rates are found in native American-Indian populations[6,7], while very low occurrences were shown in rural African and Asian groups[8,9]. Community based incidence studies of RA indicate that the disease occurs at a higher rate in Native American tribes than in Caucasian populations from Europe and North America[5]. These data support the hypothesis that RA may have originated in North America and suggest a genetic predisposition to the disease.

The peak of onset of RA is in the fifth decade of life, but it may occur as early as the second decade[10]. The prevalence of RA is 2.5 times higher in women than in men, although the ratio declines to 1 in the elderly. Erosive disease is more frequent and tends to occur earlier in men than in women and extra-articular symptoms, particularly rheumatoid nodules, are more common in men than in women[11]. Other person-related attributes, such as smoking and diet, also seem to have an impact on the course and final outcome of the disease rather than on its susceptibility[12-14].

1.1.2 Pathology

In RA, the earliest changes in the tissue surrounding the joint, the synovium, are associated with mild proliferation of the superficial lining cell layer. Normally, this lining is only 3 cell layers thick, but it can increase to up to 30 cell layers in RA. The thickened intima in RA consists mainly of activated intimal macrophages and fibroblast-like synoviocytes[15]. This stage of inflammation is associated with hyperplasia and edema. Cellular infiltration occurs early in disease and initially

consists mainly of T lymphocytes. Antibody producing B cells are usually found in more advanced stages of inflammation[16]. At the same time, multinucleated giant cells and mast cells are also present[17]. As a consequence of inflammation, the synovium becomes hypertrophic and villous projections of synovial tissue (ST) protrude into the joint cavity, where it overgrows and invades the underlying cartilage and bone[18]. Proliferating ST at the synovium-cartilage junction is often referred to as *pannus*.

1.1.3 Genetic and environmental factors

Although the cause (or causes) of RA remain(s) elusive, it is generally accepted that genetic and environmental factors contribute to the disease.

Familial clustering of RA as well as the fourfold increased concordance in monozygotic twins compared with dizygotic twins support a contribution of genetic factors to disease occurrence. Genetic modeling studies suggest that genetic factors may account for as much as 60 % of disease susceptibility[5]. The best association of RA is that with the human leukocyte antigen (HLA) class II molecules, especially with the HLA-DRB1 locus[19]. In 1987 the 'shared epitope hypothesis' was put forward. This postulated that the association was not with HLA-DRB1 per se, but with a highly conserved sequence motif (QKRAA, QRRAA, RRAAA) present in the third hypervariable region of the β chain of the HLA-DRB1 molecule[20]. Severity of disease appears to correlate with the sequence polymorphism within the disease associated sequence stretch and the number of alleles present[21]. These data suggest that the presence of the shared epitope is associated with disease severity rather than incidence. Weak linkage was also found for tumor necrosis factor (TNF)-α, interleukin (IL)-5 receptor, interferon (IFN)-γ and IL-2 genes[22]. Polymorphisms in the genes for IL-1α, IL-1β and the IL-

1 receptor antagonist (IL-1Ra) have been associated with disease severity, rather than RA susceptibility[23].

The idea that RA might be caused by infection is far from new[24]. It is possible that in genetically predisposed individuals an environmental factor triggers the development of RA by initiating an inflammatory reaction. The mechanism of RA induction by an infectious agent could be due to molecular mimicry of specific microbial peptides with autologous molecules[25]. Bacterial products such as Mycobacterial heat shock protein (HSP)-65 and dnaJ, have been suggested to be potential cross-reactive epitopes, but no conclusive evidence has been demonstrated[26,27]. Furthermore, Epstein-Barr virus has been frequently detected in synovium of RA patients[28,29]. Thus, although there are some hints that infection may play a role in triggering or perpetuating RA, there is no overwhelming evidence to link any single known infectious agent with the disease.

1.1.4 Current therapies for rheumatoid arthritis

The first-line drugs used to treat RA belong to the class of non-steroidal anti-inflammatory drugs (NSAIDs). These drugs, of which aspirin is the best known example, provide symptomatic relief but have no impact on disease progression and have various adverse events. A new class of NSAIDS, the cyclooxygenase-2 inhibitors, show equal anti-inflammatory properties but appear to be substantially safer than classic NSAIDs[30].

Glucocorticoids, such as prednisolone and methylprednisolone, are effective anti-inflammatory agents, that rapidly suppress synovitis in RA[31]. They are superior to NSAIDs in reducing pain and morning stiffness, but long-term administration results in adverse effects. In addition to glucocorticoids, disease-modifying antirheumatic drugs (DMARDs) are used to treat more severe disease. These drugs

include methotrexate (MTX) and cyclosporin A (CsA) and reduce or prevent joint damage by interfering with the inflammatory process. MTX inhibits the enzyme dihydrofolate reductase involved in pyrimidin and purine synthesis necessary for DNA production and can thus interfere with clonal growth of immune cells[32]. CsA inhibits activation induced signaling and hampers transcriptional activation of cytokine and other genes essential for T cell proliferation. Secondary, it also affects other immune cells which are dependent on T cell derived cytokines for their activation[33]. Both molecules show a strong anti-inflammatory effect, indicated by rapid onset of clinical effect.

In recent years, therapies have been developed that specifically target pro-inflammatory cytokines involved in the disease process of RA, such as TNF-α and IL-1. Several clinical trials with these agents in patients with early or established RA have shown consistent improvement in signs and symptoms as well as function, with a dramatic slowing of radiographic damage[34]. The anti-TNF-α agents etanercept and infliximab combined with optimal doses of MTX currently constitute the most interesting therapeutic regimen for the management of patients with RA failing to adequately respond to traditional DMARDs. New molecules have been constructed that have reduced side effects, but these still have to be approved before they can be used in clinical practice.

1.1.5 Current hypothesis on the etiology of rheumatoid arthritis

1.1.5.1 Role of autoreactive T cells in the immunopathogenesis of rheumatoid arthritis

The foundation for the hypothesis of RA as a T cell driven disease was laid when it was demonstrated that the majority of infiltrating lymphocytes in synovial fluid and tissue were T cells which displayed features of previous activation[15,35]. Furthermore, the strongest indication of a T cell driven disease, is the association

of RA with specific HLA or major histocompatibility complex (MHC) II alleles[27]. Additional evidence comes from the frequent observation that specific T cell receptor (TCR) V region genes are selectively expanded in the synovial compartment (often termed 'skewing' or 'bias')[36-43]. Characterization of clonally expanded $CD4^+$ T cells revealed that these cells acquire a distinct functional profile (loss of the co-stimulatory CD28 molecule, overproduction of IFN-γ, cytotoxicity, less susceptible to apoptosis) which enables them to function as proinflammatory cells, indicating a possible role in disease pathogenesis[44,45]. Further evidence for a role of T cells in RA comes from the many putative autoantigens that have been identified as candidate antigens by the use of *in vitro* proliferation assays with T lymphocytes from RA patients. Type II collagen[46,47], cartilage gp39[48], aggrecan[49], cartilage link protein[50], heat shock proteins[26] and BiP[51,52] have been implicated by virtue of T cell reactivity and a T helper 1 (Th1) T cell response in RA.

The rationale that RA is a Th1 driven, T cell mediated disease is supported by the analysis of infiltrating synovial T lymphocytes. These cells are biased towards a Th1-like phenotype and predominantly produce IFN-γ[53,54]. The amounts of T cell derived mediators expressed in rheumatoid synovial tissue are small but physiologically relevant to contribute to immune responses, fibroblast activation and bone destruction[55]. Additionally, activated T cells might directly participate in the pathogenesis of bone erosions by the expression of receptor activator of the nuclear factor (NF)-κB ligand (RANKL)[56]. They can also promote the production of cytokines, particularly TNF-α, and metalloproteinase by macrophages and fibroblast-like synoviocytes through cell-membrane interactions[57]. Since soluble factors thus induced may in turn contribute directly to T cell activation, positive feedback loops could be created. Therefore, disease perpetuation can be induced and sustained by chronic activated T cells.

Other evidence for the central role of T cells in RA evolves from the extensive investigation of animal models of RA. In 3 commonly used animal models of RA: collagen induced arthritis, streptococcal cell wall arthritis and particularly adjuvant arthritis, Th1 T cell responses are central to the initiation and maintenance of the disease[58,59]. Arthritis is transiently transferable by T cells, cannot be induced in T cell deficient animals, and the antigenic peptides which stimulate disease-causing T cells have been elucidated[58]. However, in collagen induced arthritis and many other forms of arthritis, B cells are also essential for disease development[58].

Finally, T cell directed immunotherapies showed beneficial effects in RA patients. Cyclosporin A is a general T cell suppressor which has proven to suppress disease activity in RA[60]. T cell depletion by total lymphoid irradiation[61] or lymphocyte-apherese[62,63] has demonstrated clinical improvement of the disease. Suppressive therapies that induce T cell modulation such as administration of non-depleting anti-CD4 monoclonal antibodies[64] or cytotoxic T lymphocyte-associated antigen-4 immunoglobulin (CTLA-4Ig) antibodies[65] that block co-stimulation, in addition to T cell vaccination[66] and T cell receptor peptide vaccination[67] indicate a central role for T cells in RA pathogenesis.

In conclusion, several lines of evidence suggest a critical role for T cells in RA. However, the unimpressive levels of T cell cytokines in synovium, the irreproducible pattern of oligoclonality and the failure to identify RA specific pathogenic autoantigens have prompted researchers to re-examine these issues.

1.1.5.2 Other cell types involved in the rheumatoid disease process

An important clue that B cells could play a role in RA, was the identification of 'rheumatoid factors' in blood of these patients. Rheumatoid factors (RF), which are autoantibodies specific for the constant region of immunoglobulin (Ig) G, can

be detected in the majority of RA patients and their presence predicts a more aggressive, destructive disease course[68]. IgG aggregates can trigger complement activation which led to the hypothesis that tissue damage in RA was attributable to the local deposition of immune complexes[69]. Recently, the contribution of autoantibodies and B cells to RA pathogenesis has regained interest based on the discovery of antibodies directed against citrulline modified peptides and proteins (anti-CCP antibodies)[70]. Final evidence for a role of B cells in disease pathogenesis is given by the success of B cell depleting antibody therapy (Rituximab) in RA[71].

Another cell type involved in the etiology of RA, is the mast cell. These cells are found in synovial infiltrates and express membrane associated receptors for the constant region of IgE and IgG[72]. These receptors enable mast cell triggering which results from Ig cross-linking. In murine models of erosive arthritis, mast cells have been shown to play a central role in immune complex mediated joint inflammation[73]. Mast cells triggered by immune complexes produce proinflammatory cytokines, including TNF-α and IL-1, and proteolytic enzymes. They are also major sources of vasoactive and chemotactic factors that facilitate the recruitment of other leukocytes to the synovial membrane[72]. Thus, mast cells are a plausible cellular link between autoantibodies, soluble mediators, and other inflammatory effectors in arthritis.

Macrophages also appear to play a pivotal role in RA because they are numerous in the inflamed synovial membrane and at the cartilage-pannus junction. It has been shown that the degree of macrophage infiltration correlates with the radiological progression of permanent joint damage[74]. These cells display features that can contribute to inflammation and joint destruction observed in acute as well as chronic RA[75,76]. Macrophages involved in tissue destruction produce significant amounts of proinflammatory cytokines and contribute to the

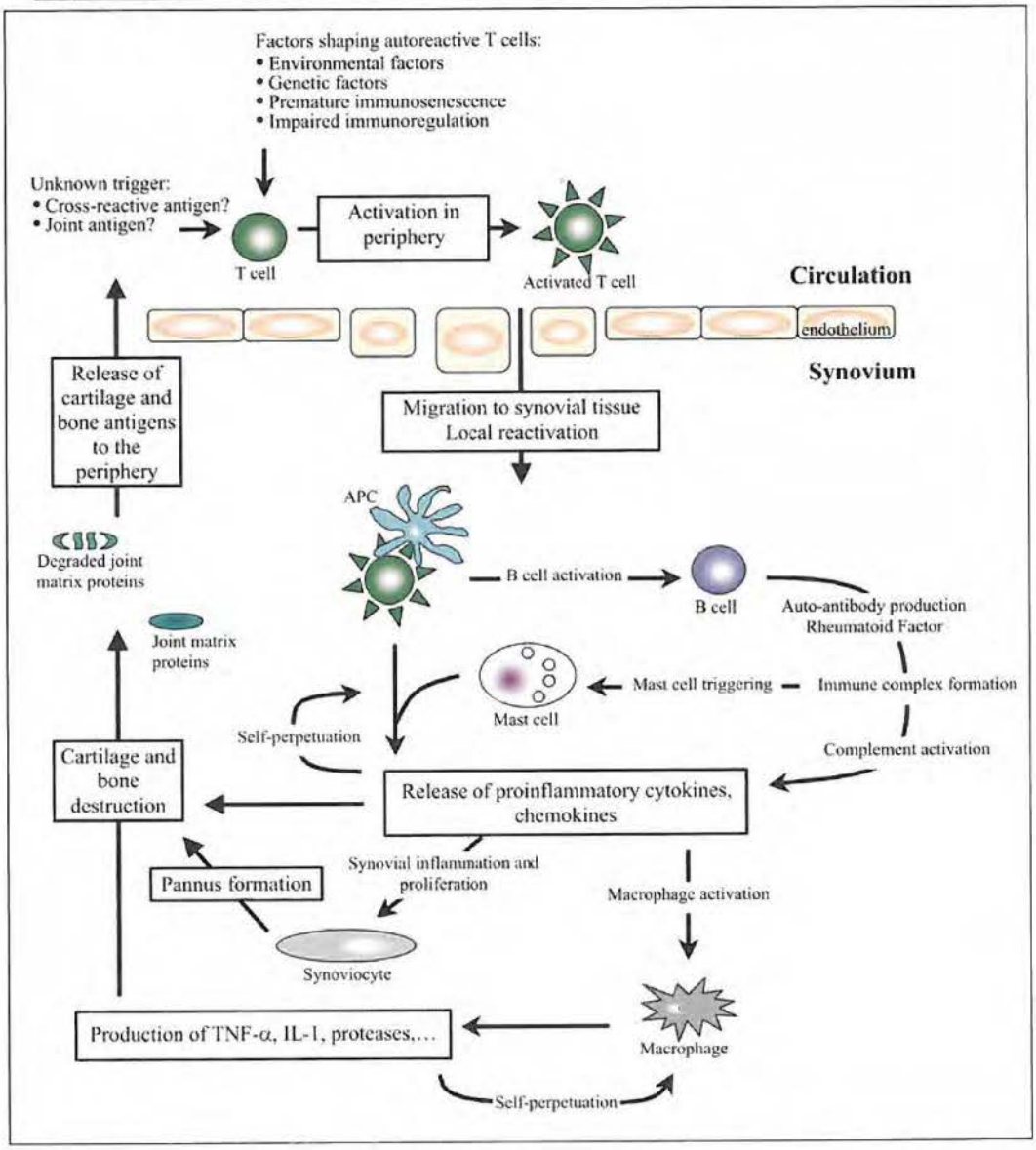

Figure 1.1. Current hypothesis of the pathogenesis of RA. *CD4+ T cells are activated in the periphery by an unknown trigger and infiltrate into the synovial tissue. In the synovium, they are reactivated and initiate the (auto)immune responses. The production of cytokines and chemokines and cell-contact dependent interactions cause infiltration and activation of other cells (B cells, macrophages, mast cells,...). Activated cells secrete cytokines and other mediators which contribute to the inflammatory self-perpetuating processes and eventual joint damage. This leads to the release of cartilage and bone derived antigens in the periphery and results in further amplification of the inflammatory response.*

production of TNF-α and IL-1 is important in disease amplification as has been shown by several experimental and clinical observations[76]. Clinical trials with anti-TNF-α monoclonal antibodies, TNF-α receptor constructs or IL-1 receptor antagonist have shown remarkable efficacy in acute RA, which is consistent with the central role of IL-1 and TNF-α[34].

Although these intensive investigations have greatly improved the insight in mechanisms that lead to synovitis, the initial trigger of RA has still not been elucidated. Multiple pathways, both antigen specific and antigen independent, could be involved in the complex disease that RA is. These pathways are not mutually exclusive and different mechanisms probably dominate at various phases of the disease. A summary of the current hypothesis of the pathogenesis of RA is given in figure 1.1.

1.1.5.3 Repercussion of impaired immunoregulation

Although autoreactive T cells are commonly found in healthy individuals, the occurrence of autoimmune diseases is rather low. The maintenance of self-tolerance can be kept by passive mechanisms such as T cell anergy or deletion, and immunological ignorance[77]. However, it can still be anticipated that under conditions leading to upregulated antigen processing during inflammation, an autoreactive response is induced, which is followed by a suppressive response when the initial trigger is cleared[78]. Although for a long time a matter of debate, new evidence indicates that T cell mediated suppression or regulation might have a role in modulating immune responses[79-84]. This protects most individuals from chronic autoimmunity, while in a minority, these processes may be dysregulated which could lead to a chronic autoreactive response.

A broad range of regulatory T cells has been implicated in RA. These cells are often decreased in number, or show a comprised function. A recently described

population of regulatory cells is the $CD4^+CD25^+$ regulatory T cell (Treg) population. These cells are anergic and can suppress other T cells upon TCR mediated activation[80]. An abnormal function of $CD4^+CD25^+$ cells has been reported in RA patients. This aberration could be reversed by anti-TNF-α therapy[85]. Others however, reported functional but decreased numbers of Treg cells in RA patients[86,87]. This inconsistency could indicate that Treg cells play a role in determining the patient's fate towards either a favorable or unfavorable clinical course of the disease. Other regulatory T cell subsets have been implicated in RA. A reduced number of regulatory Tr1 cells was observed in blood and SF of RA patients[88]. Additionally, spontaneous remissions of arthritis in children were seen to coincide with regulatory self-HSP60 specific T cell responses[89]. Finally, regulatory Natural Killer T (NKT) cells have been reported to be numerically and functionally deficient in a whole range of diseases characterized by autoreactive tissue damage, including RA[90-92]. This population of cells will be discussed in more detail later.

In patients with RA, the many autoreactive T cells may well result from a healthy state in which these cells were present, but strictly regulated. An unknown trigger might have lead to an escape of these cells from their regulatory network, shifting the normal tolerant state to a novel autoaggressive state[93].

1.2 Natural Killer T cells

Natural Killer T (NKT) cells, a newly identified subpopulation of lymphocytes, have been implicated in immune responses controlling various diseases including infection, cancer, autoimmunity and transplantation. The features of these cells and their importance in regulation of autoimmunity suggest that NKT cell based therapies might be an interesting approach for the treatment of autoimmune diseases.

1.2.1 NKT cell characteristics

1.2.1.1 General NKT cell features

The term "NK T cells" was first used to define a subset of T cells that expressed the NK cell associated marker NK1.1 (CD161). It is now generally accepted that the term "NKT cells" refers to CD1d-restricted T cells co-expressing a heavily biased, semi-invariant TCR and NK cell markers[94]. This TCR consists in humans of an invariant TCR Vα24Jα18 chain combined with a variable TCR Vβ11 chain (Vα14Jα281 and Vβ8, Vβ7 or Vβ2 chains on mouse NKT cells)[83,84,95-97]. These properties distinguish NKT cells from conventional $CD4^+$ and $CD8^+$ T cells, which use a diverse TCR to recognize peptide antigens bound by polymorphic MHC molecules (Fig 1.2).

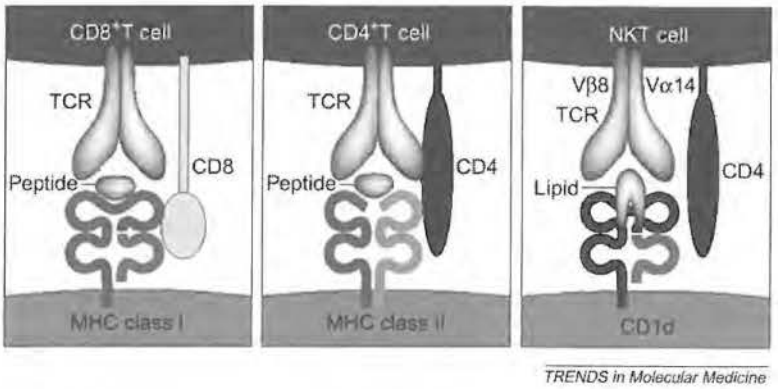

Figure 1.2. Antigen presentation and recognition properties of $CD4^+$ T cells, $CD8^+$ T cells and NKT cells[98]. *MHC class I and II molecules present peptide antigens to $CD8^+$ and $CD4^+$ T cells respectively. The non-polymorphic molecule CD1d presents glycolipid antigens to NKT cells. T cells interact with these complexes through T cell receptors, which are diverse in the case of $CD4^+$ and $CD8^+$ cells, and semi-invariant for NKT cells. Reprinted, with permission, from Trends in Molecular Medicine, Volume 8, © (2002), by Elsevier.*

In humans, only 0.2% of peripheral blood T cells are NKT cells. They are also found in the human liver but their numbers are lower than in liver of mice[99-101]. Another striking feature is their expression of markers associated with recently activated or memory T cells ($CD45RO^+CD45RA^-CD25^+CD62L^-CCR7^-$ in humans)[102,103].

Interestingly, NKT cells in germ-free mice and in cord blood also show this activated/memory surface phenotype[103,104], which suggests that previous exposure to microbial antigens is not the cause of this phenotype. In addition, these cells might recognize an endogenous, but not yet identified, antigen.

Human and mouse NKT cells segregate into $CD4^+CD8^-$ and $CD4^-CD8^-$ (double negative, DN) subsets which differ in their functional properties. About 50% of human invariant NKT cells are $CD4^+$ with high donor-to-donor variability, and CD8α expression is common, but only very few $CD8\beta^+$ NKT cells exist[105,106]. The $CD4^+$ subset potently produces both Th1 and Th2 cytokines whereas the DN population selectively produces Th1 cytokines IFN-γ and TNF-α and preferentially upregulates perforin in response to IL-2 or IL-12[105,107]. Additionally, some chemokine receptors are differentially expressed on the subsets (CCR4 on $CD4^+$ NKT cells; CCR1, CCR6 and CXCR6 on DN NKT cells)[108]. These findings were recently supported by microarray data[109].

1.2.1.2 Recognition and presentation of glycolipid antigen

NKT cells are activated by glycolipid antigens presented in the monomorphic MHC I-like molecule, CD1d[95]. The CD1d molecule is highly conserved among mammalian species[84,110]. It is primarily expressed on cells of hematopoietic origin, including thymocytes, B cells, macrophages and dendritic cells (DC) and can be induced in T cells upon activation[111]. The binding cleft of the CD1d molecule is narrower but deeper than that found in conventional MHC class I and class II molecules and consists of two binding pockets. The non-polar lining of the antigen binding groove makes the molecule ideal for the presentation of hydrophobic antigens, such as glycolipids[112].

After assembly with β2-microglobulin in the endoplasmatic reticulum (ER), CD1d is rapidly transported from the Golgi apparatus to the plasma membrane along

the secretory pathway[113] (Fig 1.3). Internalization from the plasma membrane is dependent on the CD1d cytoplasmatic tail, which directs trafficking through early and late endosomes to lysosomes[114]. Normal endosomal targeting of CD1d, presence of CD1d molecules in lysosomes and internalization of the glycolipid antigen by the APC are essential for successful antigen presentation[115,116].

Glycosylphosphatidylinositol (GPI, Fig 1.4) was the first molecule identified to be associated with CD1d in major amounts *in vivo*, but it could not stimulate NKT cells[117]. It was therefore hypothesized that GPI could act as a chaperone, shielding the hydrophobic ligand-binding groove during CD1d trafficking until the physiological NKT cell ligand is encountered in an endosomal compartment[118].

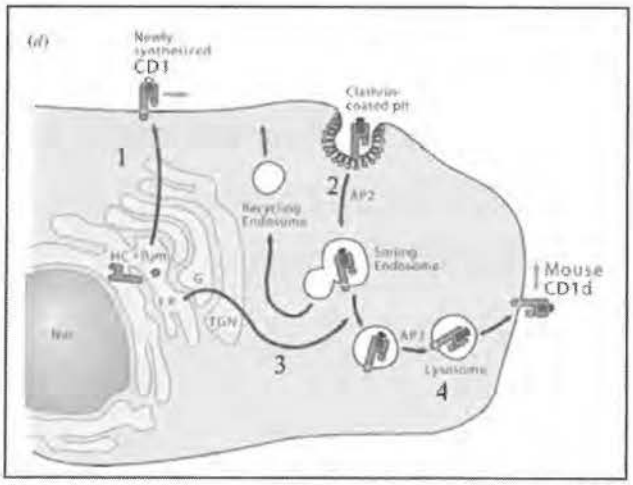

Figure 1.3. Model for the intracellular mechanisms involved in the loading of glycolipid antigens onto CD1d molecules[84]. *CD1d molecules may be loaded in the endoplasmatic reticulum (ER) by phospholipid chaperones such as glycosylphosphatidyl-inositol (GPI), and follow the secretory pathway to the cell surface (1). They can then enter endosomal compartments via recycling of membrane proteins (2). Additionally, CD1d molecules may associate with MHC class II chaperones such as Ii, which directly transport CD1d to the endosomal compartments (3). After removal of chaperones, endosomal CD1d molecules may be loaded with endogenous or exogenous glycolipid antigens (4). Reprinted, with permission, from the Annual Review of Immunology, Volume 22, ©(2004) by Annual Reviews.*

NKT cell research has expanded enormously with the discovery of α-galactosylceramide (α-GalCer, Fig 1.4), a glycolipid that binds to CD1d molecules and selectively activates both mouse and human NKT cells, during a screen for novel antitumor molecules[119]. α-GalCer was originally isolated from the sea sponge *Agelas mauritianus*[120]. Since α-glycosphingolipids are not present in mammalians, α-GalCer probably mimics self-antigens that are recognized by NKT cells. As a result of this discovery, α-GalCer loaded, genetically engineered, CD1d-tetramers have been extensively used to study NKT cell biology[100]. A truncated form of α-GalCer, OCH (Fig 1.4), could also activate NKT cells, but induced a higher production of Th2 cytokines in NKT cells than α-GalCer[121]. Recently, it was reported that a subset of α-GalCer reactive mouse NKT cells could respond to GD3, a ganglioside commonly found on tumors of neuroectodermal origin. This study was the first demonstration of a natural ligand for mouse NKT cells[122]. Furthermore, it has now been reported that both human and mouse NKT cells can also recognize a endogenous lysosomal glycosphingolipid, isoglobotrihexosyl-ceramide, identifying it as a possible natural NKT cell antigen[123].

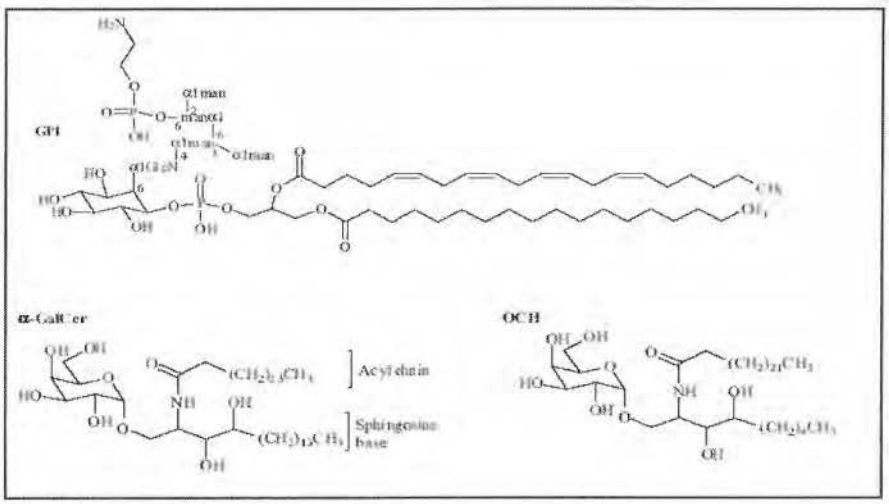

Figure 1.4. Structure of glycolipids that can bind to CD1d[124]. *Printed with permission. GPI: glycosylphosphatidylinositol; α-GalCer: α-galactosylceramide.*

1.2.1.3 Effector functions of NKT cells

The most striking property of NKT cells is their capacity to secrete large amounts of cytokines (IFN-γ, IL-4, IL-2, IL-5, IL-10, IL-13, GM-CSF and TNF-α) within minutes after TCR stimulation. This feature distinguishes them from naïve MHC class I and II restricted T cells that acquire their ability to secrete cytokines during proliferation after primary stimulation. Unlike conventional T cells, NKT cells activate IL-4 and IFN-γ transcription during thymic development and populate the periphery with both cytokine loci previously modified by histone acetylation[125]. Abundant mRNA transcripts for IL-4 and IFN-γ were detected in resting NKT cells which may allow the rapid production and secretion of these cytokines[125]. Upon TCR engagement, NKT cells also have cytotoxic activities through the release of perforins and granzymes and by the expression of membrane bound members of the TNF family (such as FasL)[126].

Quickly upon activation, NKT cells become undetectable when assessed by flow cytometry with α-GalCer loaded CD1d-tetramer staining. Although this feature was first attributed to activation induced cell death, it was recently shown to result from downregulation of the TCR[127,128]. After activation the cells dramatically expand to even up to 10-fold their normal numbers within 2-3 days of stimulation before contracting to baseline levels on subsequent days[127,128]. Activated NKT cells are relatively resistant to apoptosis due to the upregulation of several anti-apoptotic genes[129].

1.2.2 Thymic development of NKT cells

Due to the unavailability of a specific marker for NKT cells, it was first thought that NK1.1$^+$ T cells could develop independent of the thymus[130]. Most recent studies however, conclude that NKT cells are a thymus dependent population that has undergone TCR β selection[131] (Fig 1.5). This TCR β selection triggers the re-

expression of Recombination Activating Genes to allow for TCR α chain rearrangement and leads to the expression of CD4 and CD8 on the thymocytes. The TCR α chain of NKT cells is invariant at the amino acid level, but can vary at the nucleotide level, which suggests that the characteristic NKT TCR gene rearrangement is achieved randomly alongside TCR β and α chain rearrangement of mainstream precursors[131].

The divergence of $CD4^+CD8^+$ thymocytes to the NKT cell lineage is likely to be triggered by positive selection. Inhibition of CD1d trafficking through endosomes, or inhibition of endosomal proteases, all result in the failure of NKT cell positive selection[132,133], thereby demonstrating the importance of the selecting ligand. This molecule is a glycolipid self-antigen presented in a CD1d context which is confirmed by the NKT cell deficiency of $CD1d^{-/-}$ animals[102]. Interestingly, the CD1d/glycolipid complex must be presented to the developing NKT cell by hematopoietically derived $CD4^+CD8^+$ thymocytes, rather than thymic epithelial cells responsible for conventional positive selection[134]. Possibly, $CD4^+CD8^+$ cells provide essential signaling for entry to the NKT lineage which is not available on the epithelial cells. Negative selection of NKT cells is mediated by dendritic cells, occurs at an earlier stage and the window of susceptibility is far narrower for NKT cells than for other T cells[135,136]. Additionally, NKT cells require expression of RelB and NF-κB inducing kinase by a thymic stromal cell for terminal differentiation[137].

Only 30 % of the NKT cells that are exported to the periphery express a more mature phenotype, which suggests that NKT cells undergo a late phase of peripheral development[138]. Furthermore, it was reported that the number of human $CD4^+$ NKT cells in peripheral blood is mainly determined by thymic output, while $CD4^-$ NKT cells undergo extensive peripheral expansion[139]. However, both subsets develop their functions in the periphery.

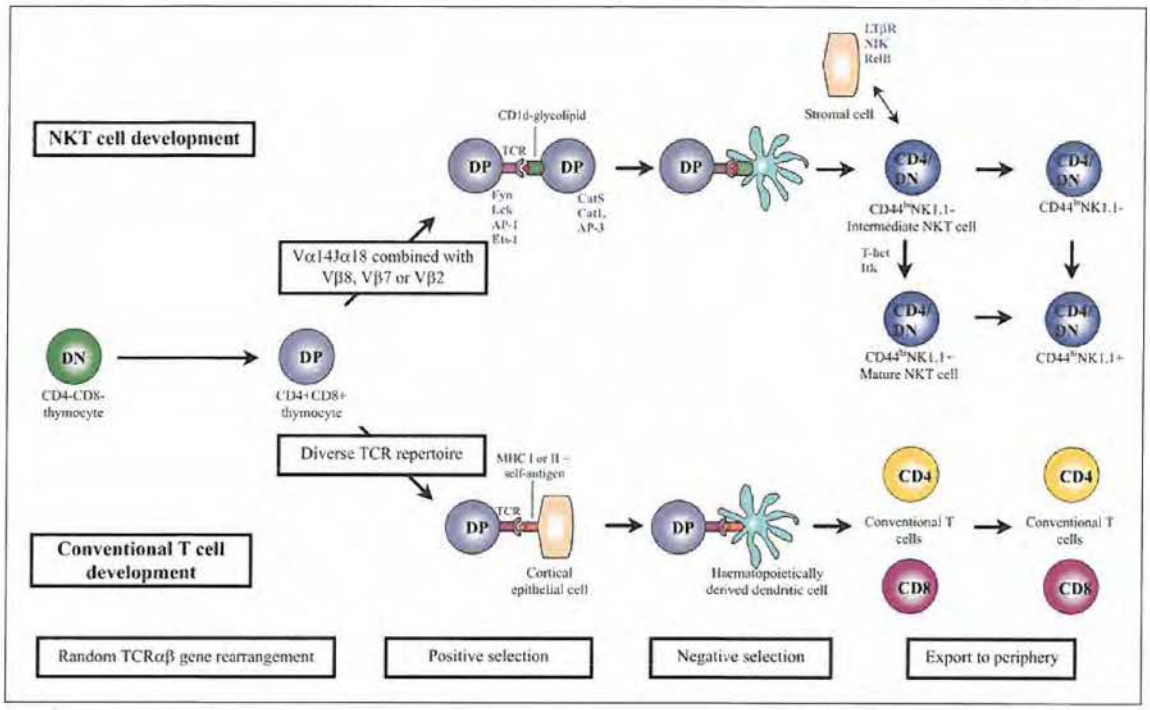

Figure 1.5. Current concepts of NKT and conventional T cell development in the thymus. *Factors involved in NKT cell development are indicated in blue. DN: double negative; DP: double positive; TCR: T cell receptor; hi: high expression; lo: low expression; LTβR: lymphotoxin β receptor; NIK: NF-κB inducing kinase; AP-1: activator protein 1; catS: cathepsin S; catL: cathepsin L; AP-3: adaptor protein 3.*

1.2.3 Regulatory functions of NKT cells *in vivo*

Due to their capacity to produce large amounts of IL-4, it was hypothesized that NKT cells were necessary for the generation of Th2 responses *in vivo*. However, this hypothesis was abandoned since β2 microglobulin, CD1d and Jα18 deficient mice were all able to elicit normal Th2 responses[124]. Nevertheless, numerous studies have shown that NKT cells can influence the Th1/Th2 balance in immune responses against infectious agents, tumors, alloantigens and self antigens.

NKT cells contribute to host responses against a variety of pathogens, including bacteria, fungi, protozoa, helminthes and viruses[140]. In most cases, NKT cell

protection against disease is dependent on the production of IFN-γ. They can also contribute to pathogen clearance by activation of macrophages, B cells and recruitment of neutrophils[141,142]. Additionally, in the response to helminth antigens, NKT cells were critically important for the generation of a Th2 response[143]. It has been speculated that the NKT TCR may be directly involved in the recognition of pathogen derived antigens, but evidence to support this notion is not yet available. Interestingly, recent studies have revealed that NKT cells are highly susceptible to infection by human immunodeficiency virus (HIV)[144-146]. In addition to their role in the natural course of infection, many studies have demonstrated protective effects of α-GalCer induced NKT cell activation against infection.

Another important role for NKT cells is found in anti-tumor immunity. Studies using chemical mutagenesis suggest that NKT cells contribute to natural tumor immunosurveillance[147]. Anti-tumor effects of α-GalCer have been observed against various tumors of different origins and their metastases[148,149]. The effect was dependent on NKT cell induced IL-12 production of DC that, together with NKT cell derived IFN-γ, secondarily stimulates NK and $CD8^+$ cells[148]. These cells function as direct anti-tumor effectors. Improved efficiency of tumor rejection and prolonged IFN-γ responses were observed when α-GalCer pulsed DC were used instead of α-GalCer administration alone[150]. Numerical NKT cell deficiencies and sometimes loss of IFN-γ production in cancer patients have been reported, which suggests a correlation between advanced cancer and impaired NKT cell function[151,152]. Paradoxically, NKT cells have been reported to also suppress tumor-specific $CD8^+$ cytotoxic T cell responses, resulting in tumor recurrence[153]. Thus, NKT cells can either promote or inhibit the development of protective anti-tumor responses.

The regulatory activity of NKT cells also seems to be implicated in the induction or maintenance of immune tolerance. The involvement of NKT cells was demonstrated in a model of immune privilege in the eye, known as anterior chamber-associated immune deviation[154]. Furthermore, NKT cells were required for the induction of allograft tolerance and survival in mouse models of transplant graft acceptance[155-157].

In addition to their role in infections, cancer and tolerance, NKT cells help to maintain tolerance to self-antigens and can thereby prevent autoimmunity. This feature will be discussed in more detail below.

1.3 Relevance of NKT cells in autoimmune diseases

Many autoimmune diseases are characterized by Th1 polarized T cell responses and therefore, a role for NKT cells in the regulation of autoimmune diseases has been proposed. Additionally, reduced NKT cell numbers have been reported in autoimmune prone mice as well as in patients suffering from autoimmune diseases. Furthermore, estrogen promotes IFN-γ production by NKT cells, which suggests a possible contribution to the sexual dimorphism found in autoimmune diseases[158].

1.3.1 Type 1 Diabetes

Studies of type 1 diabetes have provided the most evidence that NKT cells are involved in autoimmune regulation. A defect in the number and function of NKT cells in NOD mice has been reported. This type of diabetes could be ameliorated by enrichment of NKT cell numbers through adoptive transfer or transgenic overexpression of the invariant TCR[159,160]. The genetic control of NKT cell numbers in NOD mice was recently mapped to type 1 diabetes loci[161]. CD1d deficient mice developed diabetes earlier, had a greater disease penetrance and more severe disease[162,163]. In agreement, upregulation of CD1d expression restored the

immunoregulatory function of NKT cells and prevented autoimmune diabetes[164]. Protection conferred by NKT cells was associated with a Th2 shift within the pancreatic islets, and IL-4 has been implicated as a key mediator of immunoregulation[159,165]. Stimulation of naïve T cells expressing a transgenic, diabetogenic TCR with their auto-antigen in the presence of NKT cells did not block the initial activation of the pathogenic T cells. However, both the production of IL-2 and IFN-γ and later proliferation were inhibited[166]. These findings suggest that NKT cells may avert and ameliorate type 1 diabetes by preventing the differentiation of autoreactive T cells into effector cells.

In addition to a role in the natural course of type 1 diabetes, activation of NKT cells by administration of α-GalCer also prevented the onset and recurrence of diabetes in NOD mice[167-169]. Protection was associated with the induction and recruitment of tolerogenic DC[169]. It also coincided with suppression of pathogenic autoreactive T and B cells, and in the generation of tolerogenic islet autoantigen specific T cells with a protective cytokine production profile[167,168].

Conflicting results have been found regarding the numerical reduction and dysfunction of NKT cells in human type 1 diabetes[170-172]. However, this might be attributed to different methods to detect NKT cells and to differences in the patient populations tested. Moreover, gene expression profiles of regulatory NKT cells from identical twins discordant for type I diabetes showed multiple differences[173].

1.3.2 Experimental autoimmune encephalomyelitis and multiple sclerosis

Murine NKT cells correlate with the pathogenesis of experimental autoimmune encephalomyelitis (EAE). SJL mice tend to develop chronic EAE and were shown to have NKT cells that are reduced in number and have a defective IL-4

production[174]. Transgenic overexpression of the invariant TCR in NOD mice protected from EAE. This was associated with a striking inhibition of antigen-specific IFN-γ production, but was independent of IL-4[175]. Additionally, studies using α-GalCer induced NKT cell stimulation confirmed the capacity of NKT cells to modulate the disease[176-178]. However, the efficacy of α-GalCer treatment depended on the administration route, timing and dose[178,179]. In an effort to overcome these problems, the use of combination therapy of α-GalCer and CD86 blocking antibodies or the use of a truncated form of α-GalCer, OCH, was shown to selectively induce a Th2 response by the NKT cells and resulted in a reduced development of EAE[121,177,180].

Multiple sclerosis (MS) is a Th1 mediated autoimmune disease which is directed to myelin antigens in the central nervous system[181]. A decrease in Vα24 mRNA was demonstrated in peripheral blood of MS patients[182] that seemed to coincide with the relapse state of the disease[183]. Additionally, the IL-4 secretion of DN NKT cells was reduced[184]. However, $CD4^+$ NKT cell lines obtained from patients in remission, showed a strong Th2 bias compared to patients in relapse[185]. These results support an immunoregulatory function of NKT cells in MS.

1.3.3 Rheumatoid arthritis and its animal models

NKT cells are now reported to be reduced in a whole range of diseases that are characterized by autoreactive tissue damage, including RA[90]. In addition, the invariant TCR transcripts were decreased in the rheumatoid synovium[186]. It was reported that NKT cells did not expand upon stimulation with α-GalCer in some RA patients[91,92]. Furthermore, the expression levels of soluble CD1d were lower in RA patients[187]. However, little is known about the cytokine profile and the properties of these cells in the joint. Recently, it was reported that collagen induced arthritis could be suppressed by activation of NKT cells with OCH, but not with α-GalCer[188]. Neutralization of IL-4 or IL-10 with monoclonal antibodies

abolished disease protection by OCH, which indicates a critical role for these cytokines in disease protection.

1.3.4 Other autoimmune diseases

Lupus-prone mice as well as patients suffering from systemic lupus erythematosus (SLE) have reduced numbers of NKT cells, which suggests a protective effect of these cells on lupus development[90,189]. However, a possible pathogenic role of NKT cells has been supported by experiments showing that administration of α-GalCer exacerbated disease[190]. Transfer of activated NKT cells induced an autoimmune-like inflammation in young lupus-prone mice[191] and treatment with an anti-CD1d blocking antibody resulted in disease amelioration[190]. Finally, it has been shown that NKT cells might be implicated in the regulation of a murine model of colitis[192] as well as in inflammatory bowel disease[90] and Wegener's granulomatosis[193].

1.4 Therapeutic application of α-GalCer based therapies

The striking conservation of the NKT/CD1d system among mammalian species offers a great opportunity for the use of α-GalCer as therapeutic agent in human autoimmune diseases[95]. Preliminary human trials with α-GalCer have already been performed. In a phase I clinical trial using α-GalCer as a treatment for human cancers, no adverse events were reported, even at high doses[194]. However, biological effects (increased serum TNF-α and GM-CSF levels) depended on pretreatment NKT cell numbers rather than on the dose of α-GalCer. The number of NKT cells rapidly decreased after administration of the glycolipid and recovery to the pretreatment level was not observed within a week[194]. A more efficient effect was found when α-GalCer pulsed DC were used in a trial to treat cancer patients. NKT cell numbers were significantly increased above pretreatment levels after a transient decrease within 48 hours. Again, no serious treatment related

adverse events were observed during the study period[195]. The same group has recently shown that therapeutic activation of NKT cells by α-GalCer pulsed DC in human subjects resulted in highly coordinated secondary activation of acquired and innate immunity[196]. This led to a modulation of NK, B and T cell numbers and increased serum IFN-γ. The high frequency of therapy-induced, clinically apparent inflammatory responses observed at tumor sites suggested that a relevant anti-tumor response was occurring. Although these preliminary studies show promising results on the therapeutic use of α-GalCer in human cancer patients, more research will be necessary to fully comprehend the consequences of an α-GalCer mediated NKT cell activation in autoimmune diseases.

1.5 Conclusions

Much progress has recently been made in the understanding of the NKT cell biology. The importance of the immunoregulatory function of NKT cells was often demonstrated in animal models of several human diseases. Furthermore, the decreased frequency and the altered properties observed in patients suffering from autoimmune diseases implicate a (major) role of these cells in the regulation of autoimmune responses. Although the underlying mechanism of NKT cell dysfunction is not clear, it is likely that defects in their thymical development or in antigen synthesis may form the base of the NKT cell deficiency observed in many autoimmune diseases. Therapeutic approaches using α-GalCer or its analogues provide promising tools to treat autoimmune diseases. However, careful analysis of the *in vivo* consequences of α-GalCer administration or NKT cell activation should be performed to exclude unwanted Th1 responses that may worsen disease rather than suppress it. Hence, a more detailed insight in the molecular and cellular mechanisms involved in NKT cell function should help the development of new therapeutic strategies for autoimmune diseases.

1.6 Aims of the study

Rheumatoid arthritis is considered as T cell mediated autoimmune disease, although the precise T cell directed antigen that triggers the disease is not known. Since autoreactive T cells are present in healthy individuals as well, loss of peripheral tolerance may partially account for the onset of autoimmunity. Regulatory T cells are an active mechanism to maintain self-control. However, the exact contribution of regulatory T cells to the onset of autoimmune diseases are not fully understood.

In this study, the involvement of a specific regulatory T cell population, NKT cells, in RA will be highlighted. The first part focuses on NKT cells and their characteristics in the blood and synovial fluid of patients suffering from RA. In a second part, to obtain more insight in the possible application of the NKT cell system for the treatment of autoimmune diseases, the *in vitro* regulatory capacity of these cells will be investigated. Finally, the effects of commonly used medications on NKT cell function will be assessed *in vitro*. In the next chapters, following issues will be addressed.

I. Analysis of the frequency and functional characteristics of NKT cells in RA patients and healthy controls

NKT cells have been implicated in the regulatory immune mechanisms controlling autoimmunity. However, their precise role in the pathogenesis of RA is not fully elucidated. To determine whether RA might result from a loss of self regulation, we will study by flow cytometry whether the number of NKT cells in blood of healthy controls and in blood and synovial fluid of RA patients differs.

Furthermore, the functional properties of NKT cells derived from blood as well as synovial fluid will be evaluated. Reactivity towards the prototypic NKT cell ligand, α-GalCer, will be assessed in proliferation assays. This can reveal whether NKT cells from RA patients may respond differently or insufficiently to antigenic stimulation. Since cytokines produced by NKT cells play a major role in the function of these cells, cytokine production of blood and synovial fluid derived NKT cells will be assessed by intracellular staining. Differences in cytokines produced by cells obtained from RA patients or from healthy controls might account for the loss of active self control in RA. The presence of NKT cells in synovial tissue will be evaluated by PCR. These data will add to the understanding of the role of NKT cells in the pathogenesis of RA.

II. Study of NKT cell regulation *in vitro*: analysis of the regulatory capacity of NKT cells

Activation of NKT cells by *in vivo* administration of α-GalCer resulted in disease amelioration in animal models of autoimmunity. Furthermore, preliminary data obtained from open label clinical trials in human patients showed no serious adverse events of α-GalCer administration. However, there is little information on NKT cell regulation of human autoimmune reactions.

To evaluate possible direct regulatory effects of NKT cells on antigen reactive T cells, NKT cell clones will be isolated from blood of healthy controls and RA patients. These cells will be co-cultured with Tetanus Toxoid (TT) reactive T cell clones. The regulatory effects of NKT cells on the proliferative capacity and cytokine profile of the TT reactive cells will be analyzed by proliferation assays and intracellular cytokine staining. This method can provide information about a direct effect of NKT cells on antigen reactive T cells. This might also reveal

whether NKT cells in addition to their altered reactivity and cytokine profile have an impaired regulatory capacity in RA patients.

To evaluate the possible use of α-GalCer as therapeutic agent to control autoimmune diseases, we will use *in vitro* approaches to analyze the regulatory capacity of NKT cells. TT reactive T cell lines from PBMC of healthy controls will be generated in the presence or absence of the NKT cell stimulus, α-GalCer. The frequency of TT reactive T cells and the Th profile of these cells will be used to determine the effect of simultaneous stimulation of NKT cells on T cell priming.

Since the efficacy of α-GalCer treatment in mice appeared to depend on the timing of the administration, the effect of a preceding NKT cell stimulation will be compared with that of simultaneous activation of NKT cells and Toxic-Shock-Syndrome Toxin (TSST) reactive cells. Again, to obtain insight in the regulatory capacity of the NKT cells, differences in cell expansion and cytokine profile will be determined by flow cytrometric analyses. The results obtained from these experiments can reveal more insight in the regulatory mechanisms of NKT cells and might have an impact on α-GalCer based therapeutic applications for autoimmune diseases.

III. Effects of immunomodulatory treatments on NKT cell function and expansion *in vitro*

Immunosuppressive drugs such as cyclosporin A (CsA), methotrexate (MTX) and methylprednisolone (MP) are frequently used to RA treat patients. These molecules are known to inhibit T cell function and activation. However, their effect on the function of regulatory T cells has not been elucidated yet.

In this final part, PBMC of healthy donors will be stimulated with α-GalCer in the presence of CsA, MTX and MP. The *in vitro* expansion of NKT cells will be examined by flow cytometric analysis. Furthermore, the effects of the drugs on the NKT cell cytokine profile will be assessed by intracellular staining. This will provide additional clues about the effect of CsA, MTX and MP on NKT cell function.

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Chapter 2

General materials and methods

2.1 Cell culture based techniques

2.1.1 Cell culture media and antigens

Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were isolated from heparinized blood and synovial fluid by Ficoll-Hypaque (Sigma-Aldrich, Bornem, Belgium) density gradient centrifugation. PBMC and SFMC were cultured in RPMI 1640 medium supplemented with L-glutamine, 10 mM HEPES buffer, 1 mM sodiumpyruvate, 1 % non-essential amino-acids (Invitrogen, Merelbeke, Belgium) and 10% heat-inactivated fetal bovine serum (FBS, Hyclone Europe, Erembodegem, Belgium), which from now on is referred to as culture medium.

α-GalCer was kindly provided by Kirin Brewery Ltd (Gunma, Japan)[1]. Tetanus Toxoid (TT) was obtained from the Rijksinstituut voor Volksgezondheid en Milieu (RIVM, Bilthoven, The Netherlands). Phytohemagglutinin (PHA) and Toxic-Shock-Syndrome-Toxin-1 (TSST) were purchased from Sigma Diagnostics (Sigma-Aldrich, Bornem, Belgium). The anti-CD3 monoclonal antibody 2G3 is a house made antibody[2].

2.1.2 Flow cytometric phenotype analysis

Expression of cell surface markers was analyzed by flow cytometry. At least 2 x 10^5 cells were suspended at a density of 1 x 10^6 cells per ml PBS with 2% FBS and 0.09% NaN_3, stained for 30 min at 4°C and subsequently washed. Fluorescein isothiocyanate (FITC)-labeled, peridinin chlorophyll protein (PerCP)-labeled and phycoerythrin (PE)-labeled antibodies directed against CD3, CD4, CD8, CD16, CD25, CD56 and mouse anti-human IgG1 were obtained from BD Biosciences (Erembodegem, Belgium). Mouse IgG1 and IgG2a antibodies were used as isotypic control antibodies. Anti-T cell receptor (TCR) Vα24, TCR Vβ2 and TCR Vβ11 antibodies were purchased from Serotec Ltd (Oxford, UK). The frequency of

invariant NKT cells was estimated by counting at least 1 x 10^5 PBMC and using three-color anti-Vα24/anti-Vβ11/anti-CD3 staining. Cells were analyzed on a FACS Calibur flow cytometer by means of the Cellquest software (BD Biosciences, Erembodegem, Belgium).

2.1.3 Expansion and culture of Vα24$^+$Vβ11$^+$ NKT cells

PBMC and SFMC were cultured in the presence of 100 ng/ml α-GalCer (Kirin Brewery Ltd, Gunma, Japan) at a density of 7.5 x 10^5 cells per ml culture medium in a 24-well culture plate (2 ml per well, Nalge-Nunc, Hereford, United Kingdom). After 7 days, autologous antigen presenting cells were prepared by pulsing 1 x 10^7 autologous PBMC per ml culture medium with 100 ng/ml α-GalCer. After 3 hours of incubation, PBMC were irradiated at 6600 Rad using a ^{137}Cesium source (CIS Biointernational, Gif-sur-Yvette Cedex, France). Next, the cells were restimulated with 1x10^6 autologous antigen presenting cells and supplemented with 2 U/ml recombinant human IL-2 (rhIL-2, Roche Diagnostics, Brussels, Belgium). On day 7 post restimulation, NKT cells were isolated using PE-labeled anti-TCR Vα24 antibodies and anti-PE coated magnetic particles according to the manufacturer's instruction (EasySep, Stemcell technologies, Meylan, France). Briefly, cells (minimum 5 x 10^6 cells) were suspended at a concentration of 1 x 10^8 cells per ml PBS buffer containing 2% FBS and 1 mM EDTA. Fc blocking antibody and PE-conjugated anti-TCR Vα24 antibody were added at 100 µl antibody per ml and the suspension was incubated at room temperature (RT) for 15 min. Cells were washed with 10-fold excess buffer and resuspended to the original volume (1 x 10^8 cells/ml buffer). Next, EasySep PE selection cocktail was added (100 µl/ml) and cells were incubated at RT for 15 min. Then, 50 µl/ml EasySep Magnetic Nanoparticles were added and after 10 min of incubation, the cell suspension was brought to a total volume of 2.5 ml with buffer. The tube was then placed in the EasySep magnet for 5 min and subsequently, the supernatant (containing Vα24- cells) was poured off. This procedure was repeated 5 times

resulting in a highly purified (>95%) Vα24$^+$ cell fraction. The efficacy and the yield of the isolation was dependent on the frequency of Vα24$^+$Vβ11$^+$CD3$^+$ cells and the amount of cells in the starting material. Generally, about 75% of the NKT cells in the starting material were isolated. These NKT cells were then seeded at a density of 5 x 10^4 cells per well in a microtiter plate (200 µl culture medium per well).

The reactivity of the isolated NKT cells towards α-GalCer was tested using a split-well technique. Briefly, each well was split into 4 aliquots and restimulated in duplicate with 1 x 10^5 irradiated, antigen-pulsed or non-pulsed autologous PBMC. After 48 hours, proliferation capacities were measured and α-GalCer reactive T cell lines (TCL) were identified using a standard ^{3}H-thymidine incorporation assay. During the last 16 hours of culture, cells were pulsed with 1 µCi ^{3}H-thymidine (Amersham, Buckinghamshire, UK) and subsequently harvested with an automated cell harvester (Pharmacia, Uppsala, Sweden). Incorporated radioactivity was measured with a Beta-plate liquid scintillation counter (Wallac, Turku, Finland). A NKT cell line was considered antigen-reactive when mean counts per minute (cpm) in the presence of α-GalCer exceeded 1000 cpm and the stimulation index (mean counts with α-GalCer / mean counts without α-GalCer) was higher than three[3].

2.1.4 Isolation of antigen reactive T cell lines and clones

To obtain TT reactive TCL, freshly isolated PBMC were cultured at 4 x 10^4 cells per well together with 6x10^4 irradiated, autologous PBMC in culture medium supplemented with 2.5 limes flocculation units (Lf) TT /ml. After 7 days, cultures were restimulated with 1x10^5 autologous, TT pulsed, irradiated PBMC (as described in 1.3, pulsing concentration of TT: 20 Lf/ml) and cultured in the presence of 2 U/ml rhIL-2 (Roche diagnostics, Brussels, Belgium). At day 14, TT reactive TCL were identified using a standard ^{3}H-thymidine incorporation assay as

described above (2.1.3). The frequency of TT reactive T cells was set as the number of reactive TCL relative to the total number of PBMC plated.

T cell clones were prepared, using a limiting dilution procedure, as previously described[4,5]. TT or α-GalCer reactive T cell lines were plated out at 1.5 cells/ml, 5 cells/ml and 15 cells/ml in microtiter plates and stimulated with irradiated, allogeneic feeder cells and 2 µg/ml PHA (Sigma-Aldrich, Bornem, Belgium). Every 3 days, cultures were provided with fresh culture medium containing 5 U/ml rhIL-2. Growth positive clones were assessed for their antigen reactivity in a proliferation assay. TT or α-GalCer reactive T cell clones were expanded by successive rounds of restimulation with antigen pulsed, irradiated, autologous PBMC (described in 2.1.3 for α-GalCer reactive T cells and described above for TT reactive T cells).

2.1.5 Evaluation of cytokine profiles by ELISA

The cytokine production of IL-4, IL-6, IL-10, IFN-γ and TNF-α in culture supernatants was determined by the use of a sandwich ELISA based on commercially available monoclonal antibodies (CytoSets, Biosource Europe, Nivelles, Belgium) in 96 well microtiter plates (MaxiSorp, Nalge-Nunc, Hereford, United Kingdom). Duplicate wells were coated overnight with 1 µg/ml capture antibody in PBS (2 µg/ml for IFN-γ). Non-specific binding sites were blocked with 0.5% bovine serum albumine (BSA, Immunosource, Halle-Zoersel, Belgium) in PBS. Wells were subsequently washed with a 0.9% NaCl solution containing 0.1% Tween-20 (VWR International, Leuven, Belgium). Serially diluted standards (range 0-1000 pg/ml) or diluted samples (1:2 to 1:5) were incubated for 2 hours at RT, together with 0.4 µg/ml of the matching biotinylated detection antibody. After another washing step, incubation with streptavidin-conjugated horse radisch peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was performed for 30 min. Cytokines were detected by a color reaction using a

tetramethylbenzidine enzyme substrate. The reaction was stopped by the addition of 1.8 N H_2SO_4. Optical densities were measured at 450 nm and 630 nm using an ELISA reader (Biorad, Nazareth, Belgium). Net production of cytokines was determined by subtracting background levels measured in unstimulated conditions from the cytokine levels measured in stimulated conditions.

2.1.6 Evaluation of cytokine profile by intracellular cytokine staining

For intracellular cytokine detection, $2x10^6$ cells per ml culture medium were stimulated with 25 ng/ml phorbol-12-myristate-13-acetate (PMA) and 1 µg/ml ionomycine (both Sigma-Aldrich, Bornem, Belgium) in the presence of 10 µg/ml brefeldin A (Pharmingen, Erembodegem, Belgium) for 4 h at 37°C. Intracellular staining was performed as previously described[6]. Briefly, cells were washed in PBS buffer containing 2% FBS and 0.09% NaN_3 and surface stained for 15 min at RT with PerCP labeled anti-CD3, anti-CD4 or anti-CD8 antibodies or with unlabeled anti-TCR Vα24 or anti-TCR Vβ2 antibodies. The latter were subsequently stained for 15 min with PerCP conjugated rat anti-mouse IgG1 antibodies in a separate step. After an additional washing step, cells were fixed and permeabilized in a commercially available cytofix/cytoperm solution (Pharmingen, Erembodegem, Belgium) for 20 min at 4°C. Cells were washed and resuspended in a perm/wash buffer that contained 0.1% saponin (Pharmingen, Erembodegem, Belgium). Intracellular staining was performed for 30 min at 4°C with anti-IFN-γ-FITC and anti-IL-4-PE labeled antibodies or with the appropriate isotype controls (BD Biosciences, Erembodegem, Belgium). Cells were washed twice and analyzed on a FACS Calibur flow cytometer by means of the Cellquest software (BD Biosciences, Erembodegem, Belgium).

2.1.7 Evaluation of cytokine profile by ELISPOT

ELISPOT was performed as previously described[6]. Briefly, nitrocellulose bottomed plates (Millipore Corp, Bedford, MA) were coated overnight at 4°C with anti-IFN-γ or anti-IL-4 antibodies (Mabtech, Nacka, Sweden, 10 µg/ml in carbonate/bicarbonate buffer, 50 µl per well). Non-specific binding sites were blocked with culture medium for 2 hours at 37°C. Next, $2x10^5$ PBMC were stimulated with 100 ng/ml α-GalCer. After 20 hours of culture, biotinylated anti-IFN-γ or anti-IL-4 antibody (Mabtech, Nacka, Sweden, 1 µg/ml in PBS buffer containing 5% BSA and 0.05% Tween-20, 100 µl per well) was added for 2 hours. This was followed by incubation with streptavidin-alkaline phosphatase (Mabtech, Nacka, Sweden, 1 µg/ml in PBS buffer containing 5% BSA and 0.05% Tween-20, 100 µl per well) and NBT/BCIP (Nitro Blue Tetrazolium/5-Bromo-4Chloro-3-Indolyphosphate, Pierce, Rockford, IL, USA, 100 µl/well) as substrate. The number of cytokine secreting cells was calculated by subtracting the number of spots in control wells (without antigen) from the number of spots obtained in the presence of α-GalCer.

2.1.8 Cytotoxicity analysis

The cytotoxic potential of NKT cell clones was assessed by a ^{51}Cr-release assay. The jurkat T cell leukemia cell line (ATCC-TIB-152[7]) and K562 hematopoetic leukemia cell line (ATCC-CCL-243[8]) were used as targets. First, cells (1×10^7/ml culture medium) were pulsed for 2 hours with or without α-GalCer (100 ng/ml) and were subsequently labeled with 200 µCi ^{51}Cr ($Na_2Cr_3O_4$, Amersham, Buckinghamshire, UK) at 37°C for 1 hour. Labeled target cells were then washed extensively with RPMI and incubated with the effector NKT cells at various effector-to-target ratios (5/1, 10/1, 20/1) in 200 µl in a microtiterplate. After 6 hours, the supernatants were harvested and the radioactivity released was measured in a gamma counter (Cobra II 5002, Packard Instrument Company, Meriden, CT, USA). Maximum and spontaneous release of ^{51}Cr were determined in

wells containing target cells in the presence of Tween-20 (maximum release) or medium only (spontaneous release). The percentage of specific cytolysis was calculated as (experimental release – spontaneous release / maximum release – spontaneous release) x 100.

2.2 Molecular biology based techniques

2.2.1 RNA extraction and cDNA synthesis

RNA was isolated from snap frozen synovial tissue samples using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, Amsterdam, The Netherlands). For isolation of total RNA from PBMC, SFMC and isolated NKT cells, the High Pure total RNA Isolation kit (Roche Diagnostics, Brussels, Belgium) was used according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using AMV reverse transcriptase and an oligo dT primer (Promega, Madison, WI, USA).

A control PCR for the household gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed to confirm the quality of the cDNA. One microliter of cDNA was amplified in 1x PCR buffer, 0.9 units Taq polymerase, 0.02 mM dNTP mix (all from Roche Diagnostics, Brussels, Belgium), 1 µM GAPDH forward primer (5'-GCT CTC CAG AAC ATC ATC CCT GCC-3') and 1 µM GAPDH reverse primer (5'-CGT TGT CAT ACC AGG AAA TGA GCT T-3'). PCR was performed for 40 cycles (95°C, 20 sec; 55°C, 20 sec; 72°C, 40 sec) on a GeneAmp PCR system 9600 thermal cycler (Perkin Elmer, Zaventem, Belgium).

2.2.2 Analysis of clonal heterogeneity: TCR CDR3 fragment length screening

CDR3 spectratyping analysis was performed as described earlier[9]. Briefly, two microliter of cDNA was used for first-round PCR analysis performed in 1x PCR buffer, 0.9 U Taq polymerase, 0.02 mM dNTP mix (all from Roche Diagnostics, Brussels, Belgium), 1 µM forward primer specific for TCR Vα24 (5'-GAA CGG AAG

ATA TAC AGC AAC TC-3') or TCR Vβ11 (5'-TCC ACA GAG AAG GGA GAT CTT TCC TCT GAG-3') region and 1 μM reverse primer specific for TCR constant α (5'-ATC ATA AAT TCG GGT AGG ATC C-3') or constant β (5'-CTC TTG ACC ATG GCC ATC-3') region. Identical PCR conditions were used as described in 2.2.1. Obtained PCR amplicons were used in a second amplification procedure of 25 cycles using the TCR Vα24 or TCR Vβ11 specific primer as forward primer and a FAM labeled TCR constant α (5'-FAM-CTG TTG CTC TTG AAG TCC ATA G-3') or TCR constant β (5'-FAM-GTG GCA AGG CAC ACC AGT GTG GGC C-3') as reverse primer (Eurogentec, Liege, Belgium) under the same PCR conditions as described above. PCR amplicon lengths were analyzed on the 310 ABI DNA sequencer (Applied Biosystems, Warrington, UK). Fragment sizes of gene products were calculated using an internal Genescan-500 ROX labeled standard and analysis was performed with 672 Genescan Software (both from Applied Biosystems, Warrington, UK). The heterogeneity of the CDR3 spectratype profiles provides an indication of the clonality of T cell populations: monoclonal with one peak, oligoclonal with 2 to 4 peaks and polyclonal with more than 4 peaks. Identical peak lengths in monoclonal samples indicate the presence of identical T cell clones in different samples. A 350 bp fragment was obtained for the invariant TCR.

2.2.3 Sequence analysis of the invariant TCR

Purified TCR Vα24 PCR amplicons obtained from first round PCR as described above, were sequenced with a TCR constant α primer (5'- CTG TTG CTC TTG AAG TCC ATA G -3') using the Big DyeTM Terminator Cycle Sequence Ready Reaction Kit II (Applied Biosystems, Warrington, UK). PCR conditions used were as follows: 25 cycles of (96°C, 30 sec; 50°C, 5 sec; 60°C, 4 min). Fluorescently labeled PCR amplicons were purified on a sephadex G-50 M column (Pharmacia, Uppsala, Sweden), vacuum dried and resuspended in 5 μl of 1:50 25 mM EDTA/formamide. Sequences were analyzed on a ABI Prism 310 Genetic Analyser (Applied Biosystems, Warrington, UK).

2.3 Statistical analysis

Differences in the frequency of NKT cells between healthy control individuals and RA patients and between peripheral blood and synovial fluid from RA patients were analyzed using the Mann-Whitney U-test. For comparisons between matched peripheral blood and synovial fluid samples, the Wilcoxon matched pairs signed rank test was used. For all other analyses, one-way ANOVA tests were used. $P<0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

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Chapter 3

Peripheral blood, but not synovial fluid NKT cells are biased towards a Th1 phenotype in rheumatoid arthritis

Based on

Peripheral blood, but not synovial fluid NKT cells are biased towards a Th1-like phenotype in Rheumatoid Arthritis

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Abstract

Natural Killer T (NKT) cells have been implicated in the regulatory immune mechanisms controlling autoimmunity. Their precise role however in the pathogenesis of rheumatoid arthritis (RA) remains unclear. The frequency, cytokine profile and heterogeneity of NKT cells were studied in peripheral blood mononuclear cells (PBMC) of 23 RA patients and 22 healthy controls, including paired PBMC-synovial fluid (SF) samples of 7 and paired PBMC-synovial tissue (ST) samples of 4 RA patients. Flow cytometry revealed a decreased NKT cell frequency in PBMC of RA patients. NKT cells were present in paired SF and ST samples. Based on the reactivity of PBMC derived NKT cells towards α-galactosylceramide (α-GalCer), RA patients could be divided into responders (53.8%) and non-responders (46.2%). However, NKT cells isolated from SF of both responders and non-responders expanded upon stimulation with α-GalCer. Analysis of the cytokine profile of $CD4^+$ and $CD4^-$ PBMC derived NKT cell lines of RA patients revealed a significantly reduced number of IL-4 producing cells. In contrast, SF derived NKT cell lines displayed a Th0-like phenotype comparable to that of healthy controls. This suggests that synovial fluid NKT cells are functional, even in patients with non-responding NKT cells in the blood. In conclusion, since the number of $V\alpha24^+V\beta11^+CD3^+$ NKT cells is decreased and the cytokine profile of blood derived NKT cells is biased to a Th1-like phenotype in RA patients, NKT cells might be functionally related to resistance or progression of rheumatoid arthritis. A local boost of the regulatory potential of NKT cell might be useful as a candidate therapy for rheumatoid arthritis.

3.1 Introduction

Natural Killer T (NKT) cells are a distinct subset of lymphocytes that share characteristics of both T cells and NK cells. They express a semi-invariant T cell receptor (TCR) (TCR Vα24Jα18 and Vβ11 in human; Vα14Jα281 and Vβ8, Vβ7 or Vβ2 in mouse) and recognize glycolipid antigens presented by the MHC I-like molecule CD1d[1]. Two subsets can be distinguished: $CD4^+$ NKT cells that produce Th1-type and Th2-type cytokines and $CD4^-CD8^-$ (double negative, DN) NKT cells that primarily produce Th1-type cytokines[2,3]. The ability to rapidly secrete cytokines and chemokines is thought to underlie their regulatory function in a variety of diseases, including cancer and autoimmunity[4]. Although the natural ligand of NKT cells remains to be elucidated, it has been reported that the sponge derived glycolipid α-galactosylceramide (α-GalCer) is a potent activator of mouse and human NKT cells, both *in vitro* and *in vivo*[5,6]. When α-GalCer is administered to mice, it polarizes the adaptive immune response towards production of Th2 cytokines[7,8] and therefore raises the possibility that α-GalCer can temper or even prevent Th1 mediated autoimmune diseases.

Several studies have shown that NKT cells are decreased or dysfunctional in autoimmune conditions such as insulin-dependent diabetes mellitus, systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis (RA) and multiple sclerosis (MS)[9-12]. Significant therapeutic effects of α-GalCer have been demonstrated in animal models for autoimmunity such as experimental allergic encephalomyelitis (EAE)[13-15] and non-obese diabetic (NOD) mice[16,17].

Since the NKT/CD1d system is phylogenetically conserved among mammals, the results found in mice are expected to have a direct parallel in humans. The NKT cell frequency in peripheral blood mononuclear cells (PBMC) is lower in humans than in mice[1], and may therefore be a potential obstacle in translating results

from animal studies to the clinic. However, results from a phase I study in advanced cancer patients revealed that treating patients with α-GalCer can increase NKT cell numbers above pretreatment levels. This again indicates that α-GalCer could be applied for the treatment of patients with autoimmune diseases[18].

RA is an autoimmune disease characterized by a chronic inflammation of the joints, followed by progressive destruction of cartilage and underlying bone[19]. Autoreactive Th1 T cells are thought to play a major role in the disease process[20-22]. In RA patients, the frequency of NKT cells is decreased, but the functional characteristics of NKT cells have not been fully elucidated yet. Chiba et al. demonstrated that administration of a truncated form of α-GalCer to mice suffering from collagen induced arthritis, a frequently used animal model of RA, resulted in disease protection, indicating that this might be a potential therapy to enhance the numbers of NKT cells in RA patients[23].

In the present study, we analyzed the frequency, functional characteristics and heterogeneity of NKT cells in peripheral blood (PB), synovial fluid (SF) and tissue (ST) of RA patients. In parallel, we assessed these parameters in α-GalCer stimulated short-term cell lines of both PB and SF NKT cells. We found that NKT cells were decreased and had altered functional properties in PB, but they were not impaired in SF of RA patients. Our data indicate that NKT cells may be involved in the disease process of RA and that a strategy to boost the regulatory potential of NKT cells might be useful for the treatment of RA.

3.2 Materials and methods

3.2.1 Patients and healthy controls

NKT cell characteristics were examined in 23 RA patients, diagnosed according to the criteria of the American College of Rheumatology[24], and in 22 healthy subjects (mean age: 48.6 years ± 2.0, 10 males and 12 females). When RA patients presented with a swollen knee, paired PB and SF samples were obtained. Synovial tissue samples were obtained from 4 RA patients after total knee/hip arthroplasty. Patient characteristics are summarized in Table 3.1.

Table 3.1: Patient characteristics.

Patient	Age / sex	Disease duration, years	Treatment
1	54/M	5	azathriopine, MP
2	38/F	6	chlor, salazo
3	64/F	7	NSAID
4	43/F	5	NSAID
5	46/M	1	salazo
6	46/M	<1	salazo
7	52/M	11	NSAID
8	53/F	11	NSAID
9	49/M	10	NSAID
10	52/F	4	NSAID
11	69/F	36	salazo
12	65/F	<1	untreated
13	35/M	<1	untreated
14[§]	57/F	4	MTX
15[§]	46/M	2	aTNF, salazo
16[§]	41/M	10	salazo
17[§]	41/M	13	MTX
18[§]	60/M	2	leflunomide
19[§]	43/F	5	MTX
20[*]	63/M	17	salazo
21[§,*]	65/F	4	salazo, chlor
22[*]	62/F	12	leflunomide
23[*]	54/F	17	MP

[§]: synovial fluid sample; [*]: synovial tissue sample; MP: methylprednisolone; chlor: hydroxychloroquine; salazo: salazopyrine; NSAID: non steroidal anti inflammatory drugs; MTX: methotrexate; aTNF: anti-TNF treatment

3.3 Results

3.3.1 Frequency of Vα24⁺Vβ11⁺CD3⁺ NKT cells in rheumatoid arthritis

The frequency of Vα24⁺Vβ11⁺CD3⁺ NKT cells in PBMC of RA patients and healthy controls was analyzed by flow cytometry (Fig 3.1). Significantly fewer Vα24⁺Vβ11⁺CD3⁺ NKT cells were found in PBMC of RA patients (0.03% ± 0.01) as compared to healthy controls (0.11% ± 0.03; $p<0.01$). We simultaneously determined the NKT cell frequency in paired blood-SF samples from 7 RA patients. Although a tendency towards a higher frequency was observed in the SF (0.08% ± 0.03) compared to that of the concordant PBMC samples (0.05% ± 0.02), this finding could not be demonstrated for all patients. These data indicate that the NKT cell frequency is decreased in the blood of RA patients, but not increased in the SF as compared to the blood of these patients.

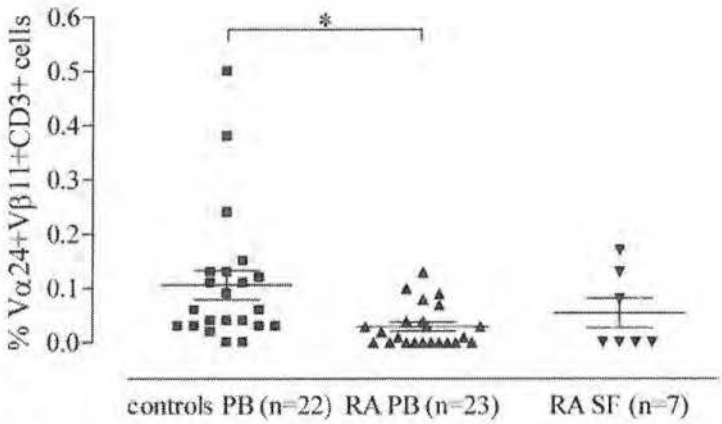


Figure 3.1. Frequency of NKT cells in RA patients and healthy controls. *NKT cell frequency in freshly isolated PBMC of 22 healthy controls and 23 RA patients and in SFMC of 7 RA patients was determined by flow cytometry. Cells were stained with anti-Vα24, anti-Vβ11 and anti-CD3 mAb. Error bars indicate SEM, (* p<0.01). PB: peripheral blood; SF: synovial fluid.*

3.3.2 Cytokine profile of α-GalCer stimulated PBMC

To assess the cytokine profile of NKT cells directly *ex vivo*, we tested the reactivity of PBMC to α-GalCer in 10 RA patients and 8 healthy controls using an ELISPOT technique with IFN-γ and IL-4 read-out (described in 2.1.7). Similar to the frequency analysis by flow cytometry, a significantly decreased number of α-GalCer reactive cells was found for IFN-γ as well as IL-4 in RA patients compared to healthy controls (2.3 spots ± 0.6 vs. 24.3 spots ± 10.1 for IFN-γ and 0.2 spots ± 0.1 vs. 3.9 spots ± 1.1 for IL-4 per $2x10^5$ cells for RA and healthy controls respectively, $p<0.05$). To determine whether this diminished frequency was also associated with an altered cytokine profile, the IL-4/IFN-γ ratio was calculated as the number of IL-4 producing cells to the number of IFN-γ producing cells (Fig 3.2). The IL-4/IFN-γ ratio in RA patients was decreased compared to healthy controls (0.07 ± 0.03 in RA patients and 0.30 ± 0.10 in healthy controls, $p=0.06$). This was mainly due to a reduced number of IL-4 producing cells, since the frequency of IL-4 producing cells compared to healthy controls was relatively more reduced than that of IFN-γ producing cells. These data indicate that NKT cells derived from RA patients are biased towards a Th1-like phenotype.

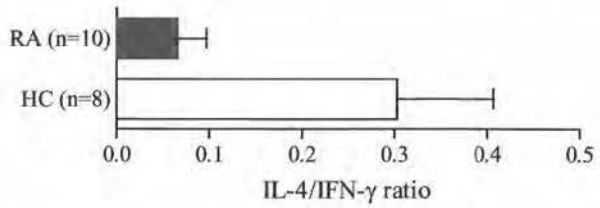


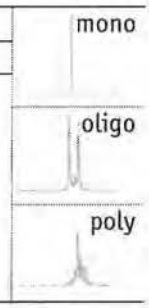
Figure 3.2. IL-4/IFN-γ ratio in α-GalCer stimulated PBMC evaluated by ELISPOT. *PBMC ($2x10^5$ cells per well) of 10 RA patients and 8 healthy controls were stimulated with α-GalCer or no antigen for 20 hours. The number of cytokine secreting cells was calculated by subtracting the number of spots in control wells (without antigen) from the number of spots obtained in the presence of each stimulating agent. The IL-4/IFN-γ ratio was set as the number of IL-4 producing cells to the number of IFN-γ producing cells. Error bars indicate SEM.*

3.3.3 Analysis of the invariant TCR in synovial tissue

NKT cells express the invariant Vα24Jα18 TCRα chain combined with a variable Vβ11 TCRβ chain. To compare the Vα24 expression profile in PBMC of RA patients and healthy controls, PBMC of 5 healthy controls and paired PBMC-SFMC and PBMC-ST samples of 4 RA patients were subjected to TCR CDR3 size analysis using primers for Vα24 and TCRα constant region (described in 2.2.2). PBMC of healthy controls showed a polyclonal peak profile or a Gaussian-like distribution for Vα24, containing a peak at 350 bp which corresponds to the invariant TCRα chain that is characteristic for NKT cells (not shown). Although PBMC of RA patients displayed an oligoclonal or monoclonal distribution which indicates a restricted usage for Vα24 (Table 3.2), the invariant TCR peak was present in all patients. We determined whether the invariant TCR could also be found in SFMC and ST samples. As in PBMC, the TCR Vα24 usage in SFMC and ST tissue samples was skewed for some patients, but polyclonal for others. Again, the invariant TCR peak was detected in SFMC and ST of all RA patients. Sequence analysis of the PCR products obtained from the CDR3 fragment length analysis confirmed that the peak size of the ST samples corresponded with the invariant TCR sequence (not shown). These data show that NKT cells are present in rheumatoid SF as well as ST samples.

Table 3.2. TCR Vα24 usage in PBMC, SFMC and ST of RA patients.

	Vα24			Vβ11		
	PBMC	SFMC	ST	PBMC	SFMC	ST
RA 1	mono	mono	NA	oligo (2)	mono	NA
RA 2	mono	poly	oligo (2)	mono	poly	oligo (2)
RA 3	mono	oligo (2)	NA	poly	oligo (2)	NA
RA 4	poly	poly	NA	poly	poly	NA
RA 5	oligo (3)	NA	mono	mono	NA	mono
RA 6	poly	NA	poly	poly	NA	poly
RA 7	oligo (3)	NA	mono	poly	NA	oligo (3)



The clonality of the TCR Vα24 and TCR Vβ11 family was assessed by CDR3 spectratyping of PBMC, SFMC and ST of RA patients. Representative figures of a monoclonal (upper right), oligoclonal (middle right) and polyclonal (lower right) profile are shown. Mono: monoclonal profile; oligo: oligoclonal profile; poly: polyclonal profile; NA: not available.

3.3.4 NKT cell reactivity to α-galactosylceramide in RA patients

To assess whether the reduced NKT cell frequency in PB of RA patients was due to an inadequate response to the glycolipid antigen, we stimulated PBMC of 9 healthy controls and 13 RA patients and SFMC of 5 RA patients with α-GalCer. At day 7, cells were restimulated with autologous α-GalCer pulsed, irradiated PBMC. The NKT cell frequency was determined by flow cytometry at day 14 (Fig 3.3). NKT cells of healthy controls expanded in response to α-GalCer to 15.8% ± 2.7, whereas the number of PB and SF NKT cells of RA patients was significantly lower after α-GalCer stimulation (8.4% ± 2.9 and 4.4% ± 1.6 respectively, $p<0.01$).

A more detailed analysis revealed that this decrease was due to the existence of 2 subpopulations of RA patients based on the NKT cell numbers reached after 14 days of α-GalCer stimulation. As shown in figure 3.4, NKT cells from 6 out 13 RA patients did not respond to α-GalCer stimulation (mean frequency after 14 days: 1.0% ± 0.2, $p<0.01$, non-responders), whereas NKT cells from the remaining 7

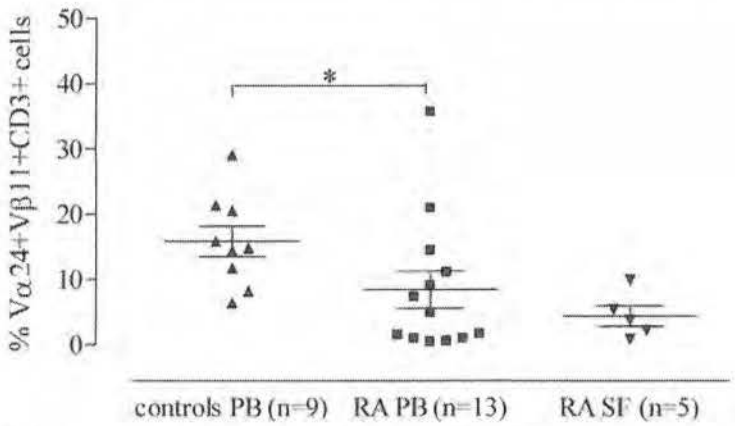


Figure 3.3. Reactivity of PB and SF derived NKT cells to α-GalCer. *PBMC (1.5×10^6 cells per well) of 9 healthy controls and 13 RA patients as well as SFMC of 5 RA patients were stimulated with α-GalCer and restimulated at day 7 with autologous, α-GalCer pulsed, irradiated PBMC in the presence of 2 U/ml IL-2. NKT cell numbers were determined by flow cytometry at day 14. Error bars indicate SEM, (* $p<0.01$). PB: peripheral blood; SF: synovial fluid.*

patients reached frequencies comparable with those of healthy controls (14.7% ± 4.0, responders). Moreover, NKT cells of responder patients appeared to have an increased capability to respond to α-GalCer since the expansion was higher than that of healthy controls (294-fold versus 149-fold respectively). No relation between disease parameters (disease duration, disease status) or treatment and (non-)responsiveness of NKT cells could be demonstrated. Remarkably, SF NKT cells, even of non-responding RA patients, did expand after α-GalCer stimulation (4.94% ± 1.90). These results indicate that the reactivity of peripheral blood NKT cells to α-GalCer is impaired in some RA patients, while it is intact and even increased in others.

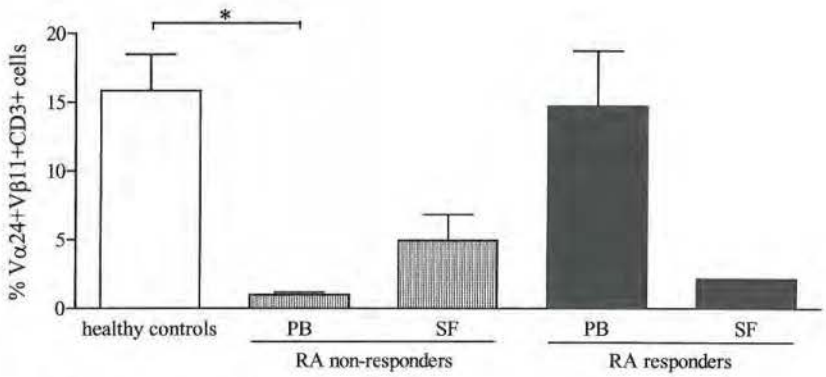


Figure 3.4. RA patients can be divided into responder and non-responder patients based on PB derived NKT cell reactivity to α-GalCer. *PBMC (1.5 x 10^6 cells per well) of 9 healthy controls and 13 RA patients as well as SFMC of 5 RA patients were stimulated with α-GalCer and restimulated at day 7 with autologous, α-GalCer pulsed, irradiated PBMC in the presence of 2 U/ml IL-2. NKT cell numbers were determined by flow cytometry at day 14. Patients were considered non-responders when the frequency of $Vα24^+Vβ11^+CD3^+$ NKT cells derived from PBMC was lower than 2% after 14 days of culture. Error bars indicate SEM, (* p<0.01). PB: peripheral blood; SF: synovial fluid.*

3.3.5 Cytokine profile of PB and SF NKT cell lines

Next, we analyzed the cytokine profile of PB derived NKT cells of 5 healthy controls and 5 RA patients, and SF derived NKT cells of 5 RA patients by intracellular staining of 14-day old, α-GalCer stimulated cultures gated on $Vα24^+$

cells. Figure 3.5 shows that the Vα24⁺ NKT cell fraction of healthy controls contained 64.5% ± 13.1 IFN-γ producing cells, 15.7% ± 6.9 IL-4 producing cells and 19.7% ± 6.4 cells producing both IFN-γ and IL-4. In contrast, PB NKT cells of RA patients consisted of significantly more IFN-γ producing and significantly less cells producing both IFN-γ and IL-4 (92.5% ± 2.7 and 6.1% ± 2.3 respectively, $p < 0.05$). Remarkably, SF derived NKT cells displayed a cytokine profile similar to that of healthy controls, although the number of IL-4 producing cells tended to be lower and the number of cells producing both IFN-γ and IL-4 was somewhat higher (5.3% ± 5.3 and 28.7% ± 6.7 respectively). No differences were found between cytokine profiles of NKT cells of α-GalCer responding or non-responding patients. Furthermore, no relation with treatment or any disease parameter was found. These observations show that although NKT cells in PBMC of RA patients are biased towards a Th1-like cytokine profile, NKT cells in the SF display a Th0-like cytokine profile comparable with that of healthy controls.

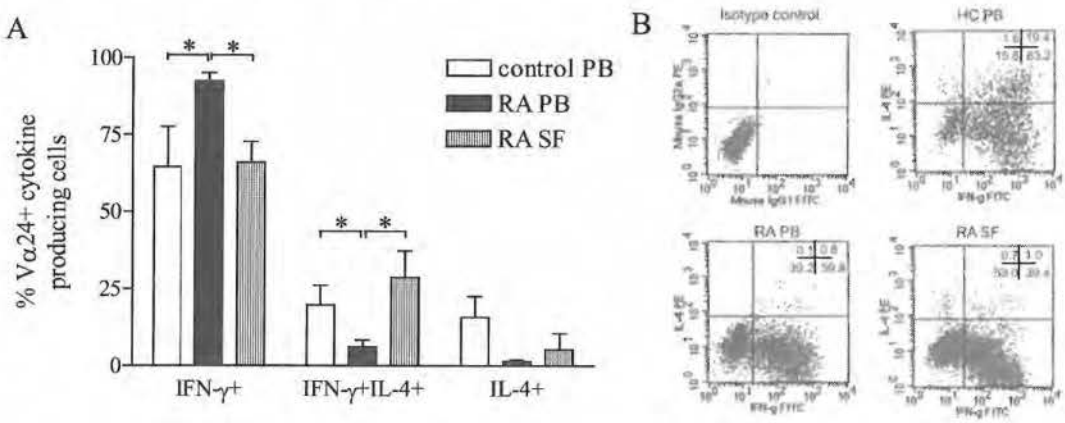


Figure 3.5. Cytokine profile of α-GalCer expanded NKT cells. A) *PBMC (1.5 x 10^6 cells per well) of 5 healthy controls and 5 RA patients as well as SFMC of 5 RA patients were stimulated with α-GalCer and restimulated at day 7 with autologous, α-GalCer pulsed, irradiated PBMC in the presence of 2 U/ml IL-2. The cytokine profile was analysed by intracellular staining and gating on the Vα24⁺ subset. Error bars indicate SEM, (* p<0.05).* ***B)*** *Representative dot plots demonstrating intracellular cytokine production of Vα24⁺ cells are shown. Upper left: isotype control; upper right: cytokine profile of HC PB Vα24⁺ cells; lower left: cytokine profile of RA PB Vα24⁺ cells; lower right: cytokine profile of RA SF Vα24⁺ cells. PB: peripheral blood; SF: synovial fluid.*

3.3.6 Cytokine profile of $CD4^+$ and $CD4^-$ NKT cell subsets in RA patients and healthy controls

The observed Th1-like bias in NKT cells of RA patients might be due to an increased number of DN NKT cells or a decreased number of $CD4^+$ NKT cells. To analyze the frequency of these NKT cell subtypes, we isolated the $V\alpha24^+$ cells of α-GalCer stimulated, 14 day old cultures derived from PBMC of 9 healthy controls and 7 RA patients by immunomagnetic selection. Positively selected cells were tested for α-GalCer reactivity to ensure the NKT cell nature of the cells. The presence of CD4 was assessed by flow cytometry. NKT cells of healthy controls consisted of 33.3% ± 6.7 $CD4^+$ NKT cells and 66.7% ± 6.7 $CD4^-$ (DN) NKT cells. The frequency of $CD4^+$ and $CD4^-$ NKT cells of RA patients did not differ significantly from that of healthy controls (49.8% ± 6.3 and 50.2% ± 6.3 respectively, data not shown).

Figure 3.6 shows the cytokine profile of each NKT cell subset determined by intracellular staining. PB derived $CD4^-$ NKT cells of healthy controls predominantly consisted of IFN-γ producing cells (IFN-γ+: 57.6% ± 8.8; IL-4+: 19.4% ± 6.6; IFN-γ+IL-4+: 23.0% ± 6.0), while $CD4^+$ NKT cells contained almost as much IL-4 producing as IFN-γ producing cells (IFN-γ+: 40.1% ± 7.4; IL-4+: 25.1% ± 7.5; IFN-γ+IL-4+: 34.8% ± 6.4). However, the $CD4^+$ as well as the $CD4^-$ NKT cell fractions of RA patients contained significantly less IL-4 producing cells compared to their counterparts of healthy controls (IFN-γ+: 57.2% ± 12.9; IL-4+: 5.8% ± 1.5; IFN-γ+IL-4+: 37.0% ± 13.2 for $CD4^+$ NKT cells and IFN-γ+: 72.1% ± 12.4; IL-4+: 3.3% ± 1.9; IFN-γ+IL-4+: 24.6% ± 11.9 for $CD4^-$ NKT cells), indicating that both $CD4^+$ and $CD4^-$ NKT cells in the peripheral blood of RA patients are biased towards a Th1-like cytokine profile.

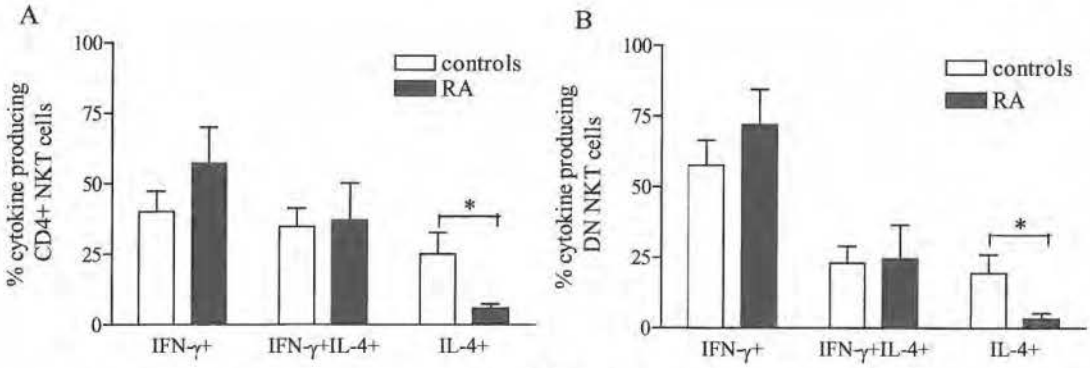


Figure 3.6. Cytokine profile of CD4$^+$ and CD4$^-$ NKT cell lines derived from PBMC of RA patients and healthy controls. *Vα24$^+$ cells of α-GalCer stimulated, 14 day old cultures of 9 healthy controls and 7 RA patients were isolated using biomagnetic selection. The cytokine profile of CD4$^+$ (**A**) and CD4$^-$ (**B**) NKT cells was assessed by intracellular staining. Error bars indicate SEM, (* p<0.05).*

To exclude that the observations in NKT cell lines of RA patients were caused by the clonal expansion of one or a few NKT cells, we analyzed the heterogeneity of the Vα24 and Vβ11 TCR by means of CDR 3 fragment length analysis. We found that the NKT cell lines of both RA patients and healthy controls displayed a monoclonal Vα24 and polyclonal Vβ11 profile (data not shown) which shows that the differences between NKT cells of RA patients and healthy controls found in response to α-GalCer are not due to a skewed outgrowth of only one or a few NKT cells.

3.4 Discussion

Several studies have provided evidence that NKT cells are involved in autoimmune conditions[27]. Attempts to increase the number of NKT cells in animal models of autoimmunity by transgenic expression of the invariant TCR or by passive transfer of NKT cells showed a protective effect against disease induction[28,29]. Additionally, administration of α-GalCer resulted in disease prevention or suppression. These studies indicate that NKT cells can play a role in the regulation of autoimmunity and that they therefore are an interesting subject for further investigation in human autoimmune diseases.

In this study we demonstrated a decreased frequency of NKT cells in PBMC of RA patients. Since we used anti-Vα24 and anti-Vβ11 mAbs to identify invariant NKT cells, it is possible that conventional T cells were also stained by this combination. However, Araki et al. have shown that the frequency of $V\alpha24^{+}V\beta11^{+}CD3^{+}$ T cells, even at low numbers, corresponded well with the NKT cell frequency determined by CD1d-tetramers[12], which supports the specificity of anti-Vα24 and anti-Vβ11 staining for NKT cells.

Several mechanisms can account for NKT cell reduction in the peripheral blood of RA patients. First, NKT cells might preferentially migrate into the joint to fulfill their regulatory function. We therefore studied the frequency of NKT cells in synovial fluid and tissue of RA patients. Our data show that the NKT cell frequency is not elevated in synovial fluid, and that the invariant TCR can be detected in both synovial tissue and fluid samples of RA patients. Preferential migration of NKT cells into the synovium may have resulted in a monoclonal or oligoclonal Vα24 profile in synovial samples. However, we did not find such a profile in the SF or ST of all patients indicating that the decrease cannot be

explained by a selective migration of NKT cells towards the joint. A similar conclusion was reached by others for RA[30] and MS[31].

A second possibility might be that the reduced NKT cell frequency is caused by a selective loss of a limited number of NKT cell clones. It was shown in mice that NKT cells exhibit a highly diverse TCRβ repertoire and a small clone size[32], hence a loss of NKT cells should result in a reduced diversity of TCR Vβ11. However, the Vβ11 profile of α-GalCer expanded PB NKT cells of RA patients was polyclonal, which suggests that RA patients do not suffer from a specific loss of NKT cells.

A third possible cause is a decreased reactivity towards the natural NKT cell ligand. To examine this possibility, we stimulated PBMC of RA patients with α-GalCer and found that in 53.8% of the patients ('responders'), NKT cells expanded upon α-GalCer stimulation and reached levels comparable to those of healthy controls. This suggests that an inadequate expression of CD1d[33] or an aberrant presentation of the natural NKT cell antigen, but not a decreased reactivity, might explain the NKT cell reduction in these responder patients. In contrast, in 46.2% of the patients ('non-responders'), NKT cells did not react to α-GalCer. This impaired NKT cell function was also reported earlier by the group of Kojo et al., who proposed that this decreased reactivity might result from an inherent NKT cell defect or a dysfunctional antigen presentation[11]. However, these authors could exclude that antigen presenting cells were dysfunctional in non-responder patients. Remarkably, SF NKT cells of both responders and non-responders expanded upon stimulation, indicating that the impaired NKT cell function in non-responders is restricted to the blood compartment.

Additional mechanisms may account for the reduced frequency, including a decreased thymical output, as has been described earlier for conventional T cells in RA[34], and a chronic overstimulation of NKT cells resulting in a decreased

frequency due to TCR downregulation after activation[35]. Moreover, it is possible that a chronic activation might also lead to non-responsiveness since it was shown that NKT cells in α-GalCer injected mice are anergic for an extended period of time[36].

When we analyzed the cytokine profiles of *in vitro* expanded NKT cells, we found that $CD4^-$ NKT cells of healthy controls mainly consisted of IFN-γ producing cells, whereas $CD4^+$ NKT cells can produce both Th1-like and Th2-like cytokines. This reflects the direct *ex vivo* situation reported by others[2,3]. We observed that PB derived NKT cells of RA patients displayed a Th1-like phenotype which was due to a decreased number of IL-4 producing cells in both the $CD4^+$ and $CD4^-$ NKT cell subset compared to healthy controls. Although these data were obtained from *in vitro* cultured cells, our data obtained from direct *ex vivo* stimulation of PBMC with α-GalCer confirm a Th1-like bias of NKT cells in RA patients. Strikingly, NKT cells in the synovial fluid do not show this Th1-like bias, but have a Th0-like profile that is comparable with that of PB NKT cells of healthy controls. A Th1-like bias of PB derived NKT cells was also found in diabetes[9] and MS[12], indicating that NKT cell dysfunction is not specific for RA but might have a major role in the etiology of autoimmune diseases. Although no relation between reactivity to α-GalCer or NKT cell cytokine profiles and drug treatment was found, a possible effect of the medication cannot be excluded.

In summary, the presence, even in non-responder patients, of functional NKT cells that display a Th0-like cytokine profile in the SF, can indicate that non-impaired NKT cells migrate from the peripheral blood towards the synovium in order to exert their regulatory function. NKT cells express a chemokine receptor profile similar to Th1-type inflammatory homing cells, which suggests that these cells perform their function mainly in the tissue[37]. However, their number and/or function are/is probably insufficient to resolve the ongoing auto-immune

reaction. Hence, a strategy to locally enhance the number of NKT cells by α-GalCer might be a potential treatment for RA. We conclude that, since the number of $V\alpha24^{+}V\beta11^{+}CD3^{+}$ NKT cells is decreased and the cytokine profile of blood derived NKT cells is biased to a Th1-like phenotype in RA patients, NKT cells might be functionally related to resistance or progression of rheumatoid arthritis and are therefore an interesting target for the treatment of RA.

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Chapter 4

Study of NKT cell regulation *in vitro*

Abstract

NKT cells are thought to play an important role in regulatory networks of the immune system. Several studies in mice have shown that overexpression or activation of NKT cells might lead to elimination of tumors and protection against autoimmunity. The regulatory potential of NKT cells in humans has not been demonstrated yet. The objective of this study was to determine the *in vitro* immunoregulatory effects of NKT cells on antigen specific T cells by using different approaches. First, the effect of NKT cell clones on Tetanus Toxoid (TT) reactive effector T cell clones was analyzed in a co-culture experiment. This revealed that addition of a $CD4^{-}CD8^{-}$ NKT cell clone of a healthy control resulted in a general decrease in proliferation and led to an increased number of IFN-γ producing, TT reactive T cells. Co-culture of RA NKT cell clones had no effect on proliferation. No major differences were observed between the cytokine profile induced by NKT cell clones of a RA patient or a healthy control. In a second approach, the effect of NKT cell stimulation prior to or during T cell activation was assessed. However, the frequencies or cytokine profiles of antigen reactive T cells were not affected by NKT cell stimulation. In the final approach, NKT cell supernatants were added to stimulated T cells and were found to strongly inhibit T cell proliferation. In conclusion, our results suggest that NKT cells may alter antigen specific T cell functions *in vitro*. Approaches using NKT cell stimulation to regulate T cells did not show any significant effect. Hence, more information about the regulatory role of NKT cells in (auto)immune reactions is essential to successfully apply NKT cells for therapeutic purposes.

4.1 Introduction

Natural Killer T (NKT) cells are a distinct subset of regulatory T cells, that express a restricted TCR consisting of an invariant Vα24Jα18 TCRα chain and a variable Vβ11 TCRβ chain. NKT cells recognize glycolipid antigens, which are presented in a non-polymorphic MHC class I like, CD1d molecule. They rapidly produce large amounts of cytokines and chemokines upon activation. This enables them to act as regulators of the adaptive immune system[1,2]. It has been reported that NKT cells can influence other immune cells such as B cells, NK cells, macrophages and dendritic cells[3-5].

Based on the expression of the CD4 molecule, NKT cells can be divided into distinct subpopulations. $CD4^+$ NKT cells produce cytokines of both Th1 (IFN-γ) and Th2 type (IL-4, IL-10) while $CD4^-CD8^-$ (double negative, DN) NKT cells mainly secrete cytokines of a Th1 phenotype[6,7]. The glycolipid α-galactosylceramide (α-GalCer) was described to be a potent activator of NKT cells, although it is not the natural ligand[8]. Injection of α-GalCer into mice induced a rapid burst of cytokine production within hours of administration and resulted in elevated NKT cell numbers after 5 days[9]. Moreover, co-administration of protein antigen with α-GalCer resulted in a Th2 directed antigen-specific T cell response, which indicates that this system might be useful to shift the balance from a Th1-dominant towards a Th2-dominant immune response[10]. Several studies have already shown that NKT cells can play a role in a range of different diseases, including infection, cancer and autoimmunity[11,12].

Decreased NKT cell numbers and NKT cell deficiencies were observed in animal models of autoimmunity and in patients with autoimmune diseases such as multiple sclerosis (MS), type I diabetes and rheumatoid arthritis (RA)[13,14]. Attempts to increase the number of NKT cells in mice by transgenic

overexpression of the invariant TCR (Vα14Jα18 in mice) or by adoptive transfer of NKT cells demonstrated protective effects against induction of diabetes or experimental allergic encephalomyelitis (EAE)[15-17]. Additionally, a number of studies evaluated the capacity of α-GalCer to modulate diabetes or EAE in mice and found that repeated injection of the glycolipid was able to prevent disease in up to 90% of the animals tested[18-21]. Disease protection was often associated with a Th2 cytokine production by NKT cells and by Th2 deviation of auto-antigen specific T cells.

The strong conservation of the NKT-CD1d system among different mammalian species allows a possible application of α-GalCer for the treatment of human autoimmune diseases. A phase I clinical trial of cancer immune therapy using α-GalCer-pulsed dendritic cells has shown a substantial and specific effect on NKT cells, which resulted in secondary immune effects such as T cell activation[22]. In contrast to results observed in mice, no adverse events were observed, even after high doses of α-GalCer administration. However, treatment efficacy of α-GalCer in mice appeared to depend on the administration dose, route and timing as well as the number of injections[19,23]. Moreover, treatment with α-GalCer resulted in disease exacerbation in some models for systemic lupus erythematosus (SLE)[24]. This illustrates the potential risk of α-GalCer administration by promoting unwanted responses that may exacerbate disease rather than temper it. The mechanisms by which NKT cells regulate autoimmunity and may prevent cancer remain poorly understood. In addition, the precise mechanism by which NKT cells control T cell activities is not known and the effect of α-GalCer induced NKT cell stimulation on human T cell activation remains unclear.

We have studied various experimental approaches to determine whether human NKT cells may influence the functional properties of T cells *in vitro* (Figure 4.1). At first, we analyzed the direct effect of NKT cell clones on Tetanus Toxoid (TT)

reactive, effector T cell clones (approach 1). To this end, we examined the proliferative capacity and cytokine profile of the effector T cell clones upon co-culture with NKT cell clones. In parallel, we determined whether NKT cell clones of RA patients had impaired regulatory capacities. In a second approach we investigated whether NKT cell activation had an effect on the stimulation of antigen specific T cells (Fig 4.1, approach 2). Therefore, we determined the frequency and cytokine profile of antigen specific cells after co- or pre-stimulation of NKT cells at the time of antigen administration. Finally, the effect of NKT cell supernatants on T cell stimulation was assessed (Fig 4.1, approach 3). Again, the proliferation and cytokine profile of the T cells were used as read-out parameters. Our results suggest that human NKT cells have an effect on conventional T cells *in vitro*, but further analysis is necessary to fully understand the regulatory role of NKT cells in immunoregulation.

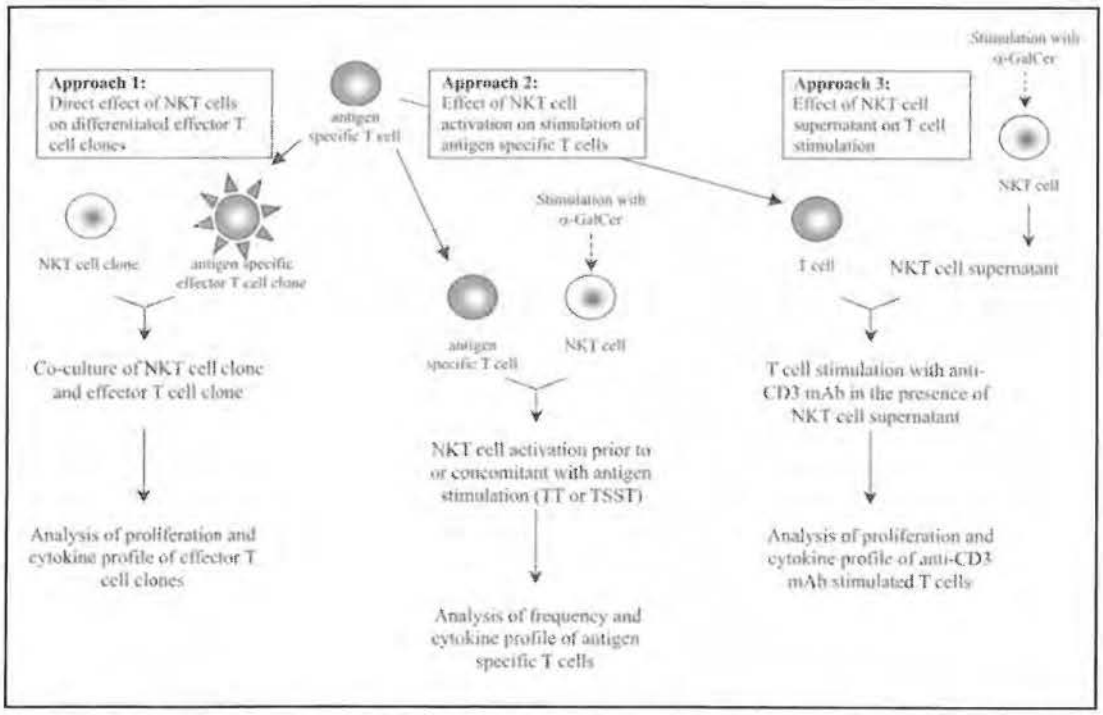

Figure 4.1. Experimental strategy to test whether human NKT cells may influence the functional properties of T cells *in vitro*.

4.2 Materials and methods

4.2.1 Co-culture of α-GalCer and Tetanus Toxoid reactive T cell clones

NKT cell clones of a healthy control subject (HC1 20C9-9) and a RA patient (RA1 Vα24-4 and RA1 Vα24-5) were used in a co-culture experiment to assess the effect of NKT cells on the proliferation and cytokine profile of antigen specific T cells. To this end, autologous, Th1-like TT reactive T cell clones were isolated from peripheral blood mononuclear cells (PBMC) of a healthy control and a RA patient by a limiting dilution assay (described in 2.2.4). Isolated TT reactive T cell clones were used as indicator cells in the co-culture experiment: $4x10^4$ indicator TT reactive T cell clones were stimulated with $1x10^5$ autologous, irradiated, TT-pulsed PBMC in the absence or presence of increasing numbers of effector NKT cells ($1x10^4$, $4x10^4$ and $8x10^4$ NKT cells). Conditions were performed in triplicate. The proliferative responses were evaluated after 3 days of co-culture, using a classical ^{3}H-thymidine incorporation assay. Culture supernatants were obtained at the time of ^{3}H-thymidine addition and the cytokine contents were tested by standard sandwich ELISA (IFN-γ, TNF-α, IL-4 and IL-10).

To specifically discriminate between cytokines produced by TT reactive T cell clones and NKT cell clones, $4x10^4$ indicator $CD4^+$ TT cells were stimulated with $1x10^5$ autologous, irradiated, TT-pulsed PBMC without or with the addition of $4x10^4$ NKT cell clones. Conditions were performed in triplicate and the samples were pooled for intracellular staining. By gating on $CD4^+$ cells, it was possible to specifically determine relative cytokine production by $CD4^+$ TT reactive T cell clones.

4.2.2 Co-stimulation of NKT cells and antigen specific cell lines

T cell lines (TCL) reactive to TT were generated from blood of 3 healthy donors by culturing $4x10^4$ PBMC per well (30 wells per condition) with 2.5 Lf/ml TT with or without 100 ng/ml α-GalCer or vehicle (buffer without α-GalCer) added. At day 7, cells were restimulated with $1x10^5$ autologous, irradiated PBMC pulsed with TT, TT + α-GalCer or TT + vehicle and supplemented with 2 U/ml rhIL-2. At day 14, TT reactive were identified and the frequency of TT reactive T cells was estimated as described in 2.2.4. The cytokine profile was determined by intracellular staining of TT reactive TCL. Based upon the relative number of cells producing IFN-γ, IL-4 or both, TCL were arbitrarily divided into a Th0 (% IFN-γ+IL-4+ > % IFN-γ ≥ % IL-4), Th0/1 (% IFN-γ > % IFN-γ+IL-4+ > 15%) or Th1 (% IFN-γ > % IFN-γ+IL-4+ < 15%) phenotype.

4.2.3 Co-stimulation and pre-stimulation of NKT cells and antigen specific cells

For co-stimulation experiments, $1x10^6$ PBMC obtained from 2 healthy donors were cultured in 24 well plates for 1 week with 0.1 μg/ml Toxic Shock Syndrome Toxin (TSST-1, Sigma, St. Louis, MO) alone, TSST and 100 ng/ml α-GalCer or TSST and 100 ng/ml vehicle as a control condition. In pre-stimulation conditions, PBMC were cultured with 100 ng/ml α-GalCer or vehicle for 1 week followed by stimulation with 0.1 μg/ml TSST for 1 week. At day 7 of TSST stimulation, the number of $V\beta2^+$ T cells was assessed by flow cytometry and the number of cytokine producing $V\beta2^+$ T cells was determined by intracellular staining.

4.2.4 T cell stimulation in the presence of NKT cell supernatants

T cell supernatants were obtained after stimulating $1x10^5$ PBMC from 2 healthy controls and 2 RA patients with 100 ng/ml α-GalCer or 0.2 μg/ml PHA for 48 hours. Cytokine contents were determined by standard sandwich ELISA in

duplicate (IFN-γ, TNF-α, IL-4, IL-6 and IL-10). To study the effects of α-GalCer or PHA stimulated PBMC supernatants on conventional T cells, $5x10^4$ PBMC of 2 healthy donors were stimulated with 1 μg/ml anti-CD3 monoclonal antibody (anti-CD3 mAb) in the presence of increasing amounts of supernatants (5%, 10%, 20% and 50% of the culture volume). Conditions were performed in triplicate. At day 3 of anti-CD3 mAb stimulation, proliferation was measured by a standard ^{3}H-thymidine incorporation assay. Simultaneously, the cytokine profile of anti-CD3 mAb stimulated PBMC was determined by intracellular staining.

4.3 Results

4.3.1 Regulatory effect of NKT cells on antigen specific effector T cell clones

4.3.1.1 Generation and analysis of NKT cell clones

We first studied whether cultured NKT cells have an effect on the functional properties of effector NKT cell clones (Fig 4.1). To this end, NKT cell clones were isolated from 2 healthy controls and 1 RA patient by stimulation of PBMC with α-GalCer at limiting dilution conditions. T cell lines reactive to α-GalCer were identified by means of a standard proliferation assay and subsequently cloned. The expression of CD4 and CD8 as well as the cytokine profile of α-GalCer reactive clones was determined by flow cytometry. Three NKT cell clones were obtained from 2 healthy controls: 2 out of 3 were $CD4^+$ and expressed a Th0/2 cytokine profile, one NKT cell clone was DN and displayed a Th1 profile. Additionally, three NKT cell clones from 1 RA patient were isolated. Interestingly, one NKT cell clone with a Th0/1 cytokine profile stained positive for CD8. In contrast to $CD4^+$ NKT cell clones of healthy controls, $CD4^+$ NKT cells of the RA patient showed a Th0 or Th0/1 profile (Table 4.1).

Table 4.1. NKT cell clone characteristics.

Clone	CD4/CD8 expression	% IFN-γ	% IFN-γ +IL-4	% IL-4	Th profile
HC1 20C9-9	DN	86	14	0	Th1
HC2 Vα24-2	CD4	4	43	53	**Th0/2**
HC2 Vα24-4	CD4	9	47	44	**Th0/2**
RA1 Vα24-4	CD4	68	31	1	**Th0/1**
RA1 Vα24-5	CD8	41	58	1	Th0/1
RA1 Vα24-10	CD4	0	97	3	**Th0**

Expression of CD4 and CD8 on α-GalCer reactive NKT cell clones was analyzed by flow cytometry. The relative number of IFN-γ (% IFN-γ), IL-4 (% IL-4) and IFN-γ+IL-4 (% IFN-γ+IL-4) producing cells was determined by intracellular staining. TCL were arbitrarily divided into a Th0/2 (IL-4 ≥ IFN-γ+IL-4+ > 15%), Th0 (IFN-γ+IL-4+ > IFN-γ ≥ IL-4), Th0/1 (IFN-γ > IFN-γ+IL-4+ > 15%) or Th1 (IFN-γ > IFN-γ+IL-4+ < 15%) phenotype. HC: healthy control; RA: rheumatoid arthritis

To further characterize the obtained NKT cell clones, the cytotoxic potential of clone HC1 20C9-9 towards the NK target cell line K562 and the leukemia T cell line Jurkat was tested in a standard ^{51}Cr release assay at three different effector-to-target ratios (5/10/20 NKT cells to 1 K562 or Jurkat cell)(see 2.2.8). The NKT cell clone did not exhibit cytotoxicity towards the CD1d-negative K562 cell line, with or without α-GalCer pulsing (Fig 4.2). Cytotoxic activity towards the CD1d-positive Jurkat cell line reached only 16 % of maximal cell lysis at the highest effector-to-target ratio (20 NKT cells to 1 Jurkat cell). However, when α-GalCer pulsed Jurkat cells were used as target, the NKT cell clone induced marked cytolysis of the cells (Fig 4.2B).

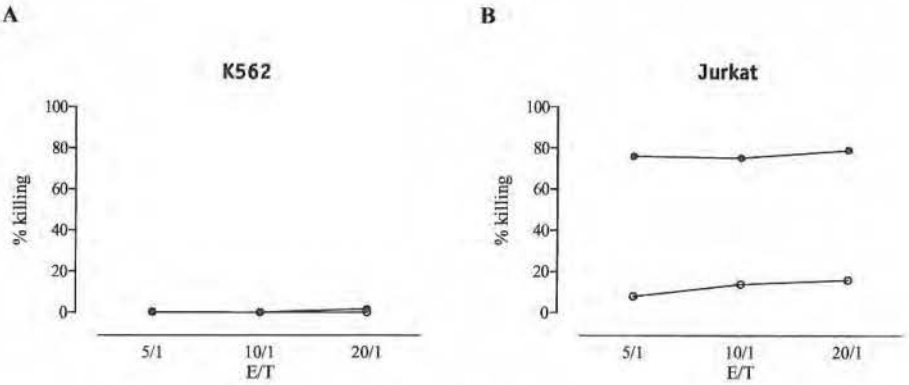

Figure 4.2. Cytotoxic potential of NKT cell clone HC1 20C9-9. *Cytotoxic activity of NKT cell clone HC1 20C9-9 towards α-GalCer pulsed (closed circles) or non-pulsed (open circles) K562 (A) or Jurkat (B) cells. Killing was evaluated in a standard ^{51}Cr release assay. Line graphs display the percentage of cytolysis at increasing effector-to-target ratio (displayed on X-axis). E/T: effector-to-target ratio.*

4.3.1.2 Effect of NKT cell clones on proliferation and cytokine profile of antigen specific effector T cells

To determine the regulatory potential of NKT cells *in vitro*, we evaluated the effect of NKT cell clones HC1 20C9-9, RA1 Vα24-4 and RA1 Vα24-5 on the proliferation and cytokine profile of antigen-specific T cell clones. For this purpose, we obtained autologous $CD4^+$ Th1-like TT reactive clones from blood of

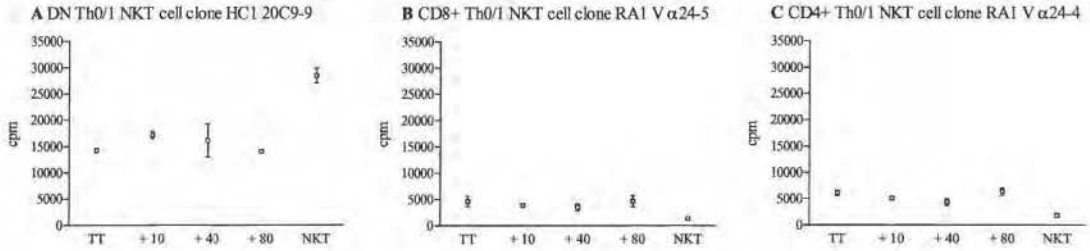

Figure 4.3. Influence of NKT cells on the proliferation of TT reactive T cells. *Cells were co-cultured for 72 hrs and analyzed by standard ^{3}H-thymidine incorporation assay. Conditions were performed in triplicate. A) Co-culture of DN Th0/1 NKT cell clone HC 1 20C9-9 and indicator cell. B) Co-culture of CD8+ Th0/1 NKT cell clone RA 1 Vα24-5 and indicator cell. C) Co-culture of CD4+ Th0/1 NKT cell clone RA 1 Vα24-4 and indicator cell. TT: TT specific indicator clone; +10-+40+-80: indicator clone co-cultured with 10-40-80x10^3 NKT cells; NKT: 40x10^3 effector clone cells; cpm: counts per minute.*

the healthy control and the RA patient. These TT reactive T cell clones were used as indicator cells. For the co-culture experiment, indicator TT cell clones were stimulated in the absence or presence of increasing numbers of NKT cell clones. Proliferative responses were evaluated at day 3 of co-culture using a classical ^{3}H-thymidine incorporation assay. Culture supernatant was obtained at the time of ^{3}H-thymidine addition and the content of IFN-γ, TNF-α, IL-4 and IL-10 was measured by ELISA.

Co-culture of the Th1-like TT reactive T cell clone and NKT cell clone HC1 20C9-9 did not influence the cytokine profile of the indicator clone (data not shown). Although highly proliferating NKT cells were added, the proliferation measured in the co-culture did not increase compared to that of the individual TT clone culture (Fig 4.3A, TT: 14216.7 ± 392.7; TT+40NKT: 16108.8 ± 3113.8; NKT: 28408.1 ± 1392.7). Addition of RA1 Vα24-4 or RA1 Vα24-5 NKT cell clones also did not alter the cytokine profile of the indicator clone (data not shown), nor did it affect the proliferation of the TT reactive T cell clones (Fig 4.3B and C). These data suggest that only the NKT cell clone of the healthy control might influence the proliferation of the indicator clone. Additionally, NKT cell clones derived from

the RA patient and the healthy control do not alter the cytokine profile of the indicator clones. However, it should be noted that the cytokine values obtained by ELISA represent the cytokine production by both TT and NKT cell clones and thus might conceal possible changes.

To specifically determine the cytokines produced by each cell type, intracellular FACS analysis can be used by gating on the $CD4^+$ TT reactive clones. To this end, indicator TT cell clones were stimulated with TT pulsed PBMC in the presence of NKT cells (described in 4.2.1) and the relative number of cytokine producing cells was determined. As a control, indicator TT cells were stimulated in the absence of NKT cells. Figure 4.4A shows that co-culture of NKT clone HC1 20C9-9 ($CD4^-CD8^-$) and the TT reactive clone ($CD4^+$), resulted in a slight increase in the number of IFN-γ producing cells (83.1% to 89.2%), which was accompanied with a decrease in the number of cells producing both IFN-γ and IL-4 (16.8% to 10.6%). A similar increase in the number of IFN-γ producing cells was found for the co-culture of the $CD8^+$ and $CD4^+$ NKT cell clones obtained from the RA patient (Fig 4.4B and 4.4C). It was not possible to phenotypically discriminate between NKT clone RA1 Vα24-4 and the TT reactive clone due to the expression of CD4 on the NKT cell clone. Our data show that NKT cell clones can slightly influence the cytokine profile of effector T cell clones and that the induced profile does not differ for NKT cell clones derived from a healthy control and a RA patient.

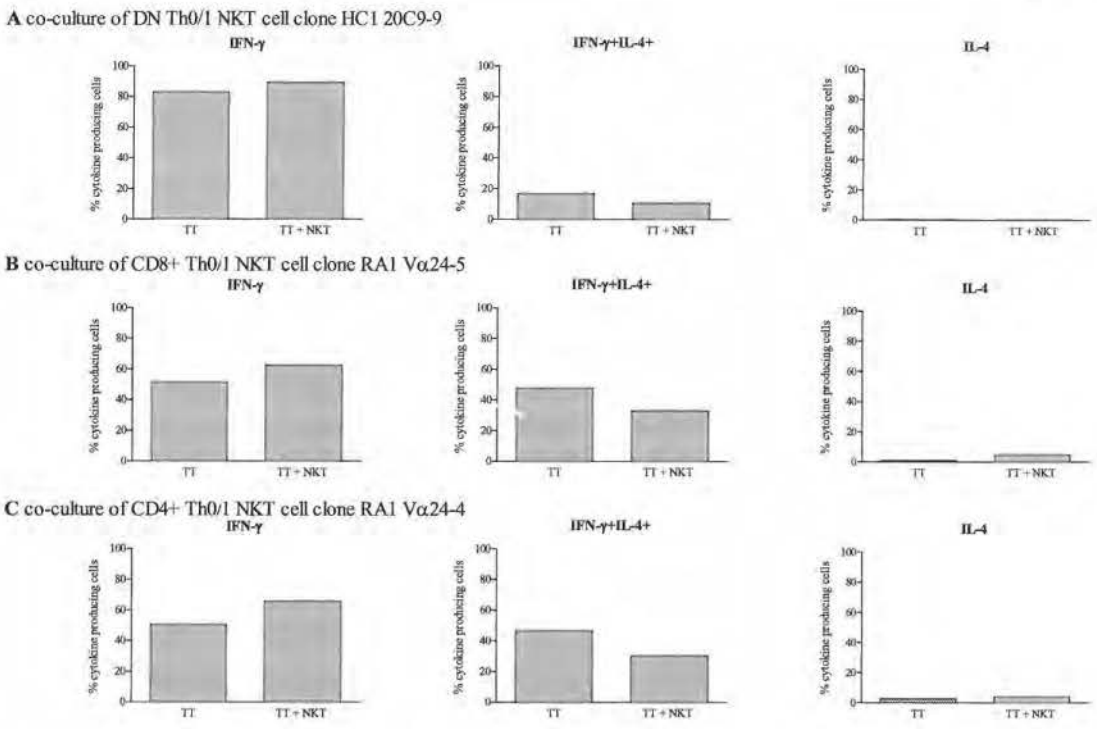

Figure 4.4. Influence of NKT cells on relative cytokine production of TT reactive T cells analyzed by intracellular staining. *A) Co-culture of DN Th0/1 NKT cell clone HC1 20C9-9 and CD4⁺ indicator cell. B) Co-culture of CD8⁺ Th0/1 NKT cell clone RA1 Vα24-5 and CD4⁺ indicator cell. C) Co-culture of CD4⁺ Th0/1 NKT cell clone RA1 Vα24-4 and CD4⁺ indicator cell. $4x10^4$ indicator TT cells were stimulated with $1x10^5$ autologous, irradiated, TT-pulsed PBMC in the absence or presence of $4x10^4$ effector NKT cells. TT: cytokine producing cells in TT reactive clone alone, gating on CD4⁺ cells; TT + NKT: cytokine producing cells in co-culture of TT and NKT clones, gating on CD4⁺ cells.*

4.3.2 Regulatory effect of co-stimulation of NKT cells and TT reactive cells

In a second approach, we examined the effect of NKT cell stimulation on the activation of antigen specific T cells (Fig 4.1). TT reactive T cell lines from 3 healthy donors were generated from PBMC in the presence of α-GalCer as described in 4.2.2. At day 14, the number of TT reactive TCL was determined by ^{3}H-thymidine incorporation. TT reactive TCL were identified in all 3 donors for all 3 conditions. No differences were found in the mean frequency of TT reactive

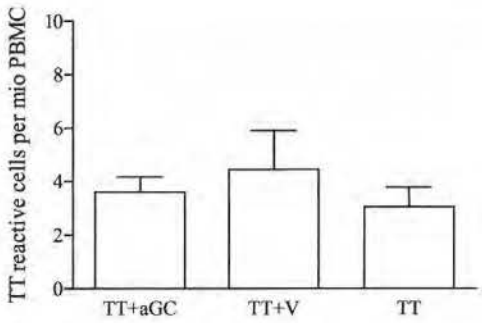

Figure 4.5. Effect of NKT cell activation at the time of antigen administration on the frequency of TT reactive T cell lines. *PBMC of 3 donors were stimulated with TT to isolate TT reactive TCL, α-GalCer or vehicle was added during TCL generation. The frequency of TT reactive T cells was set as the number of reactive TCL by the total number of PBMC plated. Error bars indicate SEM. TT + αGC: TT TCL isolated in the presence of α-GalCer; TT + V: TT TCL isolated in the presence of vehicle; TT: TT TCL isolated in absence of α-GalCer and vehicle. Error bars indicate SEM.*

cells in the different conditions tested (TT alone: 3.1 ± 0.7 TT TCL / million PBMC; TT + α-GalCer: 3.6 ± 0.6 TT TCL / million PBMC; TT + vehicle: 4.4 ± 1.5 TT TCL / million PBMC, Fig 4.5).

To determine whether NKT cell stimulation during TCL isolation affected the Th phenotype of the TT reactive TCL, we assessed the cytokine profile of each TCL by intracellular staining. Based upon the relative number of cells producing IFN-γ, IL-4 or both, TCL were divided into a Th0 (% IFN-γ+IL-4+ > % IFN-γ ≥ % IL-4), Th0/1 (% IFN-γ > % IFN-γ+IL-4+ > 15%) or Th1 (% IFN-γ > % IFN-γ+IL-4+ < 15%) phenotype (Fig 4.6). Generation of TT reactive TCL in the presence of α-GalCer resulted in lines of 3 different phenotypes (Th1: 3/12; Th0/1: 5/12; Th0: 4/12). However, TCL generation with TT only or in the presence of vehicle, resulted in only minor differences in the number of TCL of each phenotype (Th1: 4/11; Th0/1: 3/11; Th0: 4/11 for TT only and Th1: 7/16; Th0/1: 8/16: Th0: 1/16 for vehicle). These data indicate that NKT cell stimulation with α-GalCer at the time of TT reactive T cell activation, has no significant effect on the number of TCL isolated or on the cytokine profile of these cells.

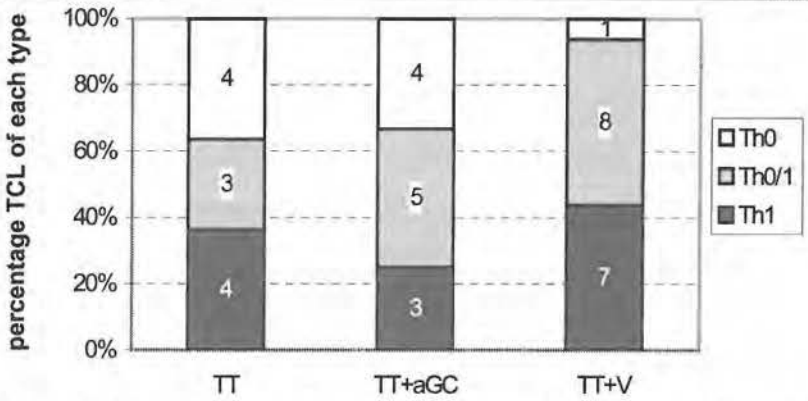

Figure 4. 6. Effect of NKT cell activation at the time of antigen administration on the cytokine profile of TT reactive TCL. *TT reactive TCL were isolated from PBMC of 3 donors without or with α-GalCer or vehicle added to the culture. Cytokine profile was assessed by intracellular staining. Data labels indicate the number of TCL of each phenotype. TT + αGC: TT TCL isolated in the presence of α-GalCer; TT + V: TT TCL isolated in the presence of vehicle; TT: TT TCL isolated in absence of α-GalCer and vehicle.*

TT reactive TCL are highly enriched in, but do not exclusively exist of TT reactive cells. Hence, the assessment of cytokine profiles by intracellular staining of a TCL does not exclude the presence of cytokine producing cells that are not TT reactive. In addition, the low number of TCL isolated in each condition hampers a significant analysis of α-GalCer induced changes. To circumvent these limitations, a modified version of approach 2 was used to analyze the effect of NKT cell activation on antigen specific T cell stimulation.

4.3.3 Effect of preceding and simultaneous NKT cell stimulation on TSST reactive cells

In the modified version of approach 2, the superantigen Toxic-Shock-Syndrome-Toxin-1 (TSST) was used as antigen, rather than TT. TSST selectively stimulates TCR Vβ2 expressing cells[25], which enabled us to specifically determine the effect of NKT cell stimulation on antigen reactive T cell activation by intracellular staining of $V\beta2^+$ cells. Supplementary, flow cytometric analysis provides significant data about cytokine profiles when sufficient cells are measured. For this experiment, PBMC of 2 healthy donors were stimulated with TSST for 7 days.

In 2 additional conditions, PBMC were stimulated with TSST and α-GalCer or vehicle. Previously, Jahng et al. reported that activation of NKT cells in mice by α-GalCer resulted in prevention of EAE when α-GalCer was administered prior but not simultaneous with EAE induction[19]. Consequently, to determine the effect of preceding NKT cell stimulation on T cells, TSST was administered to PBMC that were cultured with α-GalCer or vehicle for 1 week. After 7 days, the number of Vβ2$^+$ T cells was evaluated by flow cytometry and the cytokine profile of Vβ2$^+$ cells was analyzed by intracellular staining.

After 1 week of TSST stimulation, Vβ2$^+$ T cell levels were expanded to 51.7% ± 15.3 of the total number of T cells (Fig 4.7). A similar number of Vβ2$^+$ T cells was found in the co-stimulation conditions (TSST + α-GalCer: 48.5% ± 12.5; TSST + V: 51.9% ± 13.1). Furthermore, treatment of PBMC with α-GalCer or vehicle prior to TSST stimulation resulted in comparable Vβ2$^+$ T cell numbers (α-GalCer prior to TSST: 50.2% ± 0.1; V prior to TSST: 55.9% ± 11.7).

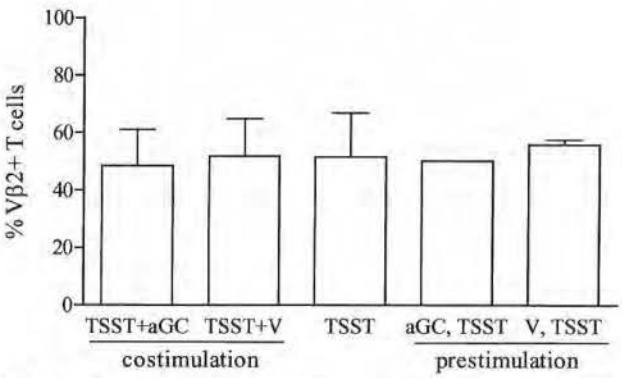

Figure 4.7. Effect of preceding or simultaneous NKT cell activation at the time of antigen stimulation on the number of TSST reactive cells. *PBMC of 2 healthy donors were stimulated with TSST alone, or in combination with α-GalCer or vehicle for 7 days. For pre-stimulation, TSST was administered to PBMC that were cultured with α-GalCer or vehicle for 1 week. At day 7 of TSST stimulation, the number of Vβ2$^+$ T cells was determined by flow cytometry. TSST + aGC: co-stimulation with TSST and α-GalCer; TSST + V: co-stimulation with TSST and vehicle; aGC, TSST: pre-stimulation with α-GalCer for 1 week before addition of TSST; V, TSST: pre-stimulation with vehicle for 1 week before addition of TSST. Error bars indicate SEM.*

The number of cytokine producing T cells was measured after 1 week of stimulation with TSST. Figure 4.8 shows that the Vβ2$^+$ fraction predominantly consisted of IFN-γ producing cells (IFN-γ: 97.8% ± 1.0; IFN-γ+IL-4+: 1.5% ± 0.6; IL-4: 0.8% ± 0.5). Similar numbers were found when PBMC were cultured in the presence of both vehicle and TSST (IFN-γ: 97.1% ± 1.5; IFN-γ+IL-4+: 1.6% ± 0.8; IL-4: 1.5% ± 0.8) or in the presence of α-GalCer and TSST (IFN-γ: 95.7% ± 0.6; IFN-γ+IL-4+: 2.9% ± 1.4; IL-4: 1.4% ± 0.8). Although stimulation of PBMC with α-GalCer prior to the addition of TSST resulted in an increase of IL-4 producing cells compared to PBMC stimulated with TSST only (IL-4: 3.7% ± 1.6), this finding was also observed for vehicle treated cells (IL-4: 2.7% ± 1.1). Our data show that NKT cell stimulation preceding to or concomitant with antigen reactive T cell activation has no effect on the number of Vβ2$^+$ cells, nor on the cytokine profile of these cells.

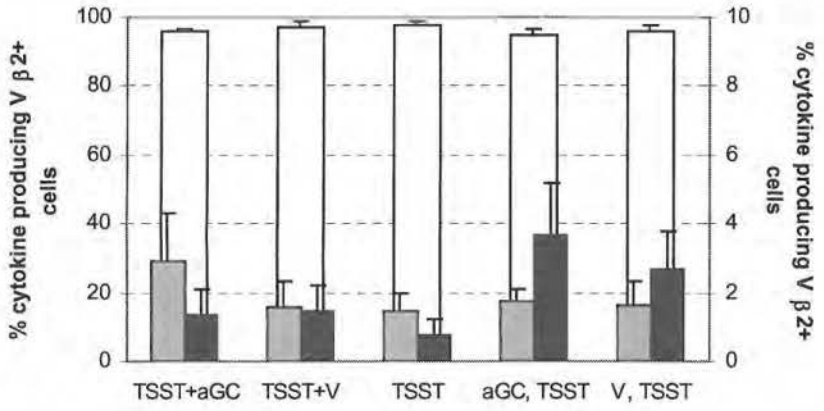

Figure 4.8. Effect of preceding or simultaneous NKT cell stimulation at the time of antigen stimulation on the number of Vβ2$^+$ cytokine producing cells. *Cytokine producing cells were assessed using intracellular staining, gating on Vβ2$^+$ cells. The relative number of IFN-γ producing cells (white bars) is plotted on the primary Y-axis, the number of cells producing only IL-4 (black bars) and the number of cells that produce both IFN-γ and IL-4 (grey bars), are plotted on the secondary Y-axis. TSST + aGC: co-stimulation with TSST and α-GalCer; TSST + V: co-stimulation with TSST and vehicle; aGC, TSST: pre-stimulation with α-GalCer for 1 week before addition of TSST; V, TSST: pre-stimulation with vehicle for 1 week before addition of TSST. Error bars indicate SEM.*

4.3.4 Effect of NKT cell derived cytokines on T cells

In the third approach, the effect of NKT cell derived cytokines on T cells was analyzed (Fig 4.1). NKT cells rapidly produce cytokines upon activation, which is thought to underlie their regulatory function. Therefore, we obtained supernatant of α-GalCer stimulated PBMC of 2 healthy donors and 2 RA patients. As a control, supernatant of PHA stimulated PBMC was collected from the same donors. Cytokine contents were analyzed by ELISA. No differences were found in the cytokine content of supernatants derived from α-GalCer or PHA stimulated PBMC of the healthy donors as compared to the RA patients (Fig 4.9). IL-4 levels remained below the detection limit of the assay in both α-GalCer and PHA stimulated cultures. The cytokine levels in PHA derived supernatants of healthy controls and RA patients were higher than in those obtained after α-GalCer stimulation (compare panel A with panel B).

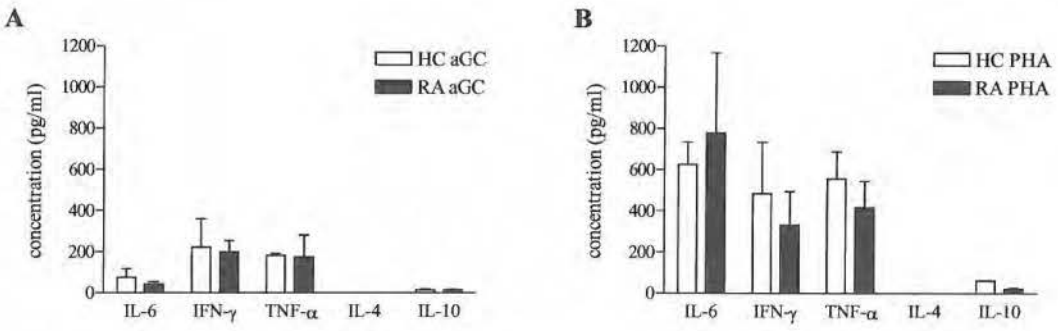

Figure 4.9. Cytokine contents of supernatants from α-GalCer stimulated PBMC. *PBMC of 2 healthy donors and 2 RA patients were stimulated with α-GalCer or PHA. After 48 hours, supernatants were obtained and the cytokine content of α-GalCer (panel A) and PHA (panel B) stimulated supernatant was determined by ELISA. HC: healthy control; RA: rheumatoid arthritis; IL: interleukin; IFN: interferon; TNF: tumor-necrosis-factor. Error bars indicate SEM.*

To study the effect of the obtained supernatants, PBMC of 2 healthy donors were stimulated with anti-CD3 monoclonal antibody (anti-CD3 mAb) in the presence of increasing amounts of supernatant obtained from the α-GalCer or PHA stimulated PBMC (5%, 10%, 20% and 50% of the culture volume). The effect on proliferation of PBMC was determined by a standard ^{3}H-thymidine incorporation assay after 3 days of anti-CD3 mAb stimulation. Figure 4.10A shows that addition of α-GalCer supernatant from healthy controls to anti-CD3 mAb stimulated PBMC resulted in a strong decrease in proliferation compared to that without supernatant. Increasing amounts of supernatant resulted in a further decrease of the proliferation of PBMC (5%: 11.5% ± 2.8; 10%: 10.0% ± 1.4; 20%: 8.6% ± 1.1; 50%: 7.8% ± 0.6). A similar finding was observed for supernatants of α-GalCer stimulated PBMC obtained from RA patients (Fig 4.9A, 50%: 7.5% ± 0.6). Supernatants of PHA stimulated PBMC suppressed the proliferation to a comparable level which indicates that the inhibitory capacity was not restricted to supernatants derived from α-GalCer stimulated PBMC (Fig 4.10B). No direct correlation between inhibition of proliferation and cytokine content of the supernatants was observed. Additionally, the effect of supernatants of α-GalCer or PHA stimulated PBMC on the cytokine profile of PBMC was determined by intracellular staining of IFN-γ and IL-4 after 5 days of anti-CD3 mAb stimulation, but no effect was observed (data not shown). These findings show that supernatants of α-GalCer stimulated PBMC as well as PHA stimulated PBMC strongly inhibit anti-CD3 mAb induced proliferation, but have no effect on the cytokine profile of these cells. Furthermore, no differences were observed between supernatants derived from healthy controls and RA patients.

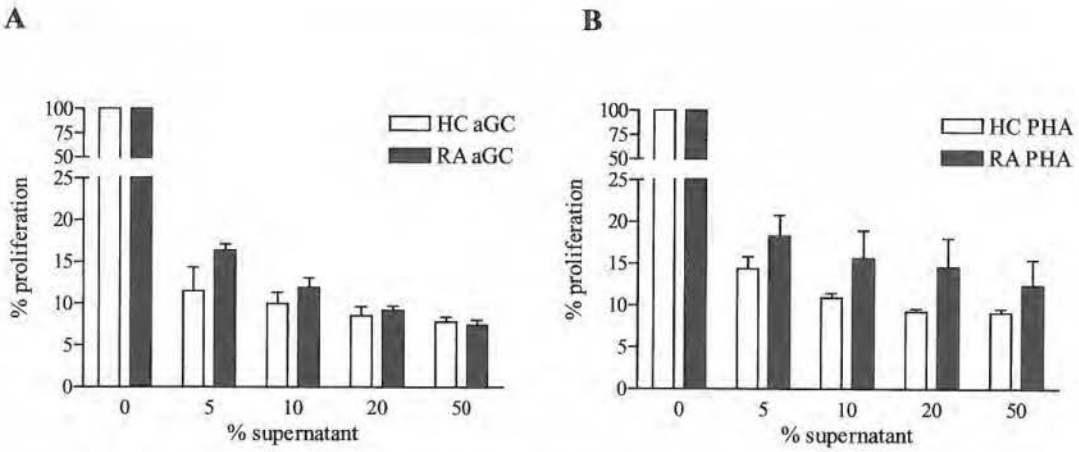

Figure 4.10. Anti-CD3 mAb induced proliferation of PBMC after addition of α-GalCer and PHA stimulated cell supernatants. *PBMC of two healthy donors were stimulated with anti-CD3 mAb in the presence of increasing amounts of supernatants derived from α-GalCer stimulated (panel A) or PHA stimulated (panel B) PBMC of two healthy donors and two RA patients. Proliferation was measured with a standard ^{3}H-thymidine incorporation assay after 3 days of anti-CD3 mAb stimulation. The percentage proliferation compared to stimulated PBMC without supernatant (100%) is presented. Error bars indicate SEM.*

4.4 Discussion

NKT cells have been implicated in the regulation of adaptive immune responses, including those directed against autoantigens. Treatment of mice with autoimmune diseases by enhancing the number of NKT cells or activating NKT cells by α-GalCer resulted in a decrease of symptoms and eventually in recovery of disease[26]. However, the precise mechanism by which NKT cells control T cell activities is not known and the effect of α-GalCer induced NKT cell stimulation on human T cell priming remains unclear. In an effort to determine whether NKT cells may influence functional properties of T cells *in vitro*, we applied different experimental approaches (summarized in Fig 4.11).

The first part of our study demonstrates that human NKT cells can have a direct effect on antigen specific, effector T cells. We found that the general proliferation did not increase when antigen reactive T cell clones were cultured in the presence of the DN NKT cell clone derived from a healthy donor. This effect was not observed in the presence of RA $CD4^+$ and $CD8^+$ NKT cell clones. However, NKT cells of both RA patient and healthy donor altered the cytokine profile of

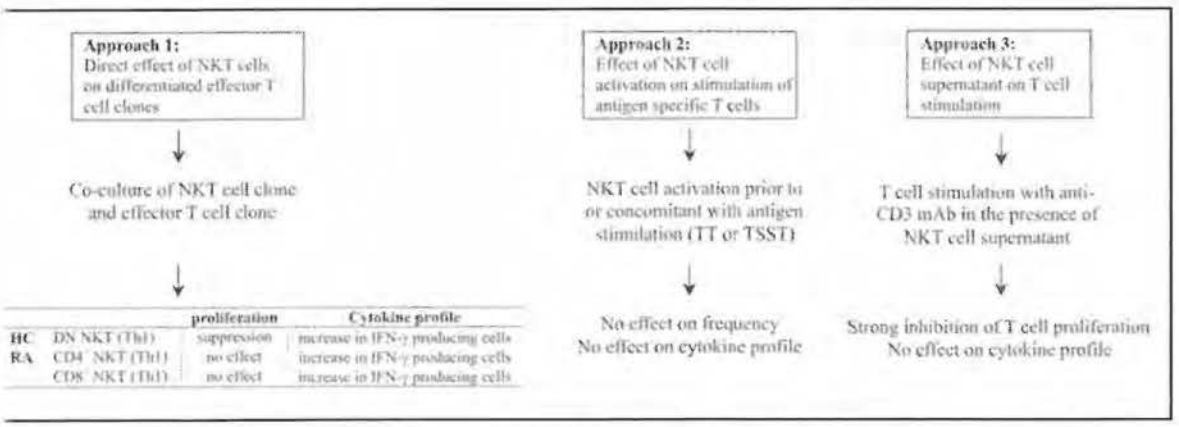

Figure 4.11. Summary of the results obtained by the various approaches.

antigen specific T cell clones. This effect appeared to depend on the cytokine production of the NKT cell itself, since addition of the Th1-type NKT cell clones influenced mainly the number of IFN-γ producing TT reactive T cells. Our observations are in partial agreement with those of others who have shown that only DN NKT cells could inhibit the proliferation[27]. Although we could not determine which cell type was responsible for the suppression, this observation suggests that the DN NKT cell clone in our system might inhibit the proliferation of the TT reactive T cell clone. Furthermore, Ho et al. also showed that activated CD1d-restricted NKT clones did not affect cytokine production of activated T cells[27]. However, these authors tested the cytokine profile of EBV peptide activated PBMC rather than antigen specific T cell clones. Hence a difference in the antigen specific T cells used may underlie these contrasting findings. Our data show that RA and HC NKT cell clones differ in the ability to suppress proliferation, but influence cytokine profiles of antigen specific cells in a similar way. This might be explained by the comparable cytokine profile of the NKT cells, even though they belong to different NKT subtypes which have different effects on antigen specific proliferation[27]. Therefore, to fully determine whether NKT cell clones of RA patients have impaired regulatory capacities, the effect on antigen specific T cells should be analyzed by using NKT cells of the same subtype.

If the regulatory effect of NKT cells was only defined by the cytokines they produce, addition of NKT cell supernatants to T cell cultures might have a similar effect on T cells as a co-culture with NKT cells. However, our results revealed a strong inhibitory activity of NKT cell supernatants on stimulated PBMC, but showed no influence on the cytokine profile. This could indicate that not only the cytokine production determines the effect of NKT cells on the cytokine profile, but that additional mechanisms like cell-cell contact or activation of other immune cells might also be involved. Additionally, effector T cell clones might be more resistant to suppression than PBMC, which were used in this assay.

In contrast to others, we did not find a correlation between the level of cytokines produced by NKT cells and the level of suppression induced by the supernatant[28]. The similar inhibitory capacity of α-GalCer and PHA stimulated supernatants might be due to activation of a broad range of cells upon NKT cell stimulation resulting in a spectrum of secreted cytokines comparable to that after stimulation with the mitogen PHA[22].

In a second approach, we assessed the effect of α-GalCer induced NKT cell activation on antigen stimulation, since this was reported to give rise to amelioration of autoimmune diseases in mice[14]. We analyzed whether α-GalCer administration could influence the cytokine profile of TT reactive TCL, by adding the glycolipid at the time of antigen stimulation with TT. This experiment showed no significant effects of NKT cell activation on TT reactive T cell stimulation. Furthermore, we tested the effect of preceding or concomitant stimulation with α-GalCer on TSST specific cells and found that co-stimulation did not result in significant changes in the cytokine profile of TSST specific cells. Although a slight Th2 stimulating effect was observed upon α-GalCer treatment prior to TSST stimulation, the cytokine profile of the T cells did not differ significantly from that of cells activated in the presence of vehicle. Our findings are in contrast with those observed in mice, where it was found that activation of NKT cells by α-GalCer could direct conventional T cells towards a Th2 phenotype[10]. However, T cells obtained from α-GalCer injected mice are often compared with vehicle treated mice only. This therefore may not give a full picture of α-GalCer mediated effects. It is not clear why vehicle treated cells behave differently than cells that have been activated with their antigen alone. Possibly, some of the vehicle components might be responsible for this effect. The exact composition of the vehicle, however, is not known.

Although the precise mechanism of Th2 induction by α-GalCer is not known, several possibilities might explain our observations. First, NKT cell regulation can act indirectly on antigen specific T cells by manipulation of other cells that interact with T cells. A number of cell types could be responsible for the overall effect of α-GalCer. It is assumed that α-GalCer injection promotes the preferential accumulation of tolerogenic dendritic cells (DC) to the regional lymph nodes, which may dampen the inflammatory process[29]. Results reported by Furlan et al. showed the importance of liver NKT cells in the protection against MOG induced EAE[23]. Their finding that the administration route determined the effect of α-GalCer also implies a role for other cells in the regulation mechanism[23]. Hence our *in vitro* system might not contain all players that are necessary to fully enhance a Th2 deviation of the antigen specific T cells.

A second possibility might be that the antigen reactive T cells used in our system may not be subjected to NKT cell regulation. To generate antigen specific TCL, we used the bacterial antigen TT. The isolation of TT reactive TCL is based on recall responses of TT reactive memory cells. However, it was reported that NKT cell stimulation did not affect initial T cell responses. It rather inhibited the differentiation into effector antigen specific cells resulting in an anergic phenotype, although α-GalCer had to be present at the initial activation of antigen specific cells to induce the effect[30]. This suggests that the TT reactive, memory T cells in our assay might no longer be affected by NKT cell regulation. Additionally, when TT reactive T cells were subject to NKT cell mediated regulation, they might have become anergic and might therefore not be detectable. The other antigen used to determine the effect of NKT cell stimulation on antigen reactive cells, was TSST. Bacterial superantigens like TSST bind directly to MHC molecules without being previously processed and specifically to the Vβ region of the T cell receptor[31]. The antigen presenting cell (APC) is therefore not actively involved in T cell activation by superantigens. If

NKT cells exert their regulatory function via manipulation of the APC, T cells activated by antigens, such as the superantigen TSST, that circumvent the role of the APC, would not be affected by NKT cell function. This mechanism might explain the lack of Th2 induction upon NKT cell activation during stimulation with TSST. *In vitro* experiments with other (auto-)antigens might therefore provide a better understanding of NKT cell regulation.

Finally, α-GalCer treatment in mice has been shown to be influenced by a large number of variables and is therefore not always as efficient. Its efficacy has been proven in mouse models like EAE and NOD diabetes, but in an animal model of Systemic Lupus Erythematosus (SLE) administration of α-GalCer exacerbated the disease[24]. Even in the case of EAE, treatment efficacy was influenced by the precise timing of α-GalCer administration[19] and the particular mouse strain used[18]. Possibly, these problems can be overcome by using reagents that have superior immunotherapeutic activities, such as the truncated analogue of α-GalCer, OCH, or by a combination treatment of α-GalCer and molecules that promote Th2 cytokine production by NKT cells, like anti-CD86 antibodies or the cytokine IL-7[32,33].

In conclusion, our data show that NKT cells and the supernatants they produce have a direct effect on the cytokine profile and proliferation capacity of antigen specific T cells *in vitro*. However, approaches using α-GalCer stimulation to regulate T cells did not show significant effects. These findings suggest that NKT cells possess the potential to regulate (auto)immune reactions. A better understanding of the function of these cells and their regulatory capacity however, is required to successfully use NKT cells for therapeutic purposes.

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Chapter 5

NKT cells can be deviated *in vitro* towards a Th2 phenotype by low dose methotrexate and methylprednisolone treatment

Abstract

NKT cells are described to be an important regulatory T cell subset that is present at a decreased frequency in autoimmune diseases such as rheumatoid arthritis (RA). Patients with RA are commonly treated with immunosuppressive drugs, known to inhibit T cell activation and function. To determine the effect of these immunosuppressive agents on NKT cells, we stimulated PBMC of 5 healthy donors with α-galactosylceramide in the presence of cyclosporine A (CsA), methotrexate (MTX) and methylprednisolone (MP). The *in vitro* expansion and the cytokine profile of NKT cells were analyzed by flow cytometry. A dose dependent inhibition of the NKT cell expansion was observed in the presence of CsA, MTX and MP. Furthermore, addition of MP and MTX deviated the cytokine profile of NKT cells to a Th2 phenotype. These data show that immunosuppressive drugs affect the NKT cell function *in vitro*.

5.1. Introduction

Natural Killer T (NKT) cells are characterized by their co-expression of the invariant T cell receptor (TCR) Vα24Jα18 chain and the variable TCR Vβ11 chain. These cells recognize glycolipid antigens presented in a MHC-I-like, CD1d context[1,2]. The marine sponge derived molecule α-galactosylceramide (α-GalCer) is a potent activator of NKT cells, but is not the natural antigen[3]. Stimulation of NKT cells with α-GalCer results in the secretion of large amounts of both Th1 and Th2 type cytokines[4] which leads to the activation of a variety of cells of the innate and adaptive immune system *in vivo*[5].

Interestingly, co-administration of α-GalCer and protein antigen to mice induced an antigen specific Th2 immune response which raises the possibility that specific stimulation of NKT cells may be exploited for therapeutic purposes of Th1 mediated autoimmune conditions[6]. Indeed, NKT cell stimulation with α-GalCer protected mice against the induction of autoimmune conditions. Although the precise mode of action is unknown, this protection coincided with an increased expression of Th2 type cytokines such as IL-4 and IL-10 by autoantigen specific T cells[7,8]. In addition, several groups reported a decreased frequency and impaired functionality of NKT cells in autoimmune diseases including multiple sclerosis (MS), type I diabetes and rheumatoid arthritis (RA)[9-12].

RA is an autoimmune disease characterized by chronic inflammation of synovial joints resulting in destruction of cartilage and, ultimately, bone. Although the actual cause of the disease is not known, autoreactive Th1 T cells are considered to play a major role in the pathogenesis[13]. Hence, approaches to restore the Th1/2 balance could be useful for tempering RA. This suggests that attempts to stimulate the regulatory capacity of NKT cells might be beneficial. Conventional therapy in RA, including non-steroidal anti-inflammatory drugs (NSAIDS), disease

modifying anti-rheumatic drugs (DMARDS) and corticosteroids, aims to block inflammation, retard synovial proliferation and prevent joint erosion. These drugs include methotrexate (MTX), cyclosporin A (CsA) and methylprednisolone (MP), which are commonly used for the treatment of RA[14]. The mechanisms by which these immunosuppressors interfere with T cell activation and proliferation are diverse. Glucocorticoids and CsA inhibit cytokine synthesis of T cells by preventing the action of nuclear transcription factors[15,16]. In contrast, MTX exerts apoptotic effects by interfering with DNA synthesis[17]. However, the effect of these molecules on NKT cell function is not known.

Since RA patients are generally treated with immunosuppressive agents, the aim of this study was to determine the effect of these molecules on the NKT cell function. To this end, the possible contribution of these drugs to the dysfunction of NKT cells in RA was investigated. We therefore analyzed the *in vitro* effects of MTX, CsA and MP on the expansion and cytokine profile of NKT cells. Our results show that the presence of CsA, MTX as well as MP inhibited NKT cell expansion while only MTX and MP specifically altered the cytokine profile of NKT cells. These results suggest that the NKT cell function is affected by immunosuppressive drugs.

5.2. Materials and methods

5.2.1 Incubation with immunosuppressive agents

Peripheral blood mononuclear cells (PBMC) of 5 healthy donors were isolated by fycoll Hypaque density gradient centrifugation. Cells were resuspended at $1.5x10^6$ cells/ml and cultured for 16 hours in the presence of CsA (1-10-100-1000 ng/ml; Neoral-Sandimmun, Novartis Pharma, Vilvoorde), MTX (10^{-8}-10^{-7}-10^{-6}-10^{-5} M; Ledertrexate, Lederle, Louvain-la-Neuve), MP (10^{-11}-10^{-9}-10^{-7} M; Medrol, Pharmacia, Puurs) or the respective vehicles (microemulsion described in [18] for CsA; 250 mM sodium carbonate pH 9.5 for MTX and PBS for MP). After incubation, cells were washed, seeded at $7.5x10^5$ cells/ml in 24 well plates and stimulated with 100 ng/ml α-GalCer in the absence (pulse-treatment) or presence (continuous treatment) of immunosuppressive drugs. After 7 days, T cell frequencies ($CD3^+$, $CD4^+$, activated $CD4^+$, $CD8^+$, activated $CD8^+$ cells) were determined by flow cytometry. The total number of cells present in each condition was estimated by counting the number of cells in the lymphocyte gate for 30 seconds using a flow cytometer. Cells were restimulated for an additional 7 days with α-GalCer pulsed, irradiated autologous APC and supplemented with 2 U/ml recombinant human IL-2 in the presence or absence of the immunosuppressive agents. The relative cytokine profile of $V\alpha24^+$ (NKT cells) and $V\alpha24^-$ (non-NKT cells) cells was assessed by intracellular staining on day 14 of culture.

5.3. Results

5.3.1 Effect of immunosuppressive drugs on the NKT cell expansion

To determine the effect of CsA, MTX and MP on the expansion of NKT cells, we stimulated PBMC of 5 healthy donors with α-GalCer in the absence or presence of increasing amounts of the immunosuppressive drugs. In this way, we tested 2 experimental approaches. In the first approach, PBMC were treated for a period of 16 hours with the drugs and then cultured with α-GalCer in the absence of the drugs (pulse-treatment). In the second approach, PBMC were also incubated with the drugs for 16 hours, but subsequently stimulated with α-GalCer in the continuous presence of the suppressive agents (continuous treatment). After 7 days, T cell frequencies were determined by flow cytometry. T cell nor NKT cell frequencies were affected by pulse-treatment with CsA, MTX and MP (Fig 5.1). Only when PBMC were pre-incubated with the highest concentration of MTX (10 μM), a significant reduction in NKT cell expansion was observed (vehicle: 4.25% ± 0.79; 10^{-5} M: 0.56% ± 0.34; $p<0.05$, Fig 5.1B).

Continuous treatment with MTX had no effect on the frequencies of CD3, CD4, CD8 and activated T cells (data not shown). When PBMC were stimulated with α-GalCer together with continuous treatment of CsA, a decrease in the number of activated $CD4^+$ and $CD8^+$ cells was observed, which was significant at the highest dose tested (activated $CD4^+$ cells: 34.0% ± 7.7 without CsA to 8.9% ± 0.4 at the highest amount of CsA; activated $CD8^+$ cells: 6.9% ± 1.2 to 0.5% ± 0.2 respectively; $p<0.05$). In contrast, long term presence of MP during culture resulted in a significant increase in the number of $CD3^+$ cells (vehicle: 81.4% ± 1.8; 10^{-11} M: 82.4% ± 2.4; 10^{-9} M: 84.3% ± 2.6; 10^{-7} M: 93.9% ± 0.0; $p< 0.01$), but did not affect other T cell frequencies (data not shown). In addition, the total number of cells estimated by flow cytometry, dropped significantly upon

increasing concentrations of MP (vehicle: 10.9 ± 1.1 x10^3 cells; 10^{-11} M: 10.2 ± 9.0 x10^3 cells ; 10^{-9} M: 7.8 ± 1.1 x10^3 cells; 10^{-7} M: 3.9 ± 0.0 x10^3 cells; $p < 0.05$).

The NKT cell expansion was inhibited in a dose dependent way upon continuous treatment with MTX, CsA and MP during stimulation with α-GalCer (Fig 5.1). Addition of MTX, even at a concentration of 10^{-7} M, resulted in a significant reduction of the NKT cell frequency (vehicle: 3.13% ± 0.62; 10^{-8} M: 1.71% ± 0.42; 10^{-7} M: 0.21% ± 0.13; 10^{-6} M: 0.35% ± 0.13; 10^{-5} M: 0.42% ± 0.25; $p < 0.05$). Long-term presence of CsA also significantly reduced α-GalCer specific NKT cell expansion in a dose-dependent manner (vehicle: 3.13% ± 0.62; 1 ng/ml: 2.95% ± 0.96; 10 ng/ml: 2.58% ± 0.81; 100 ng/ml: 1.91% ± 0.84; 1000 ng/ml: 0.13% ± 0.03; $p<0.05$, Fig 1A). The decrease in NKT cells after continuous treatment with CsA or MTX was not due to a general decrease in cell numbers, since the total number of cells in each condition were similar (Fig 5.1A and B). Furthermore, addition of MP resulted in a significant lower number of NKT cells (vehicle: 3.13% ± 0.62; 10^{-11} M: 2.29% ± 1.12; 10^{-9} M: 0.91% ± 0.29; 10^{-7} M: 0.06% ± 0.01; $p < 0.05$, Fig 5.1C). Since the number of NKT cells is determined as the relative number of $V\alpha24^+V\beta11^+$ cells in the $CD3^+$ T cell fraction, it is not dependent of the decrease in cell number. This indicates that the decrease observed after continuous MP treatment is NKT cell specific. These findings indicate that the NKT cell expansion is inhibited by continuous treatment with immunosuppressive drugs *in vitro*.

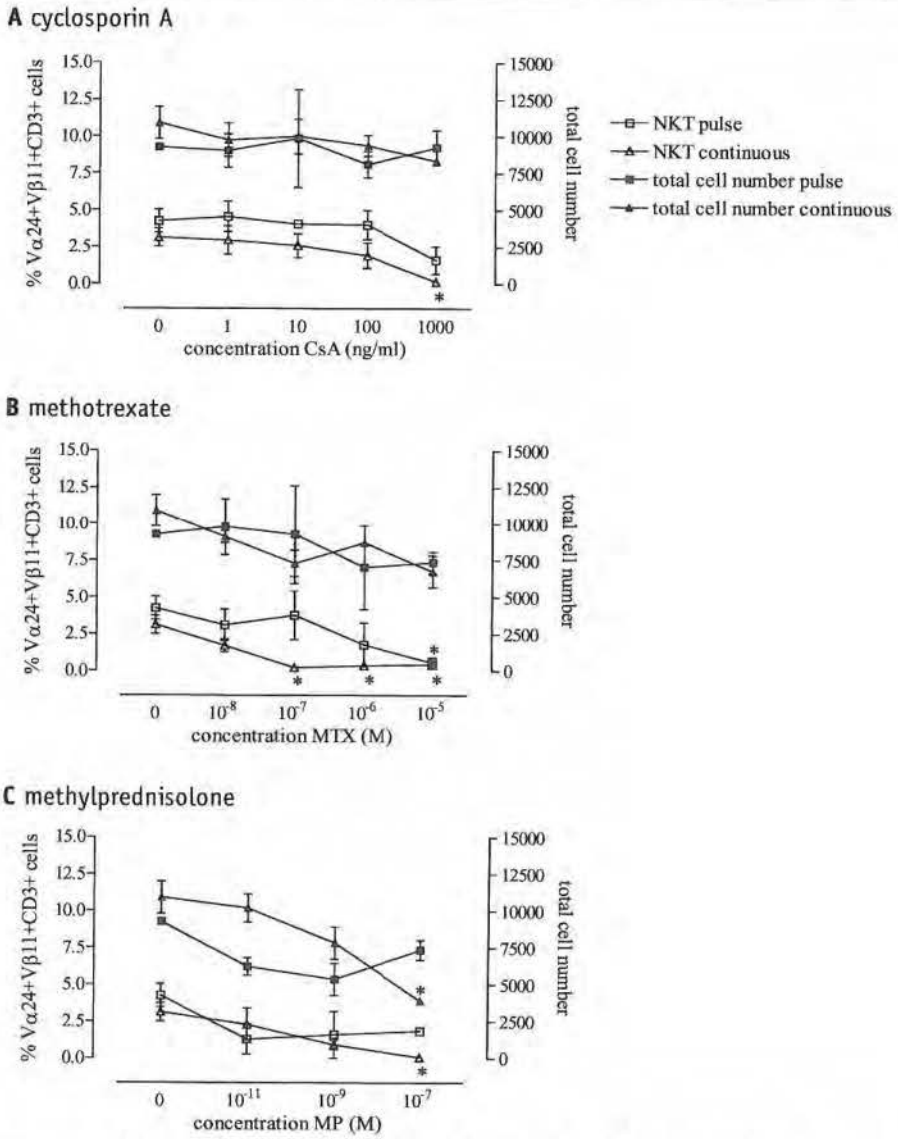

Figure 5.1. Effect of immunosuppressive drugs on NKT cell expansion. *PBMC were incubated with CsA, MTX and MP for 16 hours and stimulated with α-GalCer in the absence (pulse-treatment, squares) or presence (continuous treatment, triangles) of the drugs. After 7 days, NKT cell frequencies (depicted on left Y-axis, open symbols) and total T cell numbers (depicted on right Y-axis, filled symbols) were determined by flow cytometry. A) NKT cell frequencies and total cell numbers upon pulse and continuous treatment with CsA, B) NKT cell frequencies and total cell numbers upon pulse and continuous treatment with MTX, C) NKT cell frequencies and total cell numbers upon pulse and continuous treatment with MP. Cell numbers in each condition were determined by flow cytometry. Error bars indicate SEM. * p<0.05, significantly different compared to values without medication. CsA: cyclosporin A; MTX: methotrexate; MP: methyl-prednisolone;*

5.3.2 Effect of immunosuppressive drugs on the cytokine profile of NKT cells

To determine the effect of CsA, MTX and MP on the cytokine profile of NKT cells, cells used for the frequency analysis were restimulated with α-GalCer pulsed, irradiated autologous PBMC. Seven days after restimulation, the relative cytokine profile of Vα24$^+$ NKT cells and Vα24$^-$ non-NKT cells was determined by intracellular staining. Pulse-treatment with CsA, MTX and MP before stimulation with α-GalCer did not affect the cytokine profile of the Vα24$^+$ and Vα24$^-$ fraction (data not shown).

Continuous treatment with CsA did not influence the cytokine profile of Vα24$^+$ NKT cells nor Vα24$^-$ cells (Fig 5.2A and B). In contrast, when MTX was continuously present during culture of α-GalCer stimulated cells, the relative number of IFN-γ producing cells was suppressed (vehicle: 63.2% ± 7.0; 10^{-8} M: 34.4% ± 14.2; 10^{-7} M: 4.3% ± 2.0; 10^{-6} M: 22.2% ± 5.2; $p < 0.05$; Fig 5.2C). The number of IL-4 producing cells was significantly enhanced by MTX, but no correlation with the dose was found (vehicle: 10.3% ± 2.1; 10^{-8} μM: 30.6% ± 11.5; 10^{-7} M: 63.4% ± 5.8; 10^{-6} M: 57.0% ± 6.7; $p < 0.05$). Remarkably, this effect was restricted to the Vα24$^+$ population, since the cytokine profile of Vα24$^-$ cells did not change upon culture with MTX (Fig 5.2D). Figure 5.2E shows that continuous treatment of α-GalCer stimulated PBMC with MP significantly decreased the number of IFN-γ producing cells (IFN-γ: vehicle: 63.2% ± 7.0; 10^{-11} M: 58.7% ± 9.8; 10^{-9} M: 36.2% ± 11.6; 10^{-7} M: 24.4% ± 11.3; $p < 0.05$). This decrease was accompanied with a significant increase in the number of IL-4 producing cells (vehicle: 10.3% ± 2.1; 10^{-11} M: 14.1% ± 5.1; 10^{-9} M: 24.2% ± 7.7; 10^{-7} M: 66.1% ± 1.9; $p < 0.05$). Remarkably, we did not find a shift in the number of cytokine producing Vα24$^-$ cells (Fig 5.2F). Our data show that MTX and MP, but not CsA, can affect the cytokine profile of NKT cells *in vitro*.

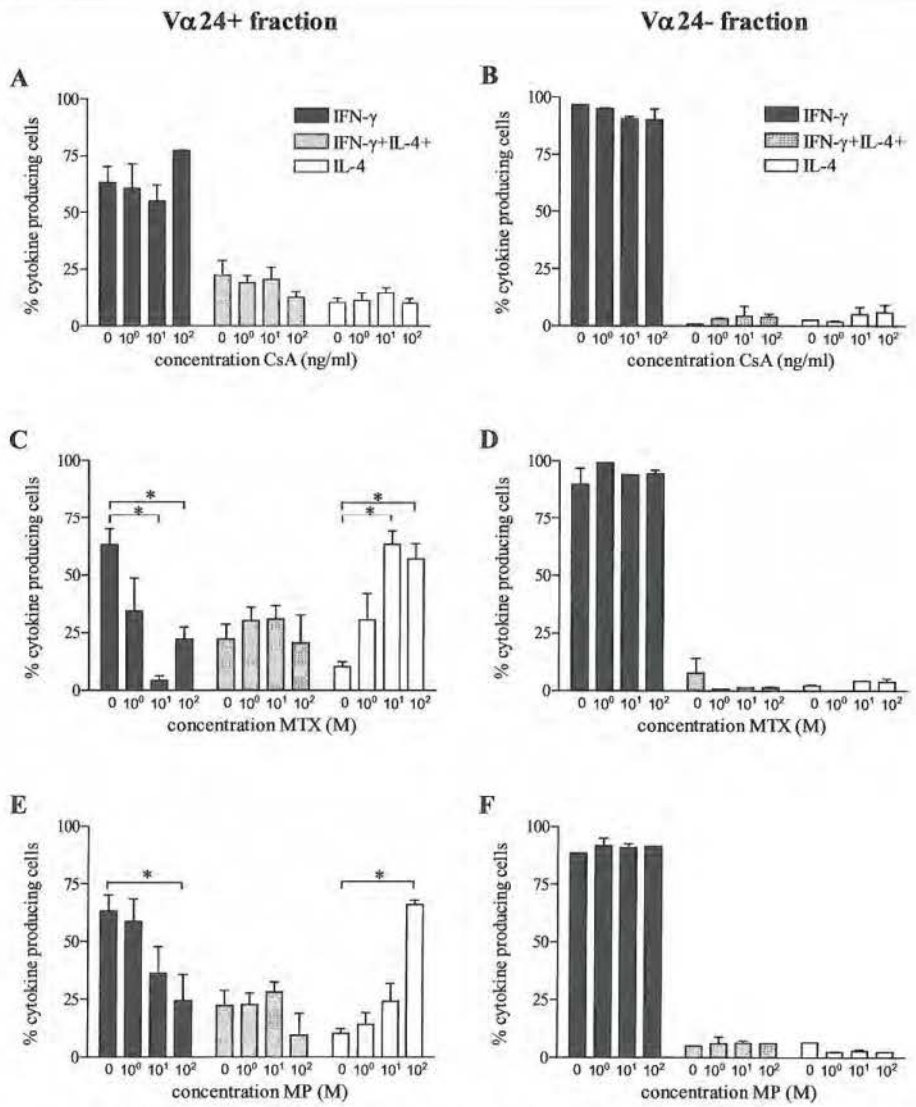

Figure 5.2. Effect of continuous treatment with immunosuppressive drugs on the cytokine profile of NKT cells. *PBMC were incubated with CsA, MTX and MP for 16 hours and stimulated with α-GalCer in the presence (continuous treatment) of the immunosuppressive agents. After 7 days, cells were restimulated with α-GalCer pulsed, irradiated autologous PBMC and cultured in the presence of the drugs. A) Cytokine profile of Vα24⁺ cells after treatment with CsA, B) Cytokine profile of Vα24⁻ cells after treatment with CsA, C) Cytokine profile of Vα24⁺ cells in the presence of MTX, D) Cytokine profile of Vα24⁻ cells in the presence of MTX, E) Cytokine profile of Vα24⁺ cells after treatment with MP, F) Cytokine profile of Vα24⁻ cells in the presence of MP. Error bars indicate SEM. * p<0.05, significantly different compared to values without medication. ** p<0.05, highest concentration significantly different compared to lowest concentration. CsA: cyclosporin A; MTX: methotrexate; MP: methylprednisolone*

5.4. Discussion

RA is a chronic inflammatory autoimmune disease that leads to progressive bone and joint erosion. Drugs that are commonly used in the treatment of RA show anti-inflammatory or immunosuppressive characteristics[14]. The goal of our study was to determine the *in vitro* effect of frequently used drugs like CsA, MTX or MP on the function of NKT cells. This study shows that CsA, MTX as well as MP inhibit NKT cell expansion when they are present during α-GalCer stimulation, but not prior to activation since pre-incubation with these molecules before antigen specific stimulation did not have any or only mild effects on NKT cell numbers. All three molecules act by inhibition of events following T cell activation, hence removal of the agents before antigen stimulation might have no effect on activation induced functions. Only when cells were pre-incubated with high amounts of CsA or MTX, the cell expansion was blocked. Moreover, it was shown that pre-treatment of antigen specific T cells with MP could inhibit glucocorticoid-induced apoptosis, which is reflected by the lack of decrease in the number of NKT cells even after incubation with high amounts of MP[19].

Our data show that incubation of PBMC with CsA during stimulation with α-GalCer inhibited the antigen specific expansion, but did not affect the cytokine profile of NKT cells. To permit a significant analysis of the cytokine profile of NKT cells, addition of exogenous IL-2 after restimulation with α-GalCer was necessary. It was reported that CsA can suppress T cell activation by preventing the induction of IL-2 gene expression which results in a decreased proliferation as well as cytokine production after mitogen stimulation[20]. Possibly, the presence of IL-2 in our experiment overcame the inhibition by CsA, leading to an unchanged cytokine profile upon α-GalCer stimulation. This is in agreement with results of Guillen et al., who reported that IL-2 can reverse the inhibitory effect of CsA[21]. An additional explanation can be that NKT cells express a $CD45RO^+$ memory

phenotype[22] and may therefore be less affected by CsA treatment. Indeed, it was demonstrated that CD45RO$^+$ T cells were less susceptible to CsA mediated inhibition than CD45RA$^+$ T cells[23]. Although we observed a decrease in the number of activated CD4 and CD8 T cells upon increasing amounts of CsA, the general cytokine profile of the total T cell population did not change. This might be explained by the finding that CsA interferes with T cell activation, but has no effect on resting T cells. It is therefore possible that the number of cells that are affected by CsA is too low to result in significant changes in the general T cell cytokine profile.

As for CsA, the presence of MTX at the time of antigen stimulation resulted in a decreased number of NKT cells. Moreover, the relative cytokine profile of NKT cells was severely affected by MTX. In contrast, only pre-incubation with the highest concentration of MTX resulted in a decreased frequency of NKT cells but had no effect on the cytokine profile. These results are in partial agreement with those of Genestier et al. who have shown that MTX selectively induces apoptosis of activated, but not resting lymphocytes[24]. However, they also found that short term exposure to MTX followed by subsequent activation of T cells resulted in deletion of these cells. Although this is in contrast with our observations, it was reported that NKT cells are relatively resistant to apoptosis due to the upregulation of several specific anti-apoptotic genes after activation[25]. These cells may therefore be less affected by MTX pre-incubation only, resulting in normal expansion levels, while continuous presence of MTX during activation may ultimately lead to apoptosis. Furthermore, our results showed a significant decrease in the number of IFN-γ producing NKT cells after α-GalCer stimulation in the presence of MTX. Remarkably, this number slightly increased upon addition of higher concentrations of MTX. It was demonstrated that addition of MTX resulted in a dose dependent, suppressive effect on IFN-γ production *in vitro*[26]. In addition, Hildner et al. reported that upon T cell priming in the presence of MTX,

IFN-γ production was suppressed at the whole concentration range tested (0.001 μg/ml - 10 μg/ml), while the production of IL-4 was increased at low concentrations[27]. The findings by Hildner et al. might explain our observations, since we measured the relative number of IFN-γ and IL-4 producing NKT cells by intracellular staining. In that way, the higher IL-4/IFN-γ ratio at low dose MTX may result from the increased IL-4 production and the suppressed IFN-γ production, whereas the higher IFN-γ/IL-4 ratio at higher concentrations of MTX might originate from the suppression of both IFN-γ and IL-4 production. Our data suggest that MTX therapy can affect the cytokine profile of NKT cells.

Treatment of PBMC with MP during stimulation with α-GalCer resulted in a significant decrease in T and NKT cell numbers. This glucocorticoid induced inhibition was also observed by Johansson et al. who reported that α-GalCer driven expansion of NKT cells was inhibited by prednisolone treatment[28]. In contrast, others have described that proliferation of NKT cells was augmented by addition of dexamethasone[29]. However, this group determined the effect of glucocorticoids after polyclonal stimulation rather than antigen specific stimulation and used *in vitro* derived NKT cells clones instead of *ex vivo* PBMC, which may account for the differences found. Our results show that the relative number of IFN-γ producing cells decreased upon MP treatment in the NKT cell fraction, although the number of IL-4 producing cells increased. Glucocorticoids are known to inhibit T cell proliferation and cytokine production by interfering with the binding or function of critical transcription factors. Hence, they may modify the Th1-Th2 cytokine profile of the cells[30]. This is in concordance with others who added dexamethasone during NKT cell stimulation in an effort to polarize the cytokine profile of Th1 biased NKT cells. They reported a strong decrease in IFN-γ secretion, although IL-4 secretion was also reduced[31]. This conflicts with our findings, but these investigators added dexamethasone to already established Th1 NKT cells, whereas we administered glucocorticoids from

the start of the experiment to freshly isolated PBMC. Finally, we found that the non-Vα24 T cell cytokine profile was not affected after MP treatment. It was reported that Th1 cytokine production was suppressed and Th2 cytokine production enhanced by glucocorticoids, although this was mainly found in activated T cells[32]. Since only the NKT cell antigen was present in our culture, non-NKT cells were not specifically activated and might therefore not be subjected to MP action. Our data indicate that low dose treatment with MP can inhibit NKT cell expansion but induces a Th2 cytokine profile of these cells.

We evaluated two different experimental approaches, where NKT cell activation was induced after short-term administration (pulse treatment) or during continuous administration of immunosuppressive drugs. Our data indicate that only treatment during stimulation inhibits NKT cell expansion and alters the NKT cell cytokine profile. The concentrations of the medication used in our *in vitro* study are comparable with those observed in the serum of RA patients treated with these agents[33-35]. However, our *in vitro* data should be supplemented with pre- and post treatment studies in patients to permit a detailed view of the effect of immunosuppressive drugs on NKT cell function *in vivo*.

Our data indicate that treatment with immunosuppressive drugs might play a role in the decreased NKT cell frequency reported in autoimmune diseases. However, several groups have shown that even in untreated patients, NKT cell numbers are low and α-GalCer driven expansion is reduced[28,36]. Additionally, we have shown that NKT cells of RA patients are biased towards a Th1 phenotype, even in those treated with MTX or MP[12]. Together, these observations indicate that the effect of immunosuppressive agents on NKT cells of patients with autoimmune diseases can only partially explain the observed dysfunctions which suggests that the NKT cell deficiency might be inherent to autoimmunity.

In conclusion, our data show that MTX, CsA and MP can inhibit NKT cell expansion *in vitro*. In addition to their general immunosuppressive function, low dose MTX and MP might deviate the cytokine profile of the regulatory NKT cells towards a Th2 phenotype and in this way temper the autoimmune inflammation.

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Chapter 6

Summary and discussion

Summary of the experimental data

Recent data indicate that autoimmune diseases such as rheumatoid arthritis (RA) might in part result from the loss of active self-control of autoreactive T cells. In this thesis, the involvement of a specific regulatory T cell population, Natural Killer T (NKT) cells, was examined in RA. In addition, the regulatory potential of NKT cells was assessed *in vitro*. This final chapter provides a concise overview and discussion of the main results of this work.

I. Analysis of the frequency and functional characteristics of NKT cells in RA patients and healthy controls

In chapter 3, the frequency and functional properties of NKT cells derived from peripheral blood of RA patients and healthy controls, and of NKT cells derived from synovial fluid of RA patients were determined. We demonstrated that the number of $V\alpha24^+V\beta11^+CD3^+$ NKT cells was significantly decreased in peripheral blood mononuclear cells (PBMC) of RA patients compared to healthy controls. Moreover, direct *ex vivo* analysis revealed a decreased IL-4/IFN-γ ratio in NKT cells of RA patients. We also demonstrated the presence of NKT cells in rheumatoid synovial fluid (SF) as well as in synovial tissue samples (ST) of RA patients. Together, our results indicate that NKT cells are present at the disease site in RA.

Next, the expansion of NKT cells in response to antigenic stimulation was tested. We first demonstrated that the proliferative capacity of NKT cells was generally decreased in RA patients compared with healthy controls. However, a more detailed analysis revealed that RA patients could be divided into responder and non-responder patients based on the reactivity of PBMC derived NKT cells towards α-galactosylceramide (α-GalCer). Remarkably, SF derived NKT cells of both

responders and non-responders expanded upon stimulation, indicating that the impaired NKT cell function in non-responders is restricted to the blood compartment.

Consistent with our *ex vivo* analysis, we observed a Th1 bias in NKT cell lines derived from the blood of RA patients, whereas NKT cell lines from healthy controls displayed a Th0 like cytokine profile. Interestingly, NKT cell lines obtained from SF of RA patients showed a cytokine profile similar to that of healthy controls. The Th1 bias in NKT cells of RA patients was found in the $CD4^+$ as well as the $CD4^-$ NKT cell subpopulation. CDR3 fragment length analysis of the NKT cell lines revealed a monoclonal Vα24 and polyclonal Vβ11 profile in healthy controls and RA patients. This indicates that the differences found between NKT cells of RA patients and healthy controls are not due to a skewed outgrowth of only one or a few NKT cells. Moreover, this suggests that there is no specific loss of NKT cell clonotypes in RA patients.

Our observations supplement earlier reports showing a reduced NKT cell frequency in RA[1,2], the presence of NKT cells in rheumatoid synovium[3] and the division into α-GalCer responder and non-responder patients[2]. However, we are the first to show a Th1 bias in blood NKT cells and a Th0 cytokine profile in SF NKT cells of RA patients. In addition, we also demonstrated that the abnormality of the NKT cells of non-responder patients appeared to be restricted to the blood compartment. Although it is not clear why blood and SF NKT cells behave differently, it is possible that NKT cells are locally reactivated or differentiated by the microenvironment, resulting in the different phenotypes[4].

Our findings are consistent with observations in other autoimmune diseases, such as type 1 diabetes and multiple sclerosis, where a reduced NKT cell frequency and a Th1 NKT cell bias was also found[5,6]. This indicates that NKT cell dysfunction is

not specific for RA, but might have a major role in the etiology of autoimmune diseases. It remains to be determined whether these abnormalities are cause of or result from the disease.

In conclusion, our results show that the number of $V\alpha24^+V\beta11^+CD3^+$ NKT cells is decreased and that the cytokine profile of blood derived NKT cells is biased to a Th1 phenotype in RA patients. This indicates that NKT cells might be functionally related to resistance or progression of rheumatoid arthritis and are therefore a possible target for the treatment of RA.

II. Study of NKT cell regulation *in vitro*: analysis of the regulatory capacity of NKT cells

NKT cells have been implicated in the regulation of adaptive immune responses, including those directed against auto-antigens. However, the precise mechanism by which NKT cells control other T cells is not known and the effect of α-GalCer on human Th differentiation remains unclear. In chapter 4, the regulatory effect of NKT cells on antigen reactive T cells was evaluated in various approaches (Fig 4.1).

In a first approach, the regulatory effect of NKT cell clones on the proliferative capacity and the cytokine profile of effector T cell clones was analyzed. In order to perform this analysis, we isolated a total of 6 NKT cell clones from 2 healthy controls and 1 RA patient. In agreement with our findings reported in chapter 3, $CD4^+$ NKT cell clones of the RA patient displayed a Th1 bias compared to the $CD4^+$ NKT cell clones derived from the healthy controls. We then assessed the effect of the isolated NKT cell clones on the proliferation and cytokine profile of Tetanus Toxoid (TT) reactive T cell clones in a co-culture experiment. This revealed that

addition of a $CD4^-CD8^-$ NKT cell clone of the healthy control reduced the proliferation, measured by ^3H-thymidine incorporation, and increased the number of IFN-γ producing TT reactive T cell clones. Co-culture of the $CD4^+$ NKT cell clones of the RA patient had no effect on proliferation, but also induced a slight increase in the number of IFN-γ producing cells. Hence, no major differences were observed between the cytokine profile induced by NKT cell clones of a RA patient or a healthy control. Although the $CD4^-CD8^-$ NKT clone might suppress proliferation, it is not clear whether this is related to the subtype or the donor. Therefore, to fully determine whether the regulatory effect of NKT cells differs between RA patients and healthy controls, one should compare regulation by NKT cells of the same subtype. In addition, we have observed only moderate effects, which could indicate that effector T cells are not (fully) susceptible to NKT cell mediated regulation and that other *in vitro* methods are necessary to analyze NKT cell regulation.

The comparable effects observed in the co-culture experiment might originate from the similar cytokine profile of the different NKT cells, even though they belong to other subtypes ($CD4^-CD8^-$ versus $CD4^+$). However, addition of NKT cell supernatants showed no influence on the cytokine profile, but resulted in a strong inhibition of the proliferation of stimulated PBMC. This indicates that not only soluble factors, but also additional mechanisms, such as cell-cell contact or activation of other immune cells, might be involved in NKT cell regulation.

The effect of α-GalCer induced NKT cell stimulation on antigen specific T cell activation was assessed in a second approach. Administration of α-GalCer at the time of stimulation with TT did not significantly affect the cytokine profile of TT reactive T cell lines (TCL), although the number of IL-4 producing TT reactive TCL appeared to increase. Additionally, the effect of NKT cell stimulation with α-GalCer on Toxic-Shock-Syndrome-Toxin (TSST) specific T cells was determined. We

found that co-stimulation did not result in significant changes of the cytokine profile, whereas α-GalCer treatment prior to antigen stimulation tended to induce a slight Th2 profile. However, the observed differences were not significant and were different for the 2 antigens used (TT and TSST). Our findings are in contrast with those observed in mice, where it was found that activation of NKT cells by α-GalCer could shift conventional T cells to a Th2 phenotype[7]. However, the efficacy of α-GalCer appeared to depend on the mouse strain used, the administration route, timing, dose and number of injections[8,9]. It is therefore possible that our *in vitro* system cannot fully mimic the mechanisms necessary for a beneficial effect of α-GalCer stimulation.

Thus, our data indicate that NKT cells may affect the cytokine profile and the proliferation capacity of antigen specific T cells. However, approaches using α-GalCer stimulation to regulate T cells did not show any significant effect. Although NKT cells might be a promising tool to control (auto-)immune responses, more insights in the biological mechanisms involved in NKT cell regulation are essential to successfully use NKT cells for therapeutic purposes.

III. Effect of immunomodulatory treatments on NKT cell function and expansion *in vitro*

The objective of the study described in chapter 5 was to determine the effect of cyclosporin A (CsA), methotrexate (MTX) and methylprednisolone (MP) on NKT cells. These products are frequently used for the treatment of RA. To this end, PBMC were incubated *in vitro* with immunosuppressive drugs and subsequently stimulated with α-GalCer. Our results show that CsA, MTX and MP dose-dependently inhibit NKT cell expansion *in vitro*. This is consistent with the earlier finding that glucocorticoid treatment inhibited NKT cell expansion[10].

Furthermore, intracellular cytokine staining revealed that MTX and MP, when present during NKT cell activation, can deviate the NKT cell cytokine profile to a Th2 phenotype. The effect is more pronounced at higher doses of immunosuppressive drugs used. This finding is in agreement with other studies which revealed that MTX and MP can suppress Th1 (IFN-γ) cytokine production and enhance Th2 (IL-4) cytokine production[11,12].

In summary, all three immunosuppressive drugs tested can inhibit NKT cell expansion. MTX and MP affect the NKT cell cytokine profile and might have a supplemental anti-inflammatory effect by the deviation of the cytokine profile of NKT cells towards a Th2 phenotype. However, our *in vitro* data should be supplemented with pre- and post- treatment studies in patients to permit a detailed view of the effect of immunosuppressive drugs on NKT cell function *in vivo*.

Concluding remarks

The work in this thesis aimed at providing more insight into the role of NKT cells in the etiology of RA. Our data show that NKT cells possess altered properties in RA patients and may therefore contribute to RA pathogenesis. Similar observations were made in a growing range of autoimmune diseases[2,3,6,13] and it therefore seems that NKT cell impairment is a general feature of autoimmune conditions rather than a RA specific trait[14,15].

Several non-mutually exclusive possibilities, which were not addressed in this work, could account for the NKT cell dysfunction. Many molecules involved in NKT cell biology have recently been identified, thereby providing information about the potential underlying mechanism of the NKT cell reduction. Defects in antigen presentation or processing pathways implicated in NKT cell differentiation and activation resulted in decreased NKT cell numbers and altered functional properties in mice[16-18]. Defective expression of candidate antigens, such as isoglobotrihexosylceramide, or their precursors might also contribute to the dysfunction observed in mice[19,20]. In addition, malfunction of the signaling pathways of the NKT cell itself has also been shown to affect NKT cell function and numbers[21-23]. In addition to molecules involved in antigen presentation, other molecules are important for the thymic development of NKT cells[24-28]. Of interest is membrane-bound lymphotoxin (LT). This molecule is indispensable for the differentiation of NKT cells and the LT β receptor had to be present on thymic stromal cells for successful NKT cell maturation[29]. Furthermore, absence of the receptor in mice leads to an impaired lympho-epithelial cross-talk in the thymus, the retention of mature T lymphocytes and is associated with autoimmune phenomena such as perivascular T cell infiltrates and autoantibodies[30]. An impaired thymic function has been suggested in RA, since the number of recent thymic immigrants is decreased in RA (Thewissen et al., unpublished results). An impaired thymic function might also underlie the decreased NKT cell

number/function observed in RA. Hence, LTβ receptor provides an interesting possible link between the observed decreased NKT cell number/function as well as the reduced thymic output of T cells in RA. Animals lacking molecules contributing to above mentioned mechanisms often show a reduced NKT cell number or altered functional NKT cell properties. However, some of these molecules are not involved in human NKT cell function or do not correlate with the autoimmune diseases. It is therefore obvious that further study of these molecules and their contribution to the development of autoimmunity is necessary. Further elucidation of NKT cell biology might also reveal novel pathways which may result in potential treatments aimed at restoring the NKT cell function and numbers.

As long as the origin of the NKT cell dysfunction remains unclear, a promising tool to enhance NKT cell numbers and function is the glycolipid α-GalCer, which has proven its potential in animal models of autoimmune diseases[31]. In this thesis, *in vitro* approaches were used to analyze the application of α-GalCer for human autoimmune diseases. Our data provide some indications that NKT cells can have a direct effect on antigen specific T cells. However, NKT cells can also control T cell activities via indirect ways, such as recruitment of regulatory dendritic cells or induction of regulatory T cells[32,33]. These indirect effects are difficult to simulate by *in vitro* methods. Hence, a better understanding of the *in vivo* nature of NKT cell regulation should supplement the data generated by *in vitro* research. This information is required for the development of therapeutic strategies targeting NKT cells.

Clinical application of α-GalCer should be employed with caution since there are several concerns that may limit translation of the preclinical studies. It is still not clear how α-GalCer-mediated NKT cell stimulation can suppress Th1 mediated autoimmune diseases and also prevent tumor metastases and infections[34].

Moreover, in many of the studies of autoimmune diseases in mice, timing and dosage of α-GalCer had a significant impact on the disease outcome. For instance, co-administration of α-GalCer and myelin antigens exacerbated the disease, coinciding with an increase of IFN-γ production by autoreactive T cells. Pre-incubation with α-GalCer however, was accompanied by an increased production of Th2 cytokines and resulted in disease amelioration[8]. This illustrates the potential risk of augmenting unwanted Th1 responses that can worsen the disease when administering α-GalCer. Additionally, although it is generally accepted that NKT cells might play a protective role in autoimmunity, some studies show that they may also contribute to autoimmune pathogenesis. In some animal models of autoimmune hepatitis, systemic lupus erythematosis and recently also in autoimmune arthritis, it was shown that NKT cells in joint tissue may play an essential role in the end-stage effector phase of RA[35]. Stimulation of these pathogenic NKT cells would again lead to exacerbation of the disease. Furthermore, α-GalCer also exacerbates allergic airway inflammation in mice, which raises the possibility that long-term treatment may promote allergic reactions. Finally, high dose administration of α-GalCer in mice has been reported to result in fatal liver damage[36]. However, it is likely that the adverse events in humans will be less pronounced since the number of NKT cells in humans is lower compared to mice. These findings clearly emphasize the need for detailed studies on the mechanisms involved in NKT cell mediated promotion of Th1 and Th2 responses and on the effect of α-GalCer in humans.

It should be noted that experimental data on the use of α-GalCer in humans are limited and are currently focused on cancer therapy[37,38]. In addition, NKT cell activation of patients with autoimmune diseases using α-GalCer might not be beneficial, since stimulation of the Th1 biased NKT cells in these patients could promote unwanted Th1 responses and thus aggravate disease. Moreover, it must be noted that augmenting the number of NKT cells using α-GalCer may solve the

decreased frequency, but provides no certainty that this will also improve the disease. Possibly, some of the potential problems associated with α-GalCer based therapy can be overcome by the use of analogues with superior activity in inducing Th2 responses (OCH) or by combination therapies with cytokines (IL-7) or antibodies (anti-CD86 antibodies) that suppress Th1 and/or enhance Th2 cytokine production by NKT cells[39,40].

Autoimmune diseases result from the breakdown of self-tolerance that protects healthy individuals from the potential harmful effects of autoreactive cells[41]. Autoreactive T cells are commonly found in normal control subjects, but rarely cause disease[42]. Hence, the mere presence of autoreactive T cells is not sufficient to induce autoimmune diseases. Consistent with this, these cells are not only present, but also display altered functional properties in patients with autoimmune conditions[43,44]. In addition, age-dependent changes in T cell homeostasis, which are also referred to as premature immunosenescence, are accelerated in patients with RA and MS[45,46]. Another general finding in a broad range of autoimmune diseases is the reduced frequency or dysfunction of regulatory T cells. These observations suggests that autoimmune diseases have a common basis, which might originate from autoreactive T cells that have gained novel autoaggressive properties and have escaped active self-control. Hence, a plausible hypothesis of the initiation of autoimmune diseases can be made. Loss of active self-control by NKT cells and other regulatory cells, may promote autoreactive T cells, that are shaped by genetic predisposition, environmental factors and premature immunosenescence, to become autoaggressive T cells. These pathogenic cells could then subsequently initiate the autoimmune disease process, which might result in different diseases such as type 1 diabetes, multiple sclerosis or rheumatoid arthritis. This would suggest that silencing of autoaggressive T cells as well as the augmentation of the regulatory response is required to adequately treat patients with autoimmune diseases.

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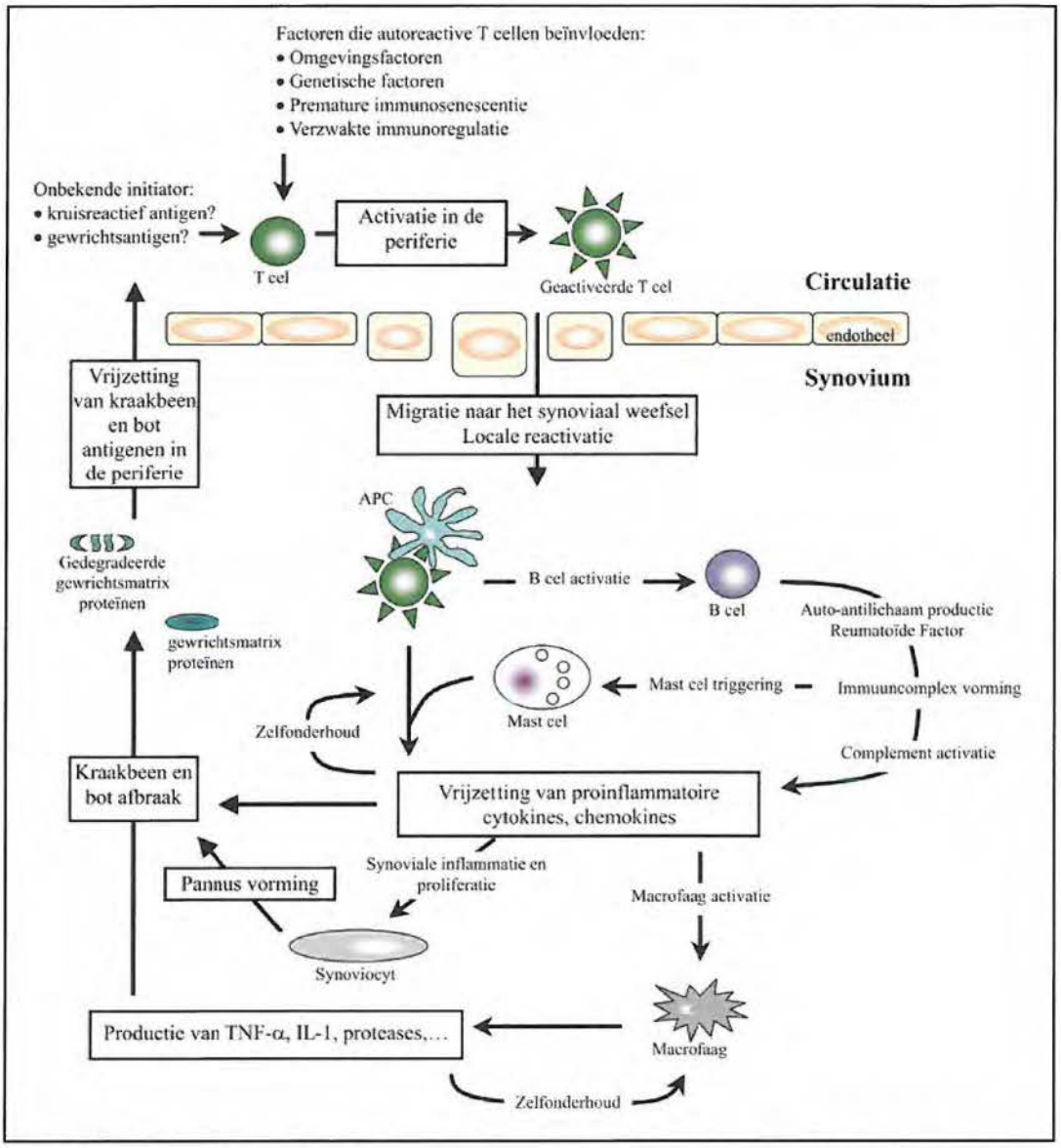
Nederlandse samenvatting

Samenvatting van de experimentele data

Reumatoïde artritis (RA) is een chronische ontsteking van de perifere gewrichten. RA treft 1% van de bevolking, en resulteert in onomkeerbare kraakbeen- en bot beschadigingen. In het ontstoken gewricht treft men een verhoogde infiltratie van mononucleaire cellen aan. Dit synoviale infiltraat bestaat vooral uit T-cellen, macrofagen en plasmacellen. Tot op heden is de precieze oorzaak van RA nog steeds niet gekend, maar er wordt meer en meer aangenomen dat autoreactieve T-cellen een belangrijke rol spelen in de initiatie van het ziekteproces. In de huidige hypothese wordt verondersteld dat T-cellen geactiveerd worden in de bloedbaan en hierdoor makkelijker door de synoviale membraan kunnen migreren (figuur 7.1). Hier worden ze vermoedelijk door lokale (auto)antigenen gereactiveerd waardoor ze pro-inflammatoire cytokines produceren. Deze cytokines zorgen voor een verhoogde proliferatie van de synoviale membraan die vervolgens ingroeit in de onderliggende kraakbeen- en botstructuren waardoor deze onherstelbaar worden beschadigd. Ten gevolge van het pro-inflammatoire milieu worden ook andere celtypes naar het synovium gelokt. Deze cellen infiltreren en accumuleren dan in het gewricht, wat resulteert in de aangehouden immuunreactie. Dit leidt dan tot de typische symptomen van RA, zoals stramheid, zwelling, vervorming en bewegingsbeperking van het gewricht.

Recente gegevens tonen echter aan dat enkel de aanwezigheid van autoreactieve T cellen niet voldoende is om autoimmune aandoeningen zoals reumatoïde artritis te veroorzaken. Het ontstaan van de ziekte kan gedeeltelijk voortvloeien uit het verlies van de actieve controle op deze autoreactive T cellen. Deze actieve controle wordt uitgevoerd door regulerende T celpopulaties, waartoe ook 'Natural Killer T' (NKT) cellen behoren. NKT cellen brengen een semi-invariante T cel receptor evenals NK cel kenmerken tot expressie en produceren grote hoeveelheden van de cytokines IFN-γ en IL-4 na activatie. Deze cellen komen verminderd voor en zijn verzwakt bij patiënten met autoimmune aandoeningen.



Figuur 7.1. Huidige hypothese van de pathogenese van RA. *$CD4^+$ T cellen worden geactiveerd in de periferie door een ongekend antigen en migreren naar het synoviaal weefsel. Hier worden ze gereactiveerd en starten ze de (auto)immuun reactie. De productie van cytokines en chemokines alsook celcontact-afhankelijke interacties leiden tot de infiltratie en activatie van andere cellen (B-cellen, macrofagen, mast cellen,...). Deze geactiveerde cellen produceren vervolgens cytokines en andere mediatoren die bijdragen tot de zelfonderhoudende processen en tot schade aan het gewricht leiden. Dit resulteert dan in de vrijzetting van kraakbeen- en botantigenen in de periferie, waardoor de ontstekingsreactie versterkt wordt.*

Stimulatie van NKT cellen in proefdiermodellen van autoimmuun ziekten resulteert in een verbetering van de symptomen tot zelfs genezing van de aandoening. Hierdoor wordt stimulatie van deze cellen aanzien als mogelijke kandidaat therapie voor de behandeling van autoimmune aandoeningen. In deze thesis werd de rol van NKT cellen in RA verder bestudeerd.

I. Analyse van de frequentie en de functionele karakteristieken van NKT cellen in RA patiënten en gezonde controles

In *hoofdstuk 3* werden de frequentie en de functionele eigenschappen van NKT cellen onderzocht. Deze cellen werden bekomen uit het bloed van gezonde controles en RA patiënten evenals uit het synoviaal vocht van RA patiënten. Aan de hand van flow cytometrie konden we aantonen dat de frequentie van $V\alpha24^{+}V\beta11^{+}CD3^{+}$ NKT cellen significant lager is in de perifere bloed mononucleare cellen (PBMC) van RA patiënten dan in die van gezonde controles. Door gebruik te maken van de ELISPOT methode kon eveneens worden gedemonstreerd dat NKT cellen van RA patiënten een verlaagde IL-4/IFN-γ ratio vertonen. NKT cellen konden ook worden waargenomen in het synoviaal vocht en het synoviaal weefsel van RA patiënten. Analyse van het T cel receptor profiel van de $V\alpha24$ familie toonde echter aan dat het profiel voor elke patiënt verschillend is. Dit suggereert dat NKT cellen niet selectief aangerijkt zijn in het gewricht. Verder werd ook de NKT cel expansie gemeten na stimulatie met het NKT cel antigen α-Galactosylceramide (α-GalCer). Op basis van de reactiviteit van NKT cellen uit het bloed konden RA patiënten worden ingedeeld in reactieve en niet-reactieve patiënten. Vreemd genoeg reageerden NKT cellen uit het synoviaal vocht van zowel reactieve als niet-reactieve patiënten wel op antigen stimulatie. Dit wijst erop dat de verzwakte NKT cel functie in niet-reactieve patiënten beperkt is tot het bloed. Meer nog, het cytokine profiel van NKT cel lijnen verkregen uit het

synoviaal vocht van RA patiënten was gelijkaardig aan dat van gezonde controles (Th0 fenotype), terwijl het cytokine profiel van NKT cellen gegenereerd uit het bloed van RA patiënten een duidelijke Th1 bias vertoonde. Uit verdere analyse bleek dat deze Th1 bias veroorzaakt werd door een verminderd aantal IL-4 producerende cellen in zowel $CD4^+$ als $CD4^-$ NKT cel lijnen. Analyse van de T cel receptor Vα24 en Vβ11 regio's van deze lijnen toonde een monoclonaal Vα24 en een polyclonaal Vβ11 profiel. Zo kon worden uitgesloten dat deze bias een gevolg was van een selectieve uitgroei van één of enkele NKT cellen. Verder kon uit deze analyse ook worden afgeleid dat RA patiënten geen specifieke NKT klonen missen in hun repertoire. Samengevat kan worden gesteld dat NKT cellen in aantal verminderd zijn en een veranderd cytokine profiel vertonen bij RA patiënten. Dit wijst erop dat NKT cellen mogelijk betrokken zijn bij het ontstaan van RA. NKT cellen vormen daarom een interessante target voor een kandidaat therapie.

II. Studie van NKT cel regulatie *in vitro*: analyse van de regulerende capaciteit van NKT cellen

In *hoofdstuk 4* werd op verschillende manieren het regulerend effect van NKT cellen op antigen reactieve T cellen nagegaan. Ten eerste werd het effect van NKT cel klonen op het cytokine profiel en de proliferatie van effector T cel klonen bestudeerd. Om deze analyse te kunnen uitvoeren werden in totaal 6 NKT cel klonen geïsoleerd van 2 gezonde controles en 1 RA patiënt. In overeenstemming met onze data uit hoofdstuk 3, vertoonden de $CD4^+$ NKT cel klonen van de RA patiënt een Th1 fenotype vergeleken met de $CD4^+$ klonen van de gezonde controles. Vervolgens werd in een co-cultuur experiment het effect van de geïsoleerde klonen op de proliferatie en het cytokine profiel van tetanus toxoid (TT) reactieve klonen nagegaan. Hieruit bleek dat de geteste $CD4^-CD8^-$ NKT cel kloon van de gezonde controle de algemene proliferatie in de cultuur kon reduceren en het aantal IFN-γ producerende TT reactieve cellen verhoogde. Co-

cultuur van de NKT cel klonen en de TT reactieve T cel kloon van de RA patiënt had geen effect op de proliferatie, maar induceerde een kleine verhoging van het aantal IFN-γ en IL-4 producerende cellen. Dit gelijkaardig effect kan mogelijk veroorzaakt worden door het vergelijkbaar cytokine profiel van de NKT cellen, ook al behoren ze tot verschillende NKT subpopulaties. Nochtans resulteerde toevoeging van NKT cel supernatans niet in een verandering van het cytokine profiel, maar wel in een sterke inhibitie van de proliferatie van gestimuleerde PBMC. Deze bevinding suggereert dat additionele mechanismen, zoals celcontact, van belang zijn bij regulatie door NKT cellen.

In een tweede benadering werd ook het *in vitro* effect van α-GalCer geïnduceerde NKT cel stimulatie op T cel priming nagegaan. Stimulatie met TT in de aanwezigheid van α-GalCer had geen significant effect op het cytokine profiel van de TT reactieve T cel lijnen. Ook het effect van een voorafgaande of gelijktijdige stimulatie met α-GalCer op toxic-shock-syndrome-toxine specifieke T cellen werd geanalyseerd. Het cytokine profiel veranderde niet bij co-stimulatie, terwijl een behandeling met α-GalCer voorafgaand aan de antigen stimulatie een kleine verschuiving naar een Th2 profiel veroorzaakte. De observaties waren echter niet significant en verschilden voor de 2 geteste antigenen. De exacte werking van NKT cel regulatie is nog niet gekend, dus mogelijk bevat ons *in vitro* systeem niet alle componenten noodzakelijk voor een bevorderlijk effect. Onze data geven aan dat NKT cellen en hun supernatantia het cytokine profiel en de proliferatie van antigen reactieve cellen kunnen beïnvloeden. Andere benaderingen, zoals NKT cel stimulatie met α-GalCer, toonden geen effect aan. Onze bevindingen duiden aan dat NKT cellen mogelijk aangewend kunnen worden voor het controleren van (auto-)immune aandoeningen. Er is echter meer inzicht nodig in de mechanismen betrokken bij NKT cel regulatie om deze cellen succesvol te gebruiken voor therapeutische doeleinden.

III. Het effect immunomodulerende agentia op de NKT cel functie en expansie *in vitro*

Het doel van de studie beschreven in *hoofdstuk 5* was het bepalen van het effect van frequent gebruikte medicatie voor de behandeling van RA, zoals cyclosporine A (CsA), metotrexaat (MTX) en methylprednisolone (MP), op de NKT cel functie en expansie. Hiertoe werden PBMC *in vitro* geïncubeerd met de immunosuppressieve agentia en vervolgens met α-GalCer gestimuleerd. Onze resultaten tonen aan dat CsA, MTX en MP de NKT cel expansie dosis-afhankelijk inhiberen als ze aanwezig zijn tijdens, maar niet voorafgaand aan, de stimulatie met α-GalCer. Er kon ook worden gedemonstreerd dat MTX en MP, wanneer ze aanwezig zijn tijdens de stimulatie, het cytokine profiel van de NKT cellen kunnen sturen naar een Th2 profiel. Dit effect was meer uitgesproken bij hogere doses van de immunosuppressiva. Samengevat geven onze resultaten weer dat de 3 geteste agentia de NKT cel expansie kunnen voorkomen, maar dat enkel MTX en MP het cytokine profiel kunnen beïnvloeden. Deze laatste 2 moleculen kunnen dus naast hun algemeen anti-inflammatoir effect nog een additioneel resultaat hebben door NKT cellen naar een Th2 profiel te sturen.

Eindconclusie

Het werk in dit proefschrift trachtte een beter inzicht te bekomen in de betrokkenheid van NKT cellen in het ontstaan van RA. Onze data tonen aan dat NKT cellen andere eigenschappen vertonen bij RA patiënten en kunnen dus bijdragen tot de pathogenese van RA. Gelijkaardige observaties zijn ook gemaakt in andere autoimmune aandoeningen. Dit wijst erop dat een verzwakte NKT cel populatie vermoedelijk een meer algemeen kenmerk is van autoimmuun ziekten, en niet specifiek is voor RA.

De oorzaak van de NKT cel dysfunctie in RA blijft nog onopgehelderd. Diverse mogelijkheden worden gesuggereerd. Deze zijn onder andere: een onvoldoende of afwijkende antigen presentatie, een verlaagde reactiviteit tegenover het natuurlijk NKT cel ligand, defecten in NKT cel ontwikkeling en een verstoorde interne NKT cel signalisatie. Verscheidene moleculen betrokken in bovenstaande processen zijn recent geïdentificeerd. De NKT cellen van proefdieren die deze moleculen missen, of niet tot expressie brengen, zijn vaak verminderd in aantal en vertonen ook veranderde functionele eigenschappen. Sommige eiwitten zijn echter niet betrokken bij de humane NKT cel functie of vertonen geen correlatie met autoimmune aandoeningen. Het is daarom duidelijk dat een verdere analyse van de NKT cel biologie nodig is om de onderliggende oorzaak van de veranderde NKT cel functie in autoimmune aandoeningen bloot te leggen. Dit zou dan kunnen leiden tot een mogelijke behandeling van autoimmuun ziekten gericht op het herstellen van het NKT cel aantal en de NKT cel functie.

Zolang de oorsprong van de NKT cel dysfunctie onbekend blijft, is het glycolipide α-GalCer een veelbelovend middel om de NKT cel functie te versterken. Het therapeutisch potentieel van deze molecule is reeds veelvuldig bewezen in diermodellen van autoimmune aandoeningen. In dit proefwerk werden *in vitro* benaderingen aangewend om de toepassing van α-GalCer bij humane autoimmuun

ziekten te evalueren. Onze waargenomen indicaties waren echter niet eenduidig. Er kon wel worden aangetoond dat NKT cellen een direct effect op antigen specifieke T cellen kunnen uitoefenen. Er zijn evenwel ook andere mechanismen waarbij NKT cellen andere T cellen kunnen controleren, zoals bijvoorbeeld het induceren van regulerende dendritische cellen. *In vitro* onderzoek kan daarom misschien niet het volledige potentieel van NKT cellen verduidelijken, maar moet aangevuld worden met een beter begrip van de NKT cel functie *in vivo*. Enkel in dat geval kunnen NKT cellen veilig gebruikt worden in klinische toepassingen.

Het toedienen van α-GalCer bij mensen moet echter met grote omzichtigheid worden benaderd, aangezien hoge doses α-GalCer bij muizen resulteerde in fatale leverschade. Daarenboven is het nog steeds niet exact duidelijk hoe één molecule zeer uiteenlopende ziekten, zoals kanker en autoimmuniteit, kan reguleren. Veel studies van autoimmune diermodellen wezen uit dat de timing en de dosering van α-GalCer de uitkomst van de ziekte beïnvloedt. Co-administratie van α-GalCer en myeline-antigenen bijvoorbeeld, resulteerde in verzwakking van de dieren, terwijl pre-incubatie een verbetering van de ziekte teweegbracht. Deze bevinding illustreert het mogelijke risico waarbij ongewenste Th1 reacties worden gestimuleerd die een verergering van de ziekte tot gevolg hebben. Deze observaties tonen ook de nood aan voor diepgaande studies over het effect van α-GalCer in humane toepassingen, evenals de mechanismen betrokken bij NKT cel gemedieerde stimulatie van Th1 en Th2 reacties. De huidige experimentele gegevens over het gebruik van α-GalCer bij mensen zijn gelimiteerd en beperkt tot kanker therapie. Verder is het ook mogelijk dat NKT cel activatie bij mensen met autoimmune aandoeningen schadelijk kan zijn aangezien de aanwezige NKT cellen een Th1 bias vertonen. Aanrijking van de NKT cellen kan het frequentieverschil wegwerken, maar geeft geen garantie dat dit leidt tot een verbetering van de ziekte. Sommige van de mogelijke problemen, geassocieerd met α-GalCer gebaseerde therapieën, kunnen worden vermeden door het gebruik

van analogen van α-GalCer met een superieure Th2 inducerende activiteit zoals OCH, of door het gebruik van combinatie therapieën van α-GalCer met cytokines of antilichamen die de productie van Th1 cytokines lamleggen en die van Th2 cytokines bevorderen.

Autoimmune aandoeningen zijn een gevolg van het verlies van de zelftolerantie die beschermd tegen de mogelijke schadelijke effecten van autoreactieve T cellen. Deze laatste worden daarentegen ook algemeen waargenomen bij gezonde individuen. Bij patiënten die lijden aan een autoimmune aandoening vertonen de autoreactieve cellen echter vaak veranderde functionele eigenschappen. Ook de leeftijdsafhankelijke achteruitgang van het immuunsysteem, ook wel premature immuunveroudering genoemd, wordt versneld waargenomen bij patiënten met RA of multiple sclerose. Daarnaast zijn ook verschillende regulerende T cel populaties aangetast bij autoimmune aandoeningen. Deze gegevens wijzen erop dat autoimmuun ziekten het gevolg kunnen zijn van het verlies van zelfcontrole gecombineerd met autoreactive T cellen die nieuwe autoagressieve eigenschappen hebben verkregen. Hieruit kan dus een logische hypothese worden opgebouwd. Het verlies van actieve zelfcontrole door NKT cellen en andere regulatorische T cel populaties induceert de transformatie van autoreactieve T cellen in autoaggressieve T cellen. De autoreactieve T cellen kunnen gemakkelijker transformeren onder invloed van genetische en omgevingsfactoren en door premature immuunveroudering. De autoagressieve cellen zullen vervolgens het autoimmuun proces initiëren, wat resulteert in het ontstaan van autoimmuun ziektes zoals type 1 diabetes, multiple sclerose en reumatoïde artritis. Deze hypothese suggereert dat zowel de autoagressieve T cellen moeten worden lamgelegd als de regulerende respons moet worden versterkt om patiënten met autoimmune aandoeningen doeltreffend te behandelen.

Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 3 augustus 1978 te Neerpelt. In 1996 behaalde ze het diploma Algemeen Secundair Onderwijs (ASO) Wiskunde-Wetenschappen aan het Don Bosco College te Hechtel. Dat jaar begon zij aan de richting Biologie aan de faculteit Wetenschappen van het Limburgs Universitair Centrum. In 1998 behaalde ze het diploma 'Kandidaat in de Biologie' aan het LUC. De licentie Biotechnologie aan de faculteit Wetenschappen van de Rijksuniversiteit Gent werd in datzelfde jaar aangevat. Haar eindwerk, getiteld 'Ontwikkeling van tumorspecifieke antilichamen voor immunotherapie: evaluatie van diabodies als bouwstenen voor recombinante en multispecifieke antistoffen', voerde ze uit aan het Departement voor Moleculair Biomedisch Onderzoek. In juli 2000 studeerde zij af als 'Licentiaat in de Biotechnologie'. Sinds 1 augustus van dat jaar is ze verbonden aan het Biomedisch Onderzoeksinstituut van het Limburgs Universitair Centrum en de Transnationale Universiteit Limburg. Ter voorbereiding van dit doctoraatsproefschrift verrichtte ze gedurende vier jaar onderzoek in het kader van reumatoïde artritis op de afdeling Immunologie. Tijdens deze periode volgde ze een doctoraatsopleiding aan de Katholieke Universiteit Leuven die begin 2005 succesvol werd beëindigd.

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You can discover more about a person in an hour of play,
than in a year of conversation
Plato