CD1d restricted NKT cells in regulation of pathogenic autoimmunity

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Abstract

Human autoimmune diseases such as reumatoid arthritis (RA) and multiple sclerosis (MS) are believed to develop when self-tolerance mechanisms are failing, with a chronic destructive inflammation as result. To experimentally study autoimmunity, we have used murine models that mimic their human counterparts; experimental autoimmune encephalomyelitis (EAE) – a model for MS, and collagen induced arthritis (CIA) and antigen induced arthritis (AIA) – models for RA.

A sub-population of T cells, termed natural killer T cells (NKT), have been suggested as having immunoregulatory features in pathogenic autoimmunity. How NKT cells are activated to perform this regulation and by which functional pathways they act is not completely understood. A number of natural antigens activating NKT cells via the antigen presenting molecule CD1d have so far been identified as glycolipids originating from either bacteria or self.

This thesis investigates the role of NKT cells in autoimmune tissue-specific inflammation. We found that NKT cells can down-regulate both the central nervous system-specific autoimmunity in EAE, and the joint-specific inflammation in experimentally induced arthritis. We also show data indicating that this regulation requires activation in the periphery, and we have identified a natural self-peptide capable of exerting this activation; A CD1d restricted peptide derived from autologous collagen type II. By vaccinating mice with this peptide we were able to ameliorate the inflammation and reduce the disease phenotype in both CIA and EAE. We also found the peptide-specific NKT cells capable of suppressing activated T cells in vitro. Taken together, this thesis show that CD1d restricted NKT cells can be activated to down-regulate pathogenic autoimmunity, and should be investigated as targets for future autoimmune therapies.

Key words: Autoimmunity, NKT cells, CD1d antigen presentation, Multiple sclerosis, Rheumatoid arthritis

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Contents

Original papers 7

Abbreviations 8

Summary of original papers 10

Paper I; CD1-dependent regulation of chronic central nervous system inflammation in experimental autoimmune encephalomyelitis 10

Background 10

Method 10

Results 11

Conclusion 11

Paper II; CD1d-dependent NK T cells play an important protective role in acute and chronic arthritis 12

Background 12

Method 12

Results 12

Conclusion 13

Paper III; Endogenous collagen peptide activation of CD1d-restricted NKT cells ameliorates tissue-specific inflammation in mice 14

Background 14

Method 14

Results 15

Conclusion 16

General introduction 17

Tolerance and autoimmunity 18

Mechanisms for keeping self-tolerance 18

Autoimmune diseases 19

Rheumatoid arthritis, RA 19

Multiple sclerosis, MS 20

Aim of this thesis 23

Animal models for autoimmunity 24

Experimental autoimmune encephalomyelitis, EAE 24

Experimentally induced arthritis, CIA and AIA 27

Collagen induced arthritis, CIA 27

Antigen induced arthritis, AIA 28

CD1d antigen presentation and NKT cells 29

CD1d antigen presentation and NKT cell activating ligands 29
CD1d restricted NKT cells and immune regulation 31
NKT cells in the regulation of EAE 34
Induced NKT cell activity to regulate EAE 34
Inherited capacity of NKT cells to regulate EAE 35
NKT cells in the regulation of experimental arthritis 37
Conclusions and reflections 39
Populärvetenskaplig sammanfattning 42
References 44
Acknowledgments 59

Original papers

This thesis is based on the following papers, which in the text are referred to by their roman numbers (I-III):

I. Anna Teige*, Ingrid Teige*, Sharam Lavasani, Robert Bockermann, Emma Mondoc, Rikard Holmdahl, & Shohreh Issazadeh-Navikas. **CD1-Dependent Regulation of Chronic Central Nervous System Inflammation in Experimental Autoimmune Encephalomyelitis**. Journal of Immunology 2004, 172(1):186-94.

II. Anna Teige, Robert Bockermann, Maruf Hasan, Katarina Olofsson, Yawei Liu, & Shohreh Issazadeh-Navikas. **CD1d-Dependent NK T Cells Play an Important Protective Role in Acute and Chronic Arthritis.** Journal of Immunology 2010, 185:345-356

III. Yawei Liu*, Anna Teige*, Emma Mondoc, Saleh Ibrahim, Rikard Holmdahl, & Shohreh Issazadeh-Navikas. **Endogenous collagen peptide activation of CD1d-restricted NKT cells ameliorates tissue-specific inflammation in mice.** Journal of Clinical Investigations 2011 Jan;121(1):249-64.

*) These authors contributed equally to this work

Abbreviations

APC	Antigen presenting cell
AIA	Antigen induced arthritis
B10	C57Bl10.Q
B6	C57Bl6
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CIA	Collage induced arthritis
CNS	Central nervous system
DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
i.p	Intra-peritoneal
IFN	Interferon
IL	Interleukin
M.t.	*Mycobacterium tuberculosis*
mBSA	Methylated bovine serum albumin
MBP	Myelin basic protein
$mCII_{707-721}$	Mouse collagen type II peptide 707-721
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NK cell	Natural killer cell
NKT cell	Natural killer like T cell
NOD mice	Non-obese diabetic mice
OVA	Ovalbumin
PLP	Proteolipoprotein
PT	Pertussis toxin
RA	Rheumatoid arthritis
s.c	Subcutaneous

T1D	Type 1 diabetes
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TNF	Tumour necrosis factor
α-GalCer	α-galactosyl ceramide

Summary of original papers

This chapter provides a short summary of the papers included in this thesis. The results are further discussed in the context with other findings and studies in sections *CD1d antigen presentation and NKT cells* and *Conclusions and reflections*.

Paper I;

CD1-dependent regulation of chronic central nervous system inflammation in experimental autoimmune encephalomyelitis

Background

CD1d restricted NKT cells had been implicated in regulating pathogenic autoimmunity, mostly investigated by activating NKT cell populations with αGalCer. In paper I we investigated whether CD1d restricted NKT cells had intrinsic regulatory properties in EAE, and by what means this regulation was carried out.

Method

We immunized wild type and CD1d deficient mice on a B6 background with MOG_{35-55} in CFA to induce EAE, and scored for clinical signs. At different time points during the disease development, we sacrificed animals from both groups to do histopathological examinations of the CNS investigating the degree of immune cell infiltration as well as demyelination. The peripheral immune system was also investigated, by measuring MOG_{35-55} specific T cell response in the spleen, assessing T cell proliferation and cytokine production after *in vitro* re-challenge with antigen. In addition, an encephalitogenic MOG_{35-55} specific T cell line was established, and used to induce EAE in CD1d deficient and wild type mice.

Results

CD1d deficient mice developed a more severe EAE compared to wild type mice when immunized with MOG_{35-55}. This was correlated with higher degree of immune cell infiltration and demyelination in the CNS. A pronounced TGF-β production in the CNS of wild type mice could be seen, which was virtually absent in the CD1d deficient mice. In addition, MOG_{35-55} specific T cells from CD1d deficient mice had an aberrant level of cytokine production when re-challenged with antigen *in vitro*, which was not correlated with elevated proliferative capacity. When EAE was induced by passive T cell transfer, no difference in clinical phenotype between CD1d deficient and wild type mice was observed. By immunizing the mice with CFA prior to passive EAE T cell transfer, the CD1d dependent regulations observed during the active EAE immunization protocol was restored, as wild type mice developed milder EAE compared to CD1d deficient mice.

Conclusion

CD1d restricted NKT cells regulated clinical manifestations of MOG_{35-55} induced EAE, and dampened the immune cell infiltration and demyelination of the CNS. This is possibly mediated through induction of TGF-β at the inflammatory lesions in the CNS. CD1d restricted NKT cells also rendered MOG_{35-55} specific T cells less capable of cytokine production in response to antigen activation, but did not influence their capacity to proliferate. CD1d restricted NKT cells needed activation in the periphery to be able to suppress encephalitogenic T cells, either in the periphery, or centrally. This could be achieved by immunization with CFA, but the specific mechanism by which this occurred is not clear.

Paper II;

CD1d-dependent NK T cells play an important protective role in acute and chronic arthritis

Background

CD1 depended NKT cells had been described as regulators of many autoimmune conditions, but their role in experimental arthritis was unclear – reports of NKT cells acting as both regulators and effector cells had been published. Hence it was of importance to clarify this matter by controlling the genetic backgrounds' influence of the congenic knock-out mice strains used in the studies, and investigate different models of experimental arthritis to further investigate the role for intrinsic CD1d dependent NKT cell regulation.

Method

By utilizing two different models for experimental arthritis, collagen induced arthritis (CIA) and antigen induced arthritis (AIA), we investigated CD1 dependent NKT cells role in both chronic and acute arthritis models. As we were interested in distinguishing between NKT cell's role as inherent regulators of immune homeostasis and as active regulators during the priming of an autoimmune inflammation, we investigated the regulatory role of NKT cells using two methods. Firstly, we induced AIA and CIA in CD1 knock out mice to study the arthritis development in mice where NKT cells were lacking from birth. Secondly, we depleted NKT cells at the time of induction of AIA and CIA, by using a depleting antibody directed towards NK1.1, a surface marked expressed on NK and NKT cells, and observed arthritis phenotype development after NK1.1 depletion. Histology evaluation of joints and T cell responses after *in vitro* re-stimulation were also assessed.

Results

CD1d knockout mice lacking NKT cells developed more severe AIA compared to wild type littermates. This was correlated with a higher degree of histopathological changes of inflammation and proteoglycan depletion in affected joints. When depleting N1.1 positive cells at the time of AIA induction, similar aberrant arthritis

was observed. In both experimental systems, peripheral T cells from mice lacking NKT cells responded with a higher IFN-γ production when re-challenged with antigen *in vitro*. T cells from mice depleted of NKT cells at the time of AIA induction also proliferated more vigorously in response to antigen. These findings were confirmed when using the CIA model, assessing chronic arthritis, as both CD1d knockout mice and mice depleted of NK1.1 expressing cells at time of CIA immunizations developed more severe arthritis compared to the control groups. The CD1d deficient mice had higher numbers of splenocytes, and when re-encountering antigen in vitro, peripheral T cells produced higher amounts of IFN-γ, but not IL-17α, TNF-α or IL-13.

Conclusion

As we observed worsened arthritis both in CD1d deficient mice, and in mice depleted of $NK1.1^+$cells at the time of arthritis induction in both arthritis models used, we concluded that NKT cells are indeed functioning as regulatory cells also in autoimmune arthritis, and are capable to suppress a pathogenic inflammation specific for joint tissue. This was also seen as a lowered capacity of antigen specific T cell production of IFN-γ, compared to a maintained IL-17α production – indicating that NKT cells altered the function of effector T cells, dampening the antigen specific Th1, but not the Th17, response.

Paper III;

Endogenous collagen peptide activation of CD1d-restricted NKT cells ameliorates tissue-specific inflammation in mice

Background

In contrast to heterologous collagen type II (CII), mouse CII is a poor arthritogen. However, several immunogenic epitopes have been identified from mCII .In this paper we aimed at characterizing the response to one of the immunodominant epitope on mouse CII, namely peptide 707-721 ($mCII_{707-721}$).

Method

Mice on B10.Q background were immunized with $mCII_{707-721}$ in CFA and peripheral T cells were re-challenged *in vitro* to investigate the T cell response. The T cell response in a variety of knockout mice on B10.Q background (i.e.$TCR\alpha\beta^{-/-}$, $TCR\gamma\delta^{-/-}$, $CD4^{-/-}$, and $CD8^{-/-}$ mice) was charecterized to identify the T cell population responding to $mCII_{707-721}$. In addition, the antigen-presenting molecule was identified by using $MHCII^{-/-}$ and $TAP^{-/-}$ mice. The antigen specific cytokine responses and TCR usage of $mCII_{707-721}$ specific cells after *in vitro* antigen re-challenge was evaluated by ELISA and PCR. The TCR signalling and co stimulatory requirements upon $mCII_{707-721}$ stimulation were considered by looking at the phosphotransferase kinase ZAP-70 on the cell surface and by using blocking antibodies towards co stimulatory molecules in the *in vitro* cultures. As CD1d had been identified as a candidate antigen presenting molecule, we also performed a CD1d - $mCII_{707-721}$ binding assay, using plate bound CD1d and biotinylated $mCII_{707-721}$ to detect bound peptide. To further address the qualities of the $mCII_{707-721}$-specific response, we established an antigen specific T cell line by cycled *in vitro* antigen simulation and resting.

The suppressive capabilities of the $mCII_{707-721}$-specific cell line were investigated in a co-culture system where anti-CD3 activated spleenocytes were co-cultured with the $mCII_{707-721}$-specific T cell line and proliferation and apoptosis was assessed.

The $mCII_{707-721}$-specific T cell line was characterized as having immunoregulatory capacity, and hence, different *in vivo* models were used to study the $mCII_{707-721}$-specific population's effect on pathogenic inflammation. B10.Q mice were vaccinated with $mCII_{707-721}$ prior to induction of CIA, EAE, DTH and ovalbumin (OVA) sensitization of the airways. The $mCII_{707-721}$ vaccinated mice were compared with mice vaccinated with a control peptide. EAE and CIA disease phenotype was evaluated, both clinically by scoring the animals for manifested symptoms, as well as by performing histopathological and immunological evaluations of the target tissues. DTH was evaluated by histological examination, and OVA sensitization was evaluated by measuring cytokines and IgE in BAL fluid.

Results

Cells responding to $mCII_{707-721}$ were found to be $CD4^+NK1.1^+TCR\alpha\beta^+$ cells, independent of both MHC I and II, and restricted to CD1d presentation of $mCII_{707-721}$ as showed by blocking of CD1d with antibodies. We also found that the $mCII_{707-721}$ peptide bound to CD1d in a concentration dependent manner. The $mCII_{707-721}$-specific CD1d restricted NKT cells produced IFN-γ, IL-4 and TGF-β in response to antigen, whereas no IL-2 or IL-10 production could be detected. They showed a restricted TCR Vα-Jα18 usage, paired with polyclonal and diverse Vβ chains, and TCR engagement and signalling in antigen activation was observed by ZAP-70 phosphorylation. The CD1d restricted NKT cells were also found to be dependent on functional co stimulation, i.e. B7.1-CD28 and CD40-CD40L, for full activation.

The $mCII_{707-721}$-specific NKT cells inhibited *in vitro* anti-CD3 activated T cell proliferation in a cell-cell contact dependent manner, and they were capable of inducing apoptosis through Fas-Fas-ligand interaction of a co-cultured anti-CD3 activated T cell line.

Mice vaccinated with $mCII_{707-721}$ were partially protected from both CIA and EAE, showing both lower clinical scores as well as less inflammation and tissue damage at site of inflammation. The alleviation of CIA was not due to a Th2 cytokine shift in inflamed joints, as cells in vaccinated mice produced reduced levels of both IFN-γ and IL-4. In line with this finding, the $mCII_{707-721}$-specific NKT cells also lowered Th1 driven DTH reaction in vaccinated mice, and reduced the Th2 response (measured by IL-4, IL-5, and IL-13 and IgE) in OVA sensitized airways of vaccinated animals compared to control group.

Conclusion

Immunization with $mCII_{707-721}$ induced an immunoregulatory CD1d restricted NKT cell population. The NKT cells responded to $mCII_{707-721}$ via CD1d presentation and were dependent on TCR and co stimulatory signalling for activation. This activation induced a clear cytokine response, but the *in vitro* suppressive functions were shown to be cell-cell contact dependent. The $mCII_{707-721}$-specific NKT cell population was shown to have the capacity to suppress pathogenic autoimmune inflammatory reactions independent of tissue specificity, as ameliorated EAE and CIA was observed after pre-vaccination. In addition, both Th1 and Th2 driven inflammatory conditions (i.e. DTH versus allergic airway inflammation) was suppressed by this NKT cell population. The results suggest that this immunological control is partly exerted via their capacity to induce apoptosis in activated T cells.

General introduction

The immune system is a highly specialized system, whose primary function is to defend its host against infectious microbes. It is generally divided in innate and adaptive immunity, with a distinction in the specificity of the immune reaction. As evident from the nomenclature, innate immunity consists of defence mechanisms that are in place even before an infection has occurred and is fully functioning and not further developed from birth, whereas the adaptive immune system is evolving during lifetime, and dependent on the challenges the individual encounters over time.

The adaptive immune system consists of cells that originate from stem cells in the bone marrow. From here, two types of adaptive immune cells –lymphocytes- emerge, giving rise to humoral immunity mediated via B cells and antibodies, or cellular immunity mediated via T cells. B cells leave the bone marrow as mature lymphocytes, whereas progenitors for T cells leave the bone marrow for the thymus, where the maturation of T cells is completed. From here, mature T cells emerge to circulate via blood and lymph, patrolling through the body. Both B and T lymphocytes carry a diverse set of receptors, generated by somatic gene rearrangements and capable of recognizing a wide variety of structures, antigens, giving rise to a highly antigen specific immune response when activated.

Innate immune cells also originate from stem cells in the bone marrow, but in contrast to adaptive lymphocytes, the innate immune response is elicited by structures shared by groups of microbes, pattern recognition, and provides the early line of defence against pathogens. Another important difference between the two branches of the immune systems is the so-called memory function of the adaptive immune response. Whereas the innate immune system has exactly the same features after an infection, the adaptive immunity evolves from and remembers an antigenic challenge, leaving the host better equipped to respond to the same antigen than before.

Both these two systems are highly integrated, dependent on each other for full activation and termination, and cannot be looked upon as isolated parties. To mediate communication between different immune cells (and with other organs and tissues), cells secrete soluble proteins called cytokines and chemokines. These are immunological mediators important for initiating, maintaining and terminating an immune reaction, and they also localizes the immune cells from the circulation to the tissue where an immune reaction is required.

Tolerance and autoimmunity

Mechanisms for keeping self-tolerance

The immune system is a powerful organ with the ability to kill cells and give rise to harmful inflammations; hence it needs to be under strict regulation. One remarkable feature of the immune system is its ability to recognize and respond to foreign antigens, while not attacking the individual's own tissues. This is called the immune system's capacity to distinguish between non-self and self, or keeping self-tolerance.

Self-tolerance is achieved through several mechanisms; one is through the elimination of T lymphocytes that express receptors capable of recognizing self-antigens presented in the thymus, a process called negative selection and maintaining central tolerance. This is a process carried out during the maturation of T cells in the thymus. As a strong and forceful immune reaction is dependent on specific T cell help, deleting auto reactive T cells is an efficient way to prevent immune responses to self-antigens. The negative selection process is however not complete, as not all antigens expressed in the periphery is presented to the maturing T cell in thymus, and self-reactive T cells do emerge from the thymus. To control these potentially dangerous self-reactive cells, other mechanisms need to be operative in the periphery – to keep peripheral tolerance to self. Peripheral T cell tolerance is maintained mainly by three known mechanisms; induced anergy leaving the T cell in an unresponsive state, deletion and cell death by apoptosis of cells reactive to self, and functional suppression of auto reactive T cells by regulatory T cells.

Regulatory T cells are believed to have the capacity to suppress auto reactivity, either by by-stander mechanisms and secreting anti-inflammatory cytokines and hereby create an anti-inflammatory milieu for further immune cell activation, or by direct cell-cell interactions, affecting both antigen presenting cells (APCs) and auto reactive T cells (1, 2). The most extensively studied regulatory T cells are the cluster of differentiation (CD)$4^{+}CD25^{+}FoxP3^{+}$ T cells, but other populations clearly exists. One is the CD1d dependent natural killer (NK) like T cell population, which has been the main research focus of this thesis. This cell population is further described in section *CD1d antigen presentation and NKT cells*.

Autoimmune diseases

When tolerance mechanisms are failing, an autoimmune disease can develop. The self-reactive cells of an individual's immune system will unhindered react towards cells and matrix of the own body, which in turn leads to a chronic inflammatory disease. Most often these disorders are tissue specific, meaning that one target organ is under attack rather than the whole body. Examples of this are rheumatoid arthritis (RA) and multiple sclerosis (MS) where epitopes and antigens in the joint and in the central nervous system (CNS) respectively are believed to be the targets for the adverse immune reaction.

Rheumatoid arthritis, RA

About 1% of the world's population is affected by rheumatoid arthritis, with a three times higher prevalence in women than in men. Although rheumatoid arthritis can occur at any age from childhood to old age, onset is most frequent between the ages of 30 and 50 (3, 4). RA is a chronic disease in which various joints in the body are inflamed, leading to swelling, pain, stiffness, and the possible loss of function and joint deformation. The pattern of joints affected is usually symmetrical, involving the hands and other peripheral joints. For the patient it is a disabling and painful condition which can eventually lead to complete immobilization and premature mortality. Diagnosis is made mainly on signs and symptoms, complemented with blood tests (screening for rheumatoid antibodies and other markers for inflammation) and imaging examinations such as ultra sound, magnetic resonance imaging analysis, and radiological examinations (5). The etiology of RA is unknown, although a combination of factors including an abnormal autoimmune response, genetic susceptibility, and environmental factors probably triggers the disease.

The disease process leading to RA is believed to begin in the synovium surrounding the joint. An abnormal immune reaction causes a chronic inflammation of the synovium, leading to collagen breakdown of the cartilage in the joint. This narrows the joint space and leads eventually bone damage. In progressive RA, the disease course is accelerated and a pannus is formed. The pannus is a growth composed of synovial tissue, which in turn produces more tissue destructive molecules and enzymes, driving the cartilage destruction, and leads to further inflammation and immune cell infiltration to the joint. Pathogenic T cells producing pro-inflammatory cytokines driving the joint inflammation, are believed to be imperative in the detrimental inflammation, but it is clear that also B-cells and antibodies binding to Fcγ receptors on macrophages are needed to

make the inflammation pathogenic with cartilage and bone destruction as a consequence (6).

Drugs commonly used to treat RA include nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), biologic response modifiers, and corticosteroids. The NSAIDs are prescribed to relive the pain caused by the underlying inflammation, but they do not modify the disease progression. The DMARDs and the biological modifiers are on the other hand indicated to slow down the disease progression by modifying the immune system. Even though the exact mode of action for its use in RA is unknown, the most commonly used DMARD is methotrexate, as this compound is available in generic forms. However, severe side effects have been reported with this drug. A number of newer and more specific biological modifiers are available, with the anti tumor necrosis factor (TNF)-α drugs being the most commonly prescribed. These drugs includes infliximab, etanercept, adalimumab , golimumab, and certolizumab, and acts to modify the immune system and dampen the tissue destruction by blocking the inflammatory cytokine TNF-α. In addition, biological compounds targeting interleukin (IL)-6, IL-1, or the activation of T or B cells are approved to treat RA. Further, the regulatory authorities in US last year approved a new DMARD, tofacitinib, a janus kinase 3 (JAK3) inhibitor, inhibiting the production of inflammatory mediators by suppressing genes regulating cytokine production in joint tissue. Finally, classical immune suppressive corticosteroids are used to dampen the joint inflammation, but the poor safety profile of these drugs prohibits long term use.

Over the past years, biological compounds with disease modifying properties have become standard care for RA in the western world, usually prescribed in addition to classical anti-inflammatory and analgesic drugs, and leading to not only symptom relief but also slower disease progression. However, there is currently no cure for RA, and the underlying cause of the inflammatory attack on the joints cannot be treated.

Multiple sclerosis, MS

MS is a relatively common neurological disease affecting more than 1 million people worldwide. Its prevalence rate varies between ethnicity and region, ranging from more than 100 per 100,000 in Northern and Central Europe to 50 per 100,000 in Southern Europe. It is more common in women and in Caucasians. In MS, the inflammatory process is believed to be triggered by T cells reacting with myelin antigens surrounding the neuronal axons in the central nervous system, which in turn leads to myelin breakdown, axonal disturbances, and impaired neuronal functions and signalling (7, 8). Affected patients go through a series of

neurological disturbances, usually with a remitting-relapsing pattern, which eventually progress to physical and cognitive disability, and increased mortality. As for RA, diagnosis is made from clinical evidence, complemented with analysis of the cerebral spinal fluid for antibodies, and magnetic resonance imaging analysis (8). 82 to 85 % of all patients present with relapsing-remitting MS, which is characterized by unpredictable acute episodes of neurological dysfunction (relapses), followed by variable recovery and periods of clinical stability. Within ten years more than 50% of patients who presented with the relapse remitting form develop sustained deterioration with or without superimposed relapses; this form of MS is known as secondary progressive MS. Approximately 15% of MS patients develop a sustained deterioration of neurological function from the beginning; this MS variant is known as primary progressive MS. Pathophysiological processes involve acute inflammatory focal lesions, gliosis, demyelination, impaired remyelination, axonal loss and neuronal loss which occur at all stages of the disease. The relative contribution of these processes changes during the course of the disease. Relapses are considered to be the clinical expression of acute inflammatory focal lesions, whereas progression is more associated with demyelination, impaired remyelination, axonal loss and neuronal loss. In primary progressive multiple sclerosis, inflammation is cortical and more diffuse.
Also very similar to RA, there is no cure available for MS, and the etiology of MS remains unknown. Current therapeutic approaches involve symptomatic treatment, treatment of acute relapses, and disease modifying therapies. Symptomatic treatment refers to all therapies applied to improve symptoms and complications caused by the disease e.g. fatigue, spasticity, ataxia, walking disability, weakness and bladder and bowel disturbances. During the last two decades, interferon (IFN) β-1a and 1b, mitoxantrone, glatiramer acetate and natalizumab have been established as disease modifying treatments; all of these treatments are administered parentally. The first oral treatment for MS, fingolimod, was recently approved by regulatory authorities in EU and US. Patients are given disease-modifying treatments with the aim to adjust or diminish the immune response and inflammatory reactions in the CNS, but classic immune suppressive treatments, such as systemic corticosteroids, are usually only given during acute relapses. Drugs available today have been shown to reduce the frequency of new relapses, and are hence believed to have the capacity to reduce disease development over time.

Both MS and RA are diseases devastating for the individual and families, with only partially effective treatments available. This shows the importance of proper regulation of the immune system and maintenance of self-tolerance. It also highlights the need of increasing the knowledge of how immune regulation can be restored when it does not function as needed and an autoimmune disease develops. As of today, the exact cause of autoimmune diseases is not known. However,

patients are usually treated with drugs targeting inflammatory mechanisms, modulating or suppressing the immune system. These drugs have the capacity to ameliorate and dampen the symptoms and to delay the progression of tissue destruction and disease, but not to completely cure the patient. More knowledge in the field of immune regulation in the context of tolerance to self-antigens will enable the scientific community to develop more effective treatments in the future, and eventually also cures, to affected individuals.

Aim of this thesis

As illustrated, there is a need to learn more about control mechanisms operating in the immune system, and how one can modulate the regulation of auto reactive immune responses.

This thesis investigates a subpopulation of T cells – CD1d dependent NKT cells - in the context of pathogenic autoimmunity. Our interests in these cells originated from studies implicating that they constitute a T cell subpopulation with regulatory capacities. The work is focused on investigating whether NKT cells have the capability to regulate and suppress autoimmune conditions, and how NKT cells are activated to exert such a regulatory function. The studies also included investigations on operating mechanisms in this suppression.

As the immune system a complex network, the objective of the work was to use *in vivo* animal models as far as possible, and to complement this with *in vitro* experiments. Hence the main emphasis has been on the regulatory function of NKT cells in animal models for two human autoimmune diseases; MS and RA. Murine models for these diseases were utilized, namely experimental autoimmune encephalomyelitis (EAE) which is a model for MS, and antigen induced arthritis (AIA) and collagen induced arthritis (CIA), experimental models for RA.

The specific aims of the thesis were:

- Investigate the natural function of CD1d dependent NKT cells as regulators in tissue specific autoimmunity, utilizing experimental autoimmune animal models.

- Characterize the T cell response towards a major antigen from mouse cartilage, collagen type II peptide 707-721 ($mCII_{707-721}$), and further investigate the regulatory capacity of this cell subset in tissue specific inflammation.

Animal models for autoimmunity

Animal models provide a useful tool in research by which we can control variability in genetic background and environment. It clearly also offers a mean to perform experiments impossible to do in human studies due to ethical reasons. As the need to learn more about mechanisms operative in pathogenic autoimmunity, and by that finding effective therapeutic target for the treatment of chronic and lethal human autoimmune diseases, is urgent, the benefits of valid animal models are evident.

Experimental autoimmune encephalomyelitis, EAE

MS is believed to be caused by an autoimmune T cell driven assault on the myelin sheets in the CNS, including infiltration of CD4 and CD8 expressing T cells. The most commonly used animal model to experimentally mimic and study autoimmune CNS inflammation and MS is Experimental autoimmune encephalomyelitis (EAE) (9). It was used as early as 1935, when Rivers et al. described experimentally induced demyelinating encephalomyelitis in monkeys (10). Today, most groups use murine models of EAE, induced by immunization with myelin derived proteins or peptides in susceptible mouse strains, resulting in activation of auto reactive, myelin specific, $CD4^+$ T cells and the herby following autoimmune inflammatory attack on the myelin sheets in CNS. Myelin proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), as well as immunogenic peptides from these, are all commonly used as EAE inducing antigens. The characteristics of the induced disease varies depending on mouse strain and immunizing agent, but typical clinical symptoms are paresis with subsequent paralysis, usually starting in the tail of the animal and moving forward to hind- and fore- legs as the disease progress. In severe cases, the disease results in complete paralysis and death. One notable contrast from human MS, is that in MS, the infiltrates are dominated by cytotoxic CD8+ T cells, whereas in the EAE model, $CD4^+$ T cells are considered to be the drivers of the disease (11, 12). The realization in the scientific community of this inconsistency has evoked the search for a $CD8^+$ T cells driven model to complement the MOG induced EAE, and this development is still in progress (13).

As EAE can be induced by passive transfer of myelin specific $CD4^+$ T cells to healthy animals, it has been regarded as a T cell mediated disease.

Encephalitogenic T cells usually exhibit a T helper 1 (Th1) or Th17 phenotype (14-20), and even though there are also reports of T helper 2 (Th2) clones causing an un-typical form of EAE in immunodeficient hosts (21, 22), Th2 cells are generally thought to be protective in EAE (23). Lesion formation in the CNS of EAE affected mice is characterized by perivascular demyelination and immune cell infiltration, where $CD4^+$ T cells and macrophages are most abundant (24). In addition to T cells, cells of the monocytic lineage have been shown to play a critical role in driving the disease and generating tissue damage (25, 26).

Different cytokines have been proven important in the regulation of EAE (27, 28). The classical Th1/Th2 paradigm with EAE being a Th1 cytokine driven disease has been shown to have some shortcomings; IFN-γ deficient mice are more susceptible to EAE with an impaired recovery, suggesting that this hallmark Th1 cytokine is not necessarily pathogenic but can even inhibit EAE development (29-31); Mice deficient in the IL-12 p35 subunit were more susceptible to EAE, whereas deficiency in the IL-12 p40 subunit rendered mice completely resistant to EAE, indicating that the Th1 driving cytokine IL-12 in its intact form is not imperative in driving the disease, but its subunit p40 is (32). Even so, Th2 cytokines (IL-4, IL-10 and IL-13) have been shown to be protective in EAE (33), indicating that a Th2 response might be beneficial for the affected host. This paradox has at least partly been explained by the identification of a third T helper cell lineage, the Th17 cells, which has been shown to drive the pathogenic inflammation in EAE (34-36). The Th17 response is characterized and sustained by the cytokines IL-17 and IL-23 (18). As IL-23 shares one subunit with IL-12, the p40, this would explain the paradoxical findings described above, and the redundancy in the IL-12 system in driving pathogenesis in EAE. In addition, it is believed that Th17 response is inhibited by IFN-γ, and hence explains the fact that mice deficient in IFN-γ express and ameliorated EAE (31).

The potent immunoregulatory cytokine transforming growth factor (TGF)-β (37) has also been shown to have the capacity to mediate regulation of EAE. This has been demonstrated both by the administration of TGF-β to EAE affected animals (38, 39), and by an association of resistance to EAE to the expression of TGF-β in the CNS (40, 41). In addition, in paper I we present data linking TGF-β production in inflamed CNS to the regulatory functions of CD1d restricted NKT cells in EAE. Another important cytokine in the context of EAE is IFN-β, as one of the most commonly used treatments for MS today is injections with IFN-β (42-44). IFN-β has been shown by our group to have regulatory properties also in EAE by inhibiting the T cell activating capacity of antigen presenting cells in the CNS (45). The exact mode of action of IFN-β-treatment in MS patients is still under investigation, but recent data points towards several points of impact on the immune system, as IFN-β-treatment in MS patients has been shown to promote

regulatory NKT cells via dendritic cell activation (46), as well as dampen the IL-1β secretion from monocytes via inhibition of signal transducers and activators of transcription-1 (STAT-1) dependent intracellular signalling (47).

In present work two different mouse models to study EAE were utilized. Firstly, the frequently used MOG peptide 35-55 (MOG_{35-55}) induced EAE in C57Bl6 (B6) mice, described by Mendel at al., have been used to study the intrinsic role of CD1d restricted NKT cells in EAE. Data from these studies are presented in paper I. In this model, B6 mice are subcutaneously (s.c) immunized with the immunodominant peptide of MOG, MOG_{35-55}, in complete Freund's adjuvant (CFA), together with an additional adjuvant injection, i.e. pertussis toxin (PT) given intra-peritoneal (i.p) (48). This results in a chronic inflammation of the CNS with infiltrating immune cells and clinical symptoms appearing after approximately 10 days. The disease manifests in nearly 100% of immunized animals and is evident histopathologically by the presence of infiltrating inflammatory cells and relatively large areas of demyelination in the CNS. This model is hence looked upon as a valid model for demyelinating autoimmunity of the CNS. To use a model suitable for mice on the B6 genetic background was important, as the B6 strain is the most characterized mouse strain for NKT cell properties and activity.

However, mice with a major histocompatibility complex (MHC) genotype Q are not susceptible to MOG_{35-55} induced EAE. Hence, in paper III, the MOG peptide, MOG_{79-96}, was used to induce EAE in C57Bl10.Q (B10.Q) mice as described by Abdul-Majid et al (49). As in the MOG_{35-55} model, mice are immunized with the MOG_{79-96} peptide in CFA and PT is given i.p as an adjuvant, resulting in CNS inflammation with demyelination and clinical manifestations. Although less widely used, this model is also considered valid to study autoimmune inflammation of in the CNS. By utilizing BQ mice in paper III we were able to investigate the effects of $mCII_{707-721}$ vaccination on both EAE and experimental arthritis, keeping the experiments focused to the same mouse strain while investigating different tissue specific inflammations.

Experimentally induced arthritis, CIA and AIA

Collagen induced arthritis, CIA

As in the case of EAE, Collagen induced arthritis (CIA) is provoked in susceptible rodents by immunization with a target tissue antigen, in this case collagen type II (CII). This causes an autoimmune attack on the joint cartilage, which is thought to mimic the events in RA patients. The model was first described in 1977, when Trentham et al. induced arthritis in rats by immunization with CII (50), and a few years later CIA in mice was reported (51). Today, it is one of the most widely used animal models for RA. The clinical phenotype of CIA can be seen in swelling and redness of peripheral joints, and permanent deformity of the affected paws is usually observed once the active inflammation subsides. The disease can be considered as chronic relapsing, as new inflammatory foci appears over time, affecting new joints, whereas others disappears leaving tissue destruction and deformity of the joint. Histopathologically the disease is characterized by infiltration of immune cells to the joints, as well as cartilage and bone destruction.

As for EAE, Th1 and Th17 cells seem to play a critical role in inducing CIA (52-54). T cells are found in the joints of arthritic mice (55), and mice carrying a targeted deletion of the TCR-β loci have been shown to be completely resistant to CIA (56). Importantly, the susceptibility to CIA is closely linked to the MHC genotype (57, 58), indicating that T cells activated by specific antigens presented on MCH drives the disease. Even so, B cells and antibody production are required for a full-blown CIA (59, 60), and in contrast to EAE, CIA cannot be induced by antigenic peptides from collagen, but requires immunization with whole CII. This points towards the importance of B cell receptor recognition of the three dimensional structure of CII in addition to T cell activation. In addition, arthritis can be induced solely by injection of collagen specific antibodies, but is then also enhanced by T cells (61, 62).

Inflammatory cytokines, produced mainly by macrophages, such as tumour necrosis factor (TNF)–α, IL-1, and IL-6, play a key role in driving the autoimmune attack in the joint, and these cytokines are today targets for approved therapeutic drugs in the treatment of RA (63-68).

In addition, IL-17 has been shown to synergistically enhance TNF-α induced pathological processes in experimental arthritis, and has been suggested as a future therapeutic target, in combination with existing drugs targeting TNF-α (69). The imperative role of IL-23 in the development CIA has also been demonstrated, as

IL-23 subunit p19 mice were shown to be resistant to CIA (70). Hence, also IL-23 is today a suggestive target for therapeutic interventions for RA (71).

Interestingly, CIA in mice is most effectively induced by immunization with heterologous CII, e.g. rat, chicken or bovine CII, whereas mouse CII provokes a weaker T and B cell response to the immunodominant epitope and induces a lower incidence of arthritis (72, 73). This could in part be due to the fact that compared to the immunodominant epitope in rat CII at position 260-270, mouse CII differs in two amino acids and binds with a slightly lower affinity to the MHC class II molecule in susceptible mouse strains (74-76). We hypothesized that another explanation, contributing to this finding, could be that an immunogenic epitope in mouse CII was inducing a T cell response with regulatory functions. A major epitope apart from the position 260-270 had been identified in mouse CII (77), and hence we further investigated this hypothesis by characterizing the immune response to this epitope. Data supporting that indeed this epitope is inducing a specific regulatory response is presented in paper III.

Antigen induced arthritis, AIA

Murine antigen induced arthritis have been extensively described by Brackertz back in 1977(78-80). It is a T-cell driven model for arthritis, caused by direct injection of an antigen into the knee joint after a pre-immunization with the antigen of choice to evoke the immune response (81, 82). Most commonly used as inducing antigen is methylated bovine serum albumin (mBSA), which is a cationic molecule allowing for antigen retention in the joint through charge mediated bindings.

The model is not as widely used as CIA to study arthritis, as it does not mimic the self-antigen driven chronic features of human RA as close, but as an acute animal model of joint inflammation targeting the cartilage, it has proven useful. The main advantages are shorter experimental incubation time, and less variability in the disease manifestation than CIA, and hence fewer animals can be used for a shorter period of time. In addition, the arthritis affects only the joint injected with the antigen, which makes it possible to use a contra-lateral joint as control.

We used this model as a mean to complement experiments in CIA, and to address the issue of whether NKT cells work as regulators or pathogenic drivers in arthritis. Reports showing dual functions of NKT cells had been published in the literature, and data indicated that the intrinsic functionality of CD1d dependent NKT cells could vary in different animal models, in different mouse strains, and during different stages of inflammation. We aimed to investigate this further by

using two different animal models for arthritis on the same genetic background (the B6 mouse), that could represent a chronic joint cartilage specific arthritis (CIA), versus a more acutely induced joint inflammation (AIA). Data presented in paper II showed that CD1d dependent NKT cells indeed worked as regulators in both arthritis models.

CD1d antigen presentation and NKT cells

CD1d antigen presentation and NKT cell activating ligands

The CD1 protein family is comprised of antigen presenting molecules, found on the cell surface of mainly hematopoietic-derived cells (83, 84). The human genome encodes five CD1 genes (*CD1A-E*) with four corresponding proteins (CD1a-d) expressed on the cell surface (85), and a fifth (CD1e) expressed as a soluble form in the lysosomal compartments (86). Based on shared sequence homology, the CD1 molecules are divided in group 1 (CD1 a, b and c), and group 2 (CD1d) (87). The group 1 CD1 molecules are found in humans but not mice, whereas group 2, CD1d, is found in both species (88). Several similarities between the classical antigen-presenting molecules MHC class I and II and the CD1 antigen presenting molecules can be found; β2-microglobulin association is required for the cell surface expression, and the overall structural architecture resembles that for MHC class I (89), whereas its localization to endosomal compartments and predominant expression on APCs are more similar to MHC class II (90, 91).

Unlike the MHC molecules, the CD1 molecules have a deep and hydrophobic antigen binding groove, comprising two pockets, and numerous studies have demonstrated the capacity of CD1 to present lipid antigens from bacterial pathogens, mounting a T cell response thought to be of importance in clearance of infections (92-98). This is in contrast to the classical MHC class I and II molecules, which present peptides to $CD8^+$ and $CD4^+$ T cells respectively. The findings that a specific immune response can be elicited to bacterial lipidic

antigens, not just peptides, have significantly broadened our view on host defence against pathogens. It also opened up a new field of research, trying to identify novel, non-classical, antigens that could trigger T cell responses. Further, in a study by Moody et al., a lipopeptide from *Mycobacterium tuberculosis* capable of activating T cells when presented by human CD1a was identified (99), and even non-lipid non-peptide small molecules with sulphur and hydrocarbon rings have been shown to be capable of eliciting a T cell response when presented by human CD1d (100).

However, the exogenous antigen α-Galactocylceramide (α-GalCer) was the first lipid antigen identified as capable of binding CD1d and activating T cells (101). It is a synthetic glycolipid, based on the structure of compounds purified from a marine sponge, and was originally screened for its pharmacological effects and anti tumour capacity (102). Not known to be produced by neither mammalian cells nor pathogenic microbes, this synthetic ligand's physiological relevance as an antigen is not known. Nevertheless, it has been a useful tool in studying CD1d restricted T cells, and a range of synthetic analogues with structural modifications that alter the functional response has been generated (103, 104). Interestingly from an autoimmune perspective, a self-glycolipid, sulfatide, from CNS in mice have been described as an antigen presented by CD1d (105), and self-phospholidids from a tumour cell line have been shown capable of activating CD1d restricted hybridomas (106). In addition, an endogenous glycosphingolipid presented by CD1d, iGb3, has been identified in mice (95, 107), however also shown not to be present in humans (108). Originally it was postulated that recognition of iGb3 occurred in the thymus during CD1d restricted NKT cell development, but it has later become evident that this antigen is probably not the selecting antigen for the development of CD1 dependent T cells (109, 110). Most exogenous glycolipid antigens presented by CD1d are comprised of a lipid backbone attached to a sugar via an α linkage (111), whereas most mammalian glycolipids have a β linkage, and it has recently been shown that during the CD1-TCR recognition of mammalian glycolipids, the interaction itself flattens the β linked glycolipid, to a structure similar to that of a α linked (112, 113), explaining the specificity for antigens with different structures.

Not much is known about CD1 presented peptide ligands activating T cells. An early study by Castano et al., showed that mouse CD1d could bind peptides with aromatic or bulky amino acids, and demonstrated that specific T cell responses were raised against these CD1d presented peptides (114). The crystal structure of the mouse CD1d molecule show that the binding groove of CD1d is deep, narrow, and hydrophobic, compatible with lipid or glycolipid antigens, but still with the possibility to form enough hydrogen bonds to bind also a peptide (89). Tangri et al. and Lee et al also worked with peptide presentation by mouse CD1d and

suggested that peptide and lipid antigens might bind to CD1d differently, occupying different binding sites (115, 116).

In paper III, we present novel data, showing that a peptide derived from autologous CII ($mCII_{707-721}$) can indeed bind to mouse CD1d. This induces a CD1d restricted NKT cell population with the capacity to down regulate a variety of tissue specific inflammations. Even though NKT cells have classically been viewed upon as reactive towards lipids presented by CD1d, this new finding should broaden the view of CD1 antigen presentation, and NKT cells' functional impact in the immune system. It will hopefully direct researchers into exploring also self antigenic peptides as CD1d presented antigens, and as activators of regulatory NKT cells with the capacity to maintain tolerance against self.

CD1d restricted NKT cells and immune regulation

The first paper identifying a CD1d restricted T cell was published by Bendelac et al. 1995. Previously, it had been known that a T cell sharing the expression of the cell surface marker NK1.1 with NK cells, and using an invariant TCR α chain (Vα14-Jα18) existed in mice (117-119), and it was now shown that these cells were reactive to CD1d (120). The homologous population in humans was described two years later as expressing a Vα24-Jα18 rearranged TCR α-chain (121). Further characterizations of these cells rendered them the name invariant NKT cells (iNKT), with reference to their invariant TCR and co expression of NK markers, or as later suggested by Godfrey et al., Type I CD1d restricted NKT cells (122).

NKT cells have been shown to be a not so homogenous population as first imagined (123). The type I NKT cells are subdivided into $CD4^+$ and double negative (DN) in mice, and in humans a population of $CD8^+$ NKT cells have been identified (124-126). Additional functional subsets have further been identified with different cytokine secreting patterns after stimulation (127). By investigating $CD4^+$ T cells in MHC class II deficient mice, Cardell et al., characterized CD1d dependent cells as being thymically derived, exhibiting an activated, memory phenotype and also added diverse TCR bearing T cells to the CD1d restricted population (128). This was confirmed four years later by Behar et al. (129). The NKT cell population bearing a diverse TCR have been referred to as Type II CD1d restricted NKT cells (122). In contrast to type I NKT cells, these cells do not respond to stimulation with αGalCer (130), and the requirement for endosomal targeting of CD1d differs between the two subsets (131). This indicates that these

two subsets recognize different sets of antigens, and studies have revealed that they also have different functional attributes (132). Tumour immunity studies have revealed that α-GalCer responsive type I NKT cells promote the immune response, whereas the type II variant NKT cells negatively regulate the tumour immunosurveillance (133-135). It is also evident that in certain infectious diseases, invariant type I NKT cells play an immune enhancing role, helping clearing the infection (136, 137), whereas the CD1d restricted response to the myelin-derived antigen sulfatide, mediated by type II NKT cells, is of negative regulatory nature (105, 138). In addition it has been shown that tolerogenic dendritic cells are acting via type II NKT cells, not involving invariant type I NKTs (139). This could indicate a natural duality between the variant and invariant NKT cells, suppressing and enhancing an immune reaction respectively. It has however been shown that also type I invariant NKT cells suppress and protect against autoimmune conditions, specifically EAE and Type 1 diabetes (T1D), (140-142) (143), indicating that this duality in function is not as clear cut as one could first imagine.

CD1d restricted NKT cells have been shown to be dependent on the expression of CD1d in the thymus to develop, and therefore this population is missing in CD1d deficient mice (144, 145). They leave the thymus as mature cells, and have been described as fast potent cytokine producers; when stimulated *in vitro*, they rapidly release large quantities of cytokines, IL-4, IFN-γ and IL-17 included (146, 147). Due to their rapid cytokine secretion potential, they have been suggested as regulators of the delicate balance between Th1 and Th2 immune responses, but research has showed that mice deficient in CD1d are capable of developing as good a Th2 response as wild type mice (144, 145). Nevertheless, they have been implicated as regulators and suppressors in pathogenic Th1 and Th17 driven inflammatory autoimmunity, both in human diseases such as T1D (148), MS (149-152) och RA (153-156), as well as in the animal model counterparts (157-161). Specifically, patients with MS has been reported to have a numerically altered NKT cell populations in the periphery, with higher numbers of circulating invariant NKT cells, but this population was shown to be unresponsive to stimulation with α-GalCer and IFN-γ, which would be consistent with an anergic state induced by previous exposure to antigen (152). In addition, it has been shown that CD4+ NKT cells expanded from MS patients in remission produced higher amounts of IL-4 compared to cells from healthy subjects or MS patients in relapse, indicating a regulatory role for these cells in MS (149). In patients with RA, reports have shown that the number of circulating NKT cells are lower compared to healthy controls (155, 162), and after treatment with rituximab, clinical remission was correlated with an increase in NKT cell frequency (162). Further, it has been shown that NKT cells are present in the affected synovia of RA patients. NKT cell lines from peripheral blood from these RA patients showed a significantly reduced number of IL-4 producing cells, whereas this was not the

case for NKT cells isolated from synovial fluid (152, 153). This shows that NKT cells might be functional in the progression of RA, and the authors suggested that providing a local boost to the regulatory potential of NKT cells might represent a useful candidate therapy for RA.

In papers I, and II, we show data that strengthens the idea of CD1d restricted NKT cells as regulators of pathogen autoimmunity and as having an intrinsic capacity to suppress and ameliorate the clinical and pathological course of both EAE and experimental arthritis (data is further discussed in sections *NKT cells in the regulation of EAE* and *NKT cells in the regulation of experimental arthritis*).

Further showing the immune regulatory capacity of CD1d restricted type II NKT cells, paper III presents the characterization of a murine NKT cell population capable of recognizing an immunodominant epitope of mouse CII ($mCII_{707\text{-}721}$) in the context of CD1d, which upon activation showed immunoregulatory properties on a variety of tissue specific inflammations. The recognition of the immunodominant peptide of mouse CII by CD1d restricted NKT cells is a novel finding as it is the first report of a self-peptide presented by CD1d to NKT cells. The $mCII_{707\text{-}721}$ specific CD1d restricted NKT cell population was shown to be $TCR\alpha\beta^+$ and $CD4^+$, and dependent on both TCR and co-stimulatory signalling for its activation in response to CD1d and $mCII_{707\text{-}721}$. Further adding importance to this finding, we observed that the NKT cells elicited in response to immunization with the CII-peptide were capable of down regulating pathogenic inflammatory autoimmunity. This was shown by an alleviated clinical course of both CIA and EAE after pre-vaccination with the peptide, with corresponding decreased inflammation in joints and CNS respectively. A Th2 cytokine shift of the inflammatory cells in vaccinated mice was not evident, but rather a decrease in both IFN-γ and IL-4 producing cells when investigating arthritic joints from vaccinated mice compared to control group. In addition, a lowered delayed-type hypersensitivity (DTH) reaction - a model for cell-mediated Th1 immunity - as well as a decreased immunoglobulin (Ig) E response in broncoalveolar lavage fluid (BAL) from sensitized mice - a model for humoral Th2 immunity – was found after vaccination with the peptide. This showed that the CD1d restricted NKT cell population specific for $mCII_{707\text{-}721}$ had suppressing effects on inflammatory responses regardless of the Th1 / Th2 dichotomy. Attempting to disclose the mechanisms behind this regulation, we found that the $mCII_{707\text{-}721}$ specific NKT cells were suppressing T cells via cell-cell contact and were capable of inducing Fas-ligand dependent apoptosis in T cells, similar to the killing of tumour cells by a-GalCer activated NKT cells previously reported (163), and their regulatory capacity was independent on cytokines. Fas-ligand depended induced apoptosis has also been described for another subset of CD1d restricted cells; CD1d restricted γδ T cells are during virus infections capable of killing regulatory T cells

through Fas-dependent apoptosis (164). It has been suggested that the balance between CD1d restricted NKT cells and CD1d restricted γδ T cells activation can be an influencing factor between self-tolerance and autoimmunity.

Our findings indicate that the NKT cell population characterized in paper III have immunomodulatory qualities. Hence activation hereof by the CII-peptide could potentially be used as treatment for harmful immune reactions, such as tissue specific inflammatory autoimmunity. It has been described that there is a high degree of conservation between man and mouse regarding the recognition of CD1d by NKT cells. Human NKT cells recognise mouse CD1d and vice versa (88, 97). This indicates that the mechanisms described above could be applicable to treat human autoimmune diseases. Clearly, paper III gives just a first hint on the therapeutic potential of this antigen, but one well worth investigating further. Future studies should include a thorough investigation on how the collagen peptide binds to mouse CD1d and interacts with the NKT cell TCR, which signalling pathways this interaction triggers, and also studies to elucidate whether human CD1d can present the mouse collagen peptide to elicit a similar response in human NKT cells, and later if such a response could have regulatory capacities on pathogenic inflammation in man.

NKT cells in the regulation of EAE

A number of studies on CD1d dependent NKT cells and their regulatory function in EAE in mice have been published to date. The majority of these studies have focused on the activation of invariant type I NKT cells by α-GalCer as a mean to regulate pathogenesis in CNS (103, 165-168). A few others, including a study from our group (paper I), have looked at NKT cell's inherited capacity to regulate EAE without α-GalCer activation (141, 142, 157, 169).

Induced NKT cell activity to regulate EAE

Studies with somewhat conflicting results have been published on the ability of α-GalCer-activated NKT cells to protect mice from EAE (103, 165-168). Pál et al. was using IL-4 and IFN-γ knockout B6 mice to show that activation of CD1d restricted NKT cells by α-GalCer could either enhance or suppress EAE respectively, whereas no effect could be seen when using wild type mice (167). From these data they concluded that IL-4 triggered by α-GalCer protected from EAE, whereas the opposite was true for IFN-γ. Supportive of this was a study

from Miyamoto et al., where a α-GalCer injection to B6 mice did not confer any protection from EAE (103). On the other hand, when using an analogue of α-GalCer which directed the NKT cell response towards producing more IL-4 and less IFN-γ, OCH, a clear effect could be seen on the disease course, with a milder EAE as outcome. In contrast to this, several groups have showed that treatment with α-GalCer protected B6 wild type mice from EAE in a CD1d dependent manner (165, 166, 168, 170), and it was demonstrated that both IFN-γ, IL-4 and IL-10 was important for the α-GalCer-mediated protection. In the above studies, the time point for injection of α-GalCer in relation to immunization for EAE, the number of injections, and the route of administration has all been shown to be of importance for conferring protection, possibly explaining the conflicting results. The protective effects of α-GalCer-activated NKT cells have been shown to be mediated by the peripheral induction of myeloid-derived suppressor cells that later migrate to the CNS to suppress the pathogenic inflammation in MOG induced EAE (170).

The above mentioned studies give some hope that a possible future pharmaceutical treatment for MS could be α-GalCer – or a Th2 inducing analogue of this compound. In addition, the data presented in paper III and discussed in previous section, show that NKT cells can be activated by the $mCII_{707-721}$ peptide, to become functional regulators in EAE, as well as other autoimmune and inflammatory conditions.

Inherited capacity of NKT cells to regulate EAE

Fritz et al. published in 2001 a study showing that passive transfer EAE in B6 TCR knockout mice could be partially inhibited by an additional transfer of $NK1.1^+$ T cells, indicating that an excessive number of NKT cells could protect from EAE (169). Mars et al. also showed in a study using Vα14-Jα18 transgenic non-obese diabetic (NOD) mice, that an increase in the number of type I NKT cells could significantly reduce the severity of EAE induced by direct immunization (142). The clinical phenotype correlated with histopathological findings in CNS, and the effect was independent of IL-4. In the same publication, they also looked at the severity of EAE in CD1d deficient NOD mice, without observing any difference as compared to wild type littermates. They concluded that this was not unexpected, as NOD mice are known to have a defective NKT cell population, both qualitatively and quantitatively. The same group later showed that the protection from EAE in the type I NKT cell enriched mice was associated with an infiltration of cytotoxic NKT cells to the CNS. Even though the regulation was independent on expression of CD1d outside the thymus, an increased level of CD1d was found on CNS resident APCs during the EAE inflammation (141). *In*

vitro recall experiments with MOG peptide suggested that the type I NKT cells prevented EAE by the inhibition of autoagressive Th1 and Th17 response. In addition, Oh et al (157) showed that type I NKT cells suppress Th1 and Th17 responses in the CNS during EAE.

In paper I we showed that CD1d deficient mice, lacking CD1d restricted NKT cells, developed a more sever and chronic EAE as compared to wild type mice when immunized with MOG_{35-55}. This correlated with a higher degree of demyelination and immune cell infiltration in the CNS. These findings illustrated that CD1d restricted NKT cells can be of importance in immune regulation of pathogenic autoimmunity. We also observed that activation of CD1d restricted NKT cells in the periphery was required for them to exert their regulatory function, as when an encephalitogenic T cell transfer was used as EAE inducing protocol, the regulation was absent. However, the regulation could be restored by an immunization with CFA of recipient mice, and we concluded that this immunization activated the regulatory pathway. Trying to reveal the mechanisms behind how NKT cells exerted this regulation, we found that the specific MOG_{35-55} response differed in characteristics between the CD1d deficient and wild type mice. Autoantigen specific T cells from mice lacking CD1d had an enhanced cytokine production when investigating both IL-4 and IFN-γ. This indicated that CD1d restricted NKT cells can control the level of cytokine production from autoantigen specific T cells, without skewing them towards a Th2 or Th1 profile (at the time point for this study, the importance of the Th17 lineage in EAE was unknown, and hence could not be addressed). We could also show that TGF-β production in the CNS, which was prominent in wild type mice, was virtually absent in the CD1d deficient CNS, suggesting that TGF-β could be one pathway by which NKT cells regulate EAE. One of the interesting findings in our study was that CD1d dependent NKT cells regulated EAE without activation by α-GalCer. We instead hypothesised on other possible activation pathways; either in an antigen specific manner by a bacterial antigen in CFA, or via non-specific, innate mechanisms in inflammation, both of which are operative during a normal "working day" for the immune system. Indeed it has later been shown that the activation of type I NKT cells *in vivo* can be CD1d and TCR independent and instead accomplished by the raised cytokine levels in an inflammatory milieu (171-173).

To further add light on the function of CD1d dependent NKT cells in EAE, Jahng et al. 2004 published their findings of a NKT cell population reactive to a myelin derived self glycolipid, sulfatide, presented by CD1d (105). These NKT cells were shown not to use the described invariant TCR, and their antigen specific activation prevented EAE. In concordance with our findings, the disease protection correlated with a decrease in both IFN-γ and IL-4 production by the MOG_{35-55}

specific T cells, and the regulation did not appear to be dependent on IL-4. This study interestingly showed that CD1d restricted NKT cells could be regulatory in EAE without operating through an exogenous ligand. It was further shown that multiple tissue specific isoforms of sulfatide, present in both myelin and pancreatic β-cells, could activate type II CD1d restricted NKT cells, showing that this is one of the naturally occurring antigens for NKT cells (138). In addition, CD1d-dependent NKT cells have been shown to be crucial mediators for conferring protection to EAE via tolerogenic dendritic cells, without the addition of an exogenous NKT cell ligand (139, 174).

Together, these studies indicate that CD1d restricted NKT cells have an inherited capacity to regulate EAE.

NKT cells in the regulation of experimental arthritis

During the last decade, studies have been published on NKT cell's role in experimental arthritis. Some of them have focused on the possible therapeutic effects of treatment with α-GalCer, whereas others have worked with different knock out animals to elucidate NKT cells' intrinsic role in arthritis. Confusingly enough, the results from these studies showed great discrepancies, some results pointing towards a suppressive role for NKT cells also in experimental arthritis (175-177), while others suggesting that NKT cells are actually drivers of the arthritogenic inflammation (178-181). Dissecting the field a bit closer, it becomes evident that results obtained are greatly dependent on the experimental protocols used, i.e. chosen arthritis model, genetic background of mice utilized, and for studies on α-GalCer, timing of administration.

NKT cells have been implicated as effector cells in antibody-induced arthritis, as Kim et al (178, 179) used serum transfers to induce arthritis in both $J\alpha281^{-/-}$ NKT cell deficient mice and $CD1d^{-/-}$ mice and showed that compared to wild type B6 mice, these knock-outs developed less ankle swelling. As the authors conclude, the serum transfer model does probably not include adoptive immune responses, but solely immune-complex induced inflammation, and hence the suppressive role of NKT cells described from other adoptive immune models was not found in the antibody induced arthritis model. Indeed, it has been shown that antibody-induced arthritis is using effector pathways independent of T-cells (178), and might therefore be unaffected by regulatory NKT cells.

In contrast, activation of NKT cells using α-GalCer or its analogues α-C-GalCer and OCH has been reported to protect mice from CIA (176, 180). Chiba et. al

activated NKT cells using both α-GalCer and OCH, the analogue known to stimulate NKT cells to produce mainly IL-4 in contrast to α-GalCer which induces both IL-4 and IFN-γ production from NKT cells. From these experiments they report reduced CIA and protection against disease via IL-4 and IL-10 when NKT cells were stimulated.

However, also utilizing the CIA model, Jα281$^{-/-}$ mice lacking the invariant NKT cells have been shown to manifest a decreased clinical arthritis score compared to wild type B6 mice (180). When using CD1d$^{-/-}$ mice (181), Ohnishi et al. also observed a decrease in arthritis scores compared to wild type, whereas Chiba et al. could only see this when using an antibody induced arthritis protocol. These reports would, in contrast to the studies activating NKT cells with α-GalCer, support the notion that NKT cells are instead pathogenic effector cells driving arthritic inflammation.

These results could, taken together, indicate that NKT cells have dual functions in arthritis; In the immune induction phase working as regulators of pathogenic self reactive T cells, and in the effector phase, where B-cells leading to antibody mediated cartilage and bone destruction is prominent, functioning as disease drivers.

To test this hypothesis further, we investigated the role of NKT cells in acute joint inflammation, using AIA as an experimental model, versus chronic arthritis, utilizing the CIA model. Results reported in paper II show that CD1d$^{-/-}$ mice lacking NKT cells develop more arthritis, both AIA and CIA, compared to wild type mice. Hence, in our lab, NKT cells seemed to be regulatory both in acute and chronic arthritis, and not responsible for driving pathogenesis. In addition, wt mice where depleted of NK1.1 expressing cells by injection with a depleting antibody at the time of arthritis induction. Paper II shows that also these mice exhibited a more severe arthritis compared to control injected mice, indicating that the regulatory mechanism whereby NKT cells exert their function needs to be active during disease initiation, and a functional pool of NKT cells for keeping the inherent immune homeostasis before disease induction might not be enough. This was further strengthened by the results previously discussed and reported in paper III, where we showed that a CD1d restricted NKT cell population could be activated by immunization with a specific collagen peptide. When vaccinating mice to evoke the NKT cell response prior to immunization for CIA, an ameliorated CIA developed compared to the control group. The collagen peptide-specific NKT cells produced IFN-γ, as well as IL-4 and TGF-β, and where capable of inducing Fas ligand dependent apoptosis of activated T cells. This indicates that the mechanisms by which this population of NKT cells exerted regulatory functions on T cells might be different from the α-GalCer-analogue activated NKT cells,

where regulatory instructions via dendritic cells in addition to regulatory cytokines are believed to play the main role (182), even though cell contact dependent regulation of B-cells has been described also for this population (183).

The conflicting results described from studies on NKT cell function in experimental arthritis could possibly in part be explained by the fact that different inbred mouse strains were used, in addition to different CIA protocols. It highlights the need for more studies to elucidate CD1d restricted NKT cell's biology in CIA.

Conclusions and reflections

As autoimmune diseases are devastating for affected individuals, the need for knowledge on their regulation is crucial. In this thesis we investigated the role of CD1d restricted NKT cells in this context. We were able to show that in the autoimmune models EAE, CIA and AIA, CD1d restricted NKT cells have a suppressive role on disease course, and tissue inflammation and degradation. This suppression is an active process, occurring during disease initiation, and requires immunological activation to be functional. In addition, we identified a CD1d restricted type II NKT cell population with specificity for a mouse CII peptide ($mCII_{707-721}$), and we demonstrated that this NKT cell population has a general immunomodulatory and regulatory effect on inflammatory conditions, EAE and CIA included. All these studies support the idea that NKT cells have regulatory functions in autoimmunity and should be regarded as potential targets when developing pharmaceutical treatments for human autoimmune diseases.

An important novel finding from the studies on the $mCII_{707-721}$ specific NKT cells was the notion that CD1d have the capacity to present a self-peptide antigen to NKT cells. That NKT cells are reactive to self-antigens has been known for some time, and recently has their capacity to regulate immune reactions been suggested to be attributed this capacity (123, 184). However, CD1d has in general been looked upon as a lipid antigen-presenting molecule, but in this thesis we show that this is not always the case, and that peptide self-antigens should be further investigated for specificity to CD1d and NKT cell activation. Whether the peptide identified in paper III binds into the hydrophobic antigen-binding cleft of the CD1d molecule is not clear, even though it was able to partly outcompete the ligand α-GalCer, and this should be one of the aims of future studies with this immunoregulatory peptide. Nevertheless, our study shows that the field might

need to revise its view on CD1d antigen presentation and the structures that could have the potential to activate regulatory CD1d restricted NKT cells. Clearly, identifying specific antigens able to enhance regulatory NKT cells function should be important in finding interesting candidates for therapeutic disease modifying interventions in autoimmune diseases. However, to develop this further, one should better investigate the capacity of these NKT cells to overcome an on-going autoimmune process. In our studies, mice were immunized with the immunoregulatory peptide prior to disease inductions. These protocols should be repeated, but with the peptide immunization performed during the disease course, where a chronic pathogenic inflammation has already been established. One should also better characterize the influence of the dose of the peptide, and the adjuvant used for enhancing the response, to optimize the induction of regulatory NKT cells. It is clear from the studies using α-GalCer to activate type I NKT cells that the protocol used for administration is strongly influencing the type of NKT cell response elicited. Treatment with α-GalCer could induce both prevention and acceleration of disease, depending on the timing of administrations, the number of administrations, and the route of administration, and this should be studied for $mCII_{707-721}$ activation as well. In addition, the capacity of human CD1 molecules to present the studied mouse collagen peptide to human NKT cells, and the type of response it would evoke, should be investigated. Even though there is a high degree of conservation amongst species in terms of NKT cells capacity to recognize CD1, there are by no means any guarantees of cross reactivity between the species in terms of $mCII_{707-721}$ recognition and cell activation, and this needs to be clarified. However, should no cross reactivity be confirmed, molecular modification of the $mCII_{707-721}$ might still prove to be a viable antigen also in a human system.

Elucidating the mechanisms by which CD1d restricted NKT cells regulate autoimmunity and inflammation will further facilitate the understanding of their role in the immune system. It has earlier been suggested that NKT cells mainly suppress autoimmunity by secreting large amounts of Th2 cytokines, in particular IL-4 and IL-10, and thereby shifting the immune response to a status regarded as less pathogenic in the autoimmune context. In our studies we found data indicating that this is not necessarily the case, and we could not show a classical Th1/Th2 shift during the manifested regulations in any of the investigated models. Instead, our results show that NKT cells might be able to induce apoptosis via Fas-FasL and suppress T cells in a cell-cell contact dependent manner; as has been described for NKT cells killing of tumour cells and γδ cells killing of regulatory T cells. This finding opens up a new view on how NKT cell mediated regulation of autoimmunity can be carried out, and should be investigated further. However, the role for cytokines and bystander suppression is still relevant, and might very well be a part of the regulatory pathway by which NKT cells act. For example, we

found TGF-β to be produced by the $mCII_{707-721}$ activated immunoregulatory NKT cells, and we also saw a parallel between lack of TGF-β expression in the inflamed CNS of CD1d deficient mice and worsened EAE. This indicates that TGF-β could be of importance when CD1d restricted NKT cells suppress autoimmunity, either by direct production by activated NKT ells, or as a down-stream mechanism. In addition, we showed that the $mCII_{707-721}$ specific NKT cells have a capacity of producing high levels of cytokines, and others have described the cytokine dependent nature of the NKT cell driven control in autoimmune models. However, modulation of the immune response through vigorous cytokine production is not the complete picture for how NKT cells exert control, and we show here that it should be complemented by the capacity of these cells to induce apoptosis in activated pathogenic T cells, and thereby end an immune reaction. Studies by other groups have also showed that NKT cells can act via tolerogenic instructions to dendritic cells. This pathway was not the focus of our work, but it should be interesting to study the peptide-specific NKT cells in this context, and investigate any possible influences on dendritic cell capacity to induce pathogenic or tolerogenic T cells. In addition, the NKT cell subset responding to the $mCII_{707-721}$ should be further described in terms of surface markers, cytokine profiling, and intracellular signalling pathways upon activation.

Taken together, our studies show that properly activated, NKT cells can indeed be beneficial in protecting against autoimmune diseases. The fact that we were able to show that NKT cells can be regulatory cells in both CIA, AIA, and EAE, as well as in other inflammatory conditions such as DTH and antigen induced airway inflammation points to the important notion that this regulation is neither restricted to a certain tissue, nor restricted to the Th1/Th2 paradigm, but it is operative during a variety of immune driven reactions. These studies provide a step on the way towards identifying a role for NKT cells for therapeutic purposes in the future, and specifically for self-peptides as possible activators of regulation through CD1 restricted NKT cells. Without doubt, more knowledge of the nature of this response and its possible translation to a human setting is needed, but the data presented here in should provide the start of such studies. Hopefully this will lead to beneficial treatments for patients with autoimmune diseases in the future.

Populärvetenskaplig sammanfattning

Immunsystemets främsta uppgift är att bekämpa infektioner och tumörer samt att läka vävnadsskador. För att göra detta har det i däggdjur utvecklats ett komplicerat system av celler och molekyler som samspelar i ett stort nätverk – immunsystemet. Läran om detta system kallas immunologi, och syftar till att öka vår kunskap om hur immunsystemet fungerar i så väl en frisk kropp som i en sjuk.

En invasion av skadliga bakterier eller andra mikroorganismer, eller en okontrollerad tillväxt av tumörceller får fatala följder för individen. Därför krävs det en kraftig reaktion för att bekämpa detta, och immunsystemet är utrustat för att kunna döda andra celler vid behov. Alla har nog känt av detta kraftfulla maskineri i form av en smärtsam inflammation, immunsystemets svar på provokation och skada. Ett så kraftfullt system kräver nogsam reglering, och för att immunsystemet i våra kroppar inte skall löpa amok finns det reglerande funktioner som kontrollerar dess aktivitet. En viktig sådan är immunsystemets förmåga att se skillnad på den egna kroppens celler och andra främmande typer av celler. Därigenom undviks att den egna kroppen angrips och dödas, utan bara sådana celler som är främmande för kroppen, ex bakterier eller tumörceller. När denna kontrollmekanism inte fungerar som den ska kan en så kallad autoimmun sjukdom uppstå. Då angriper immunsystemet felaktigt den egna kroppens vävnad, vilket kan leda till allvarliga följder för individen. Exempel på sådana sjukdomar är multipel skleros (MS), reumatoid artrit (RA) samt ungdomsdiabetes, vilka alla är allvarliga och livslånga sjukdomar. Idag finns ingen bot för dessa sjukdomar, utan man behandlar patienter med läkemedel för att försöka mildra och dämpa sjukdomens förlopp och för att lindra dess symptom. Många av de läkemedel som idag används påverkar immunsystemet relativt oprecist, och ger därför ofta oönskade bieffekter utan tillräckligt bra önskad effekt. Om vi visste mer om immunsystemet, skulle vi kunna få fram bättre mediciner mot dessa allvarliga autoimmuna sjukdomar, och vi kan även på sikt hoppas på att kunna hitta botemedel. Därför är det viktigt att studera immunsystemet och dess reglering i autoimmunitet.

Genom att använda djurmodeller som efterliknar människors autoimmuna sjukdomar kan man ställa, och få svar på, frågor som inte går att undersöka i människor. Vi kan till exempel ha bättre kontroll över geners inflytande och miljöpåverkan i våra djurexperiment. Vi kan titta på hur immunsystemet ser ut i ett djur som vi vet kommer att bli sjukt, innan sjukdomen har brutit ut, vilket är omöjligt i människor. Viktigt är även att vi kan titta på immunsystemets celler och komponenter i detalj. Genom att ta ut organ från djur och undersöka med metoder

för cellodling, eller granska organet i mikroskop, kan vi se vilka delar av immunsystemet som är aktivt och på vilket sätt det agerar. På så sätt får vi mer kunskap om hur varje del av immunsystemet fungerar, och kan förhoppningsvis på sikt utveckla läkemedel som påverkar exakt den mekanism som är fel i en autoimmun sjukdom.

I mitt arbete har jag undersökt en typ av celler i immunsystemet som kallas NKT celler. Detta är en specialiserad celltyp som man tror kan ha betydelse för hur immunsystemets andra celler uppträder och reagerar. Bland annat så har man sett att djur som inte har NKT celler har lättare för att utveckla olika typer autoimmuna sjukdomar, och därför har man dragit slutsatsen att NKT celler på något sätt kan reglera immunsystemet. Jag har tittat på NKT cellers funktion, främst i olika musmodeller för autoimmunitet. Två modeller som liknar RA, som kallas AIA och CIA, och en som liknar MS och kallas EAE, har använts i mina studier. I båda modellerna gör man mössen sjuka genom att injicera ett protein som kommer från det organet som sedan blir angripet av immunsystemet. I CIA ger man mössen en injektion av kollagen som bygger upp brosk, vilket gör att ledernas brosk blir inflammerat och bryts ned av immunceller, och mössen får svårt att gå. I EAE ger man en injektion av det ämne som ligger runt nervcellerna för att isolera och skydda dessa, myelin, vilket gör att hjärnan och ryggmärgen blir inflammerade och mössen får neurologiska symptom som t ex förlamning. När jag har undersökt om NKT celler är viktiga i dessa modeller har jag sett att dessa celler till viss del kan reglera sjukdomarna. I en studie såg jag att om möss har NKT celler utvecklas mindre EAE än om dom har blivit genetiskt modifierade till att inte ha NKT celler, vilket tyder på att NKT celler kan kontrollera sjukdomsförloppet. Det samma gäller för CIA och AIA, möss som saknar NKT celler utvecklar mer artrit än möss med ett normal fungerande immunsystem. Jag har även hittat en del av ett protein - en peptid - som man kan använda för att aktivera NKT celler. Om man vaccinerar möss med den peptiden svarar NKT celler genom att blir fler och mer aktiva. Vi upptäckte att om man aktiverade NKT celler med peptiden i möss innan vi inducerade EAE eller CIA, så blev inte mössen lika sjuka som icke-vaccinerade möss. Detta tyder återigen på att NKT celler har kapacitet att reglera autoimmuna sjukdomar. Ur ett behandlingsperspektiv är detta särskilt viktigt, eftersom man kanske skulle kunna aktivera NKT celler i sjuka människor på samma sätt. NKT celler i möss och människor liknar varandra väldigt mycket, och kan troligtvis aktiveras av ungefär samma saker. Vad man däremot inte vet så mycket om, är om NKT celler är viktiga i regleringen av autoimmuna sjukdomar i människor också, inte bara i djur. Även om det finns studier som tyder på att det kan vara så, och djurmodeller kan användas för att ge information om människans sjukdomar, behövs det mer kunskap från människor innan man vet med säkerhet. Min avhandling ger information som jag tror kan vara användbar i framtiden till att utveckla läkemedel mot autoimmuna sjukdomar.

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