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Natural killer T cell subsets and regulation of autoimmune diabetes

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Natural Killer T cell subsets and regulation of autoimmune diabetes

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Abstract

This thesis is focused on natural killer (NK) T cells. NKT cells recognize lipids and glycolipids, rather than peptides, in the contents of the antigen presenting molecule CD1d. NKT cells have a surface phenotype reminiscent of memory cells, and have been shown to rapidly produce large amounts of cytokines, such as IL-4 and IFN-gamma, upon activation. Because of their rapid response to activation, NKT cells have been suggested to play a role in several different immunological situations, such as clearance of pathogens, tumor rejection and regulation of autoimmune reactions. The broad spectrum of their activities suggested that functionally different subsets of NKT cells may exist. We have been able to demonstrate two functionally distinct splenic NKT cell populations identified by their surface phenotype and cytokine secretion profile. Previous reports of reduced autoimmune diabetes incidence in NOD mice related to an artificially increased NKT cell population, have been attributed to the enhanced production of IL-4 by classical NKT cells. We show that an overexpression in NOD mice of non-classical NKT cells, producing high levels of IFN-gamma but low amounts of IL-4, leads to prevention of autoimmune diabetes. This demonstrates that both classical and non-classical NKT cells possess immuno-regulatory functions. Finding out which mechanisms that are shared by all NKT cells and which that are not, will broaden our knowledge on NKT cell biology and increase the possibility to control the immune system in a way that may prevent diseases and autoimmunity.

Key words: CD1d, NKT cells, autoimmune diabetes

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Martin Stenström

This thesis will be defended on the 16th of December 2005
at 9 am in GK-salen at BMC, Sölvegatan 19, Lund.
Faculty Opponent will be Dr. P. Dellabona,
San Raffaele Scientific Institute, Milano, Italy

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Original papers

This thesis is based on the following original papers, which are referred to in the text by their roman numerals (**I-III**).

I. Stenström[*], M., Sköld[*], M., Ericsson, A., Beaudoin, L., Sidobre, S., Kronenberg, M., Lehuen, A. and Cardell S. Surface receptors identify mouse NK1.1[+] T cell subsets distinguished by function and T cell receptor type. *Eur. J. Immunol.* 2004. 34:56-65.

II. Stenström, M., Sköld, M., Andersson, Å. and Cardell L., S. Natural killer T-cell populations in C57BL/6 and NK1.1 congenic BALB.NK mice – a novel thymic subset defined in BALB.NK mice. *Immunology* 2005. 114:336-345.

III. Duarte[*], N., Stenström[*], M., Campino, S., Bergman, M-L., Lundholm, M., Holmberg, D. and Cardell L., S. Prevention of diabetes in nonobese diabetic mice mediated by CD1d-restricted nonclassical NKT cells. *J. Immunol.* 2004. 173:3112-3118.

[*] Contributed equally.

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Abbreviations

α–GalCer	α-galactosylceramide	LN	lymph node
APC	antigen presenting cell	PaLN	pancreatic LN
β_2m	β_2-microglobulin	mAb	monoclonal antibody
B6	C57Bl/6	MHC	major histocompatibility
C	constant		complex
CD	cluster of differentiation	NK	natural killer
D	diversity	NOD	nonobese diabetic
DC	dendritic cell	RAG	recombination activating
DN	double negative		gene
DP	double positive	SP	single positive
ER	endoplasmatic reticulum	Scid	severe combined
H-2	histocompatibility-2		immune-deficiency
HLA	human leukocyte antigen	T1D	type 1 diabetes
IDDM	insulin dependent	TAP	transporter associated with
	diabetes mellitus		antigen processing
IFN	interferon	TCR	T cell receptor
IL	interleukin	tet	tetramer
J	joining	Th	T helper
KIR	killer immunoglobulin-	V	variable
	like receptors	VLA	very late antigen

Introduction

As far back as I can remember I have enjoyed solving and understanding difficult and complicated problems, and I am quite sure that I am not the only one. I believe that it is in the human nature and it is one of the most important properties that we have been given. So when I first came in contact with the field of immunology it was love at first sight. The immune system is a system present in almost every corner of the entire human body and without it we would immediately get infected and die. In a general description the purpose of the immune system is to attack and get rid of everything that is non-self and abnormal, and doing so without damaging yourself. When you think of everything that you would encounter during a lifetime, you have to confess that a system capable of doing this must indeed be both very complicated and, hence, also difficult to understand. Still, one cannot refuse to admit that we have come a far way in our understanding of how the immune system works, considering that most of the progress has been accomplished during the last decades. Fortunately for me, there is still a lot left to understand.

This thesis is focused on a subpopulation of T lymphocytes called natural killer (NK) T cells. They have been described to play an important role in several different immunological situations, such as autoimmunity, tumor rejection, and immune responses against bacteria and parasites. They are clearly separated from conventional T lymphocytes, as they are restricted to recognizing antigens presented on the cluster of differentiation (CD) 1 molecule. While classical antigen presenting molecules present antigenic peptides this molecule present lipids and glycolipids. During the last years several aspects of lipid recognition and the immunological relevance of lipid-specific T cells have been revealed, resulting in an increased interest in the nature of NKT cells.

In this thesis we ask if functional subsets within the NKT cell population can be distinguished by distinct surface molecule expression and cytokine production profile. We determined that CD69 and CD49b expression divides the NKT cell population into two functional subsets. Secondly, as most reports on NKT cells have been done in one single mouse strain, called C57Bl/6 (B6), we asked if the genetic background could influence the composition of the NKT cell population. We compared the distribution of NKT cell subsets in B6 mice with another mouse strain, named BALB.NK. We detected a clear difference in the size and ratio of the two

functional subsets between B6 and BALB.NK mice. Finally, one of the NKT cell subsets, called invariant or $V\alpha14$ NKT cells, has been shown to have a regulatory capacity in autoimmune diabetes. We wondered if non-classical (non-$V\alpha14$) NKT cells shared the same ability. We used a transgenic mouse model with an increased non-classical NKT cell population to show that these cells also have the capacity to prevent or delay the onset of diabetes.

General view of the immune system

To protect us from pathogenic invaders and eliminate altered self-cells, the immune system has developed during the vertebrate evolution. In general the immune system can be divided into two parts, innate and adaptive immunity. These two parts do not operate independently of each other, they rather function as a highly interactive and cooperative system that together achieve a more effective response than either could on its own. However, there is a major difference in their ability to specifically strike on a given antigen. NKT cells are an unconventional population of T lymphocytes that operate on the border between the innate and adaptive immune system, in that they share some characteristics of both systems.

Innate immunity

Innate immunity is called so because it is present at birth. The innate immune system is our first line of defense. It acts fast and is dependent on pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMP). Macrophages, neutrophils, eosinophils, and basophils, each a type of cell with a slightly different function, are all cells involved in the innate immune response. Their main function is to ingest bacteria, foreign cells, parasites, and damaged and dead cells, as well as helping in attracting other immune cells. Another cell population is the natural killer (NK) cells. They are called NK because of their ability to, as soon as they are formed, kill other cells, by releasing enzymes and proteins that break the membrane of the target cell. NKT cells are a population that shares receptor expression and some functional abilities with NK cells. They are involved in the early stages of an immune response and are rapid producers of several cytokines. Innate immunity also involves the complement system, which is built up by more than 30 proteins, acting in sequence, with the potential to neutralize viruses and directly kill bacteria, or help other cells of the innate system in destroying bacteria.

Adaptive immunity

Adaptive immunity, also known as specific immunity, is not present at birth; hence you could say it is acquired. During life, as the immune system encounters different antigens, it will learn the most effective way to attack each antigen, and develop a memory for that specific antigen. The only true adaptive cell populations are T and B

lymphocytes, but the adaptive immune system also involves dendritic cells (DC), secreted antibodies and cytokines. It is called adaptive immunity because of the ability of T and B lymphocytes to rearrange their antigen receptors and clonal selection of antigen specific lymphocytes during an immune response. Because of this process the adaptive immunity will need more time to initiate an attack when encountering a new antigen, as compared to the innate response. However, when exposed to a previously seen antigen the response will be faster than the primary response. B lymphocytes produce antibodies, which are the part of the immune system that is responsible for the results of Edward Jenner, when he already in 1796 discovered that cowpox could protect against human smallpox. The two major populations of T lymphocytes are the $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells. $CD8^+$ T cells have the capacity to directly kill infected or abnormal cells, while $CD4^+$ T cells work as "helper" cells to activate other cells of the immune system. $CD4^+$ T cells are divided into two functional categories, T helper (Th) 1 cells that mainly activate macrophages to kill pathogens, and Th2 cells that are involved in the activation of B cells to produce antibodies. NKT cells are considered a subpopulation of T lymphocytes. They express a T cell receptor (TCR), originate from the same precursor cell, and are thymus dependent for their development, just as conventional T cells.

A brief history of natural killer T cells

The first reports of this unconventional T cell population came in 1987, when three papers were published on a distinct subset of T cells in mice that were CD4$^-$CD8$^-$ double negative (DN), had intermediate levels of TCR expression and a bias in their TCRβ usage towards variable (V)β8 (Budd et al., 1987; Fowlkes et al., 1987; Ceredig et al., 1987). T cells with a NK like phenotype were first reported in 1989 (Yankelevich et al., 1989), followed by another observation in 1990 (Sykes, 1990). Both reports show the presence of αβTCR$^+$NK1.1$^+$ T cells in the mouse bone marrow. These cells expressed CD3 and αβTCR as well as the pan-NK cell marker NK1.1. NK1.1$^+$ cells in the thymus were shown to be biased in their TCRβ usage towards Vβ8 and Vβ7 (Ballas and Rasmussen, 1990; Arase et al., 1992).

The interest in this T cell population was increased when it was shown that they were potent producers of immunoregulatory cytokines, such as interleukin (IL)-4, interferon (IFN)-γ, and tumor-necrosis factor (TNF) (Zlotnik et al., 1992).

In mice, the majority of these cells expressed an invariant TCR Vα chain joined with an invariant joining (J)α chain, called Vα14Jα18 (formerly known as Jα281) (Lantz and Bendelac, 1994; Makino et al., 1995), with the corresponding Vα24JαQ TCR used by similar cells in humans (Dellabona et al., 1994; Porcelli et al., 1993). The mouse Vα14Jα18 receptor was already in 1986 isolated from a suppressor T cell hybridoma (Imai et al., 1986).

The cells were given the name NK1 T cells, which later on was shortened to NKT cells. These cells were clearly distinct from conventional T lymphocytes, as they acquired an activated surface phenotype already at maturation with the capacity to rapidly secrete large amounts of cytokines upon stimulation without the need for proliferation, and expressed a limited set of α and β TCR chains (reviewed in Bendelac, 1995b; MacDonald, 1995; Bix and Locksley, 1995). In absolute numbers the TCRβ$^+$NK1.1$^+$ cell population in the mouse was shown to be a rather small population compared to conventional T cells, about 1×10^6 cells per organ, in thymus, spleen, liver, and bone marrow.

Instead of being restricted to the classical antigen presenting molecules, NKT cells that carry the Vα14Jα18 receptor were shown to be dependent on a non-classical

presenting molecule called CD1d (Bendelac et al., 1995c). Even though the majority of NKT cells in mice expresses the Vα14Jα18 receptor, a subset of CD1d-dependent NKT cells that have a non-Vα14/Vα24 TCR have been shown to exist in both mice (Cardell et al., 1995; Chiu et al., 1999; Behar et al., 1999) and humans (Behar et al., 2001; Exley et al., 2002; Fuss et al., 2004). Interestingly, the Vα24 expressing NKT cells constitute only a minor part of the total NKT cell population in humans. Later, the CD1d molecule was shown to have the capability to present lipids and glycolipids to T cells (Kawano et al., 1997; Spada et al., 1998).

At this point it was accepted that mouse TCRβ^+NK1.1$^+$ cells were a separate T cell population, with a limited TCR repertoire, expression of NK cell and memory T cell markers, CD1-restriction, and a strong cytokine-producing capacity. Similar populations have been identified in other primates (Kashiwase et al., 2003; Motsinger et al., 2003) and rats (Matsuura et al., 2000).

Antigen presentation

The function of T cells is completely dependent on their ability to recognize cells that has been infected by or taken up a pathogen or its products. T cells achieve this by recognizing fragments of pathogen-derived proteins loaded on an antigen-presenting molecule, sometimes presented by an antigen-presenting cell (APC) (Clevers et al. 1988). This interaction, between an antigen presenting molecule and the antigen recognizing TCR is crucial in several steps during T cell development, priming and activation. To become loaded and presented on an antigen-presenting molecule, the original protein is modified by an event referred to as antigen processing. The actual display of the fragment is called antigen presentation. During the evolution of antigen presentation, different molecules have developed that process and present antigens of different type and origin to T cells. The classical major histocompatibility complex (MHC) class I and II are responsible for presentation of antigens to the absolute majority of T cells. The restriction of T cells to MHC was established in 1974 (Zinkernagel, 1974) and at that time, the link between the MHC locus and the development of an immune response was already known. Additionally to the MHC class I and II, there are other presenting molecules like the CD1 molecule, which NKT cells are restricted to (Bendelac et al., 1995c).

Classical MHC class I
The restriction of T cells to MHC class I was shown by Doherty and Zinkernagel in 1975 (Doherty and Zinkernagel, 1975). The class I loci are called human leukocyte antigen (HLA)-A, HLA-B, and HLA-C in humans, and histocompatibility (H)-2K, H-2D, and H-2L in mice. The MHC class I molecule is expressed on most somatic cells. The molecule is assembled in the endoplasmatic reticulum (ER), and it consists of one transmembrane glycoprotein, the heavy chain, and a soluble protein, the light chain, called β_2-microglobulin (β_2m) (Bjorkman et al., 1987). The genes are highly polymorphic and each locus is codominantly expressed, yielding a high diversity in peptide-binding specificity. The peptide binds to the groove formed by the extracellular heavy chain $\alpha 1$ and $\alpha 2$ domains, which together with the peptide are involved in the interaction with the TCR.

Peptides loaded on the MHC class I molecule are 8-10 amino acids long (Rötzschke et al, 1990), and are generated from the intracellular compartment (Gooding and

O'Connell, 1983; Townsend et al., 1985; Moore et al., 1988), where proteins are degraded and then transported, by the transporter associated with antigen processing (TAP), into the ER. Inside the ER, the peptides are loaded onto newly formed MHC I molecules by a complex composed of several chaperons (Cresswell et al., 1999). Exogenous antigens can also be presented by MHC class I molecules, in a process known as cross presentation (Norbury et al, 1997; Wick and Ljunggren, 1999). When loaded, the peptide-MHC I complex is rapidly transported, via the Golgi apparatus, to the cell surface where it interacts with CD8^{+} cytotoxic T cells.

Classical MHC class II

The cell populations expressing the MHC class II molecule are more limited and it is mainly found on dendritic cells, macrophages, and B cells. The class II loci have been named HLA-DR, HLA-DP, and HLA-DQ, in humans, and H-2A, and H-2E in mice. The molecule consists of two transmembrane glycoproteins (Brown et al., 1993), an α and β chain, that just like the MHC class I molecule is assembled in the ER. The two distal domains, α1 and β1, build up the peptide-binding groove.

In contrast to MHC class I, MHC class II is not loaded with peptide in the ER but associates with an invariant chain, that blocks access to the peptide binding groove. The newly formed MHC class II/invariant chain complex leaves the ER to be transported and introduced into the endocytic pathway. In the endosomes and lysosomes the invariant chain is degraded and the MHC class II molecule becomes competent to bind antigenic peptides (reviewed in Guermonprez et al., 2002). Hereby, MHC class II is predominantly loaded with peptides derived from proteins internalized by the presenting cell. Compared to MHC class I, peptides loaded onto the MHC class II are longer, 13-18 amino acids (Rudensky et al., 1991; Chicz et al., 1993). When loaded, the MHC class II is expressed on the surface, where it interacts with CD4^{+} T helper cells.

CD1 – a non-classical MHC class I molecule family

In addition to MHC class I & II, several molecules structurally related to MHC class I have been found, called MHC class 1b molecules. These molecules are less polymorphic than the classical MHC class I molecule and are more specialized and interact with more limited subsets of lymphoid cells. The majority of these proteins associates with the β_2m and has genes that are linked to MHC. NKT cells, the focus

of this thesis, recognize antigen on an MHC class 1b molecule called CD1d, belonging to the CD1 family (Bendelac et al., 1995c), and are often referred to as CD1d-restricted T cells. The CD1 family proteins associate with β_2m but are not MHC linked, and present lipids rather then peptides. In mice, CD1d is expressed on dendritic cells, macrophages, B cells, mature and immature thymocytes, and peripheral T cells. Outside the lymphoid system, CD1d-expression has been observed in liver and gastrointestinal epithelium (Bleicher et al., 1990; Brossay et al., 1997; Roark et al., 1998; Mandal et al., 1998).

CD1 family genes
The CD1 molecules were first identified using monoclonal antibodies that bound to human thymocytes (McMichael et al., 1979). The structure of the CD1 family genes are comparable to MHC class I heavy chain genes, consisting of an α1, α2, and α3 domain (Martin et al., 1987). The CD1 family genes form a distinct locus of tightly clustered genes located in chromosome 1 in humans and on chromosome 3 in mice (Calabi and Milstein, 1986; Albertson et al., 1988; Moseley et al., 1989). In humans there are five isoforms of CD1 expressed (CD1a-e) that, based on sequence identities in the α1 and α2 domains, can be divided into two groups. Group 1 includes CD1a, b, and c, and group 2 includes CD1d, while CD1e is intermediate (Calabi et al., 1989). The CD1d protein is the only CD1 protein expressed in mice (Balk et al., 1991). Mice carry two homologous CD1d genes, both being very similar to the human CD1d gene. The lack of other CD1 genes in rodents is commonly believed to be due to a chromosomal break event.

The complete CD1 molecule consists of a heavy chain non-covalently paired with β_2m (Sugita et al., 1997). CD1 heavy chain is translocated into the ER, where it associates with calnexin, calreticulin and the thiol oxidoreductase Erp57 (Kang and Cresswell, 2002). The association with β_2m appears to be necessary for cell surface localization of CD1d, as the absence of β_2m in β_2m-deficient mice, leads to no surface expression of CD1d (Bauer et al., 1997; Brutkiewicz et al., 1995). Although expression of human and mouse CD1d at the cell surface without β_2m has been detected (Balk et al., 1994), the functional significance of β_2m-free CD1d is not known. The α1 and α2 domain form the binding groove that consists of a narrow and deep hydrophobic cleft (Zeng et el., 1997), formed by two interconnecting pockets in human CD1a and mouse CD1d, and by three pockets and one interconnecting hydrophobic channel in CD1b. The different CD1 molecules bind lipids with

different structures; however, some antigens promiscuously bind to several CD1 isoforms (Shamshiev et al., 2002).

Other than in mice and humans, CD1 family genes have been found in rat, rabbit, guinea pig, sheep, cow, and rhesus macaque. The low polymorphism of the CD1 proteins is not clearly understood. One reasons may be the relatively low variation in the antigen presented, compared to antigenic peptides presented on classical MHC. As a result CD1 has not needed to evolve high polymorphism to accommodate their binding.

CD1 antigen loading
The molecule that was first identified to be presented by a CD1 molecule was a cell wall component from *Mycobacterium tuberculosis* binding human CD1b (Beckman et al., 1994), revealing the lipid binding capacity of CD1 molecules. This was confirmed when also human CD1c was shown to bind microbial glycolipids (Beckman et al., 1996), and later on also human CD1a (Rosat et al., 1999). The first lipid shown to bind to CD1d was α-galactosylceramide (α-GalCer) (Kawano et al., 1997; Spada et al., 1998). Both mouse and human CD1d was shown to be able to present α-GalCer to mouse Vα14 NKT cells (Naidenko et al., 1999). Hydrophobic peptides have been shown to bind mouse CD1d (Castano et al., 1995), but it is most likely that lipids and glycolipids are the main source of antigens presented on CD1d. Lipid antigens differ from peptides in an important feature, they are not soluble in water. This makes the biology and immunogenicity of lipids different from that of peptides. Lipids are always associated with membranes or lipid-binding proteins in the tissue or fluids. So far, several lipid antigens of mycobacterial origin that can be loaded on different CD1 isoforms have been characterized. In 1997, mouse CD1d was shown to present glycolipids to T cells (Kawano et al., 1997). NKT cells can also be stimulated by exposure to CD1d in the absence of foreign antigens, which means that they are reactive to some self-antigens (Cardell et al., 1995; Bendelac et al., 1995c). Just as proteins, lipids are partially degraded, in late endosomes, into smaller molecules that are suited for CD1d loading and TCR interaction. Little is known about the enzymes that are required for generating immunogenic lipids.

The antigen loading of CD1d is TAP independent (Brutkiewicz et al., 1995), and takes place in endosomes and lysosomes, although, in some instances it may also occur on the cell surface (Shamshiev et al., 2000). Saposins, lipid-transfer proteins, have been shown to be required for the presentation of both endogenous (Zhou et al.,

2004a) and exogenous lipid antigens (Kang and Cresswell, 2004). The intracellular trafficking routes followed by CD1 molecules are distinct from those of the classical MHC molecules. Newly synthesized CD1 molecules are transported via the Golgi to the cell surface, with the exception of CD1e, before being internalized. In humans, CD1a routes to early endosomes and back to the plasma membrane, CD1b is transported to the late endosomes and traffics to the lysosomes, CD1c preferentially routes to early endosomes and to a lesser extent to the late endosomes and lysosomes, CD1d is transported mainly to the late endosomes and only partially to the lysosomes, CD1e accumulates in the Golgi and routes to the late endosomes, but it never reaches the plasma membrane. In contrast to human CD1d, mouse CD1d, which is the only molecule present in mice, is more prone to trafficking also the lysosomes. A fraction of mouse CD1d can also be directly transported to the late endosomes without first being expressed on the cell surface (reviewed in De Libero and Mori, 2005).

The Vα14 NKT cells seem more dependent on CD1d trafficking to endosomal compartments. CD1d presenting cells, which have a disrupted endosomal localization of CD1d, can still stimulate some CD1d-autoreative T cell hybridomas that express a non-Vα14 TCR, but not Vα14 expressing T cells hybridomas (Brossay et al., 1998). Similarly, the Vα14 NKT cell numbers were severely decreased in mice lacking adaptor protein AP-3, involved in the localization of membrane proteins to lysosomes (Elewaut et al., 2003b). Together, these findings suggest that Vα14 NKT cells and some non-Vα14 cells have different requirements for the trafficking of CD1d during antigen loading.

Definition of NKT cells

The original definition of NKT cells as NK1.1$^+$ $\alpha\beta$TCR$^+$ cells is clear, but its usage has with time come to cause a lot of misunderstandings. As the research on NKT cells has developed, several findings have shown that the TCRβ^+NK1.1$^+$ population is a heterogeneous population.

Clearly there exist some NK1.1$^+$ TCRβ^+ cells that are CD1d-independent (Chen et al., 1997a; Mendiratta et al., 1997; Hammond et al., 1999; Eberl et al., 1999c; Zeng et al., 1999a). These cells are mainly CD8$^+$ T cells, even though CD4$^+$ and DN cells can also be detected, and produce comparatively low levels of cytokines. Moreover, both CD8$^+$ and CD4$^+$ T cells can up regulate NK1.1, as well as CD49b, during an immune response (Assarsson et al., 2000; Slifka et al., 2000), while CD4$^+$ NK1.1$^+$ cells lose their NK1.1 expression when activated (Chen et al., 1997b).

Additionally, NK1.1 is only expressed in a few mouse strains. Because of this, several different sets of markers have been used to identify NKT cells, such as TCRβ^+CD4$^-$CD8$^-$ cells (Baxter et al., 1997), DX5$^+$CD3$^+$ cells (Moodycliffe et al., 2000), CD4$^+$CD44^{high} cells (Bendelac et al., 1994), and Ly49A$^+$CD122$^+$CD3$^+$ cells (Falcone et al., 1999). However, none of these combinations specifically stains all CD1d-restricted T cells. In my opinion, the term NKT cells should comprise all CD1d-restricted $\alpha\beta$TCR cells. In this thesis I will try to be clear on if the cell population referred to is CD1d-restricted or may include CD1d-independet cells, using the following names and definitions:

TCRβ^+NK1.1$^+$ cells	NK1.1$^+$ $\alpha\beta$ T cells, also including cells that are not restricted to CD1d and excluding some that are.
NKT cells	CD1d-restricted cells.
Vα14/Vα24 NKT cells	NKT cells that express the invariant Vα14/Vα24 TCR. Sometimes also called invariant NKT cells.
Non-Vα14/Vα24 NKT cells	NKT cells that do not express the invariant Vα14/Vα24 TCR. Sometimes also called non-classical NKT cells.

NKT cells – a subpopulation of T lymphocytes

T cells are a lymphocyte population belonging to the adaptive immune system. They all express a membrane bound TCR, with an enormous diversity established through mechanisms discussed below. The precursor cells, which are to become T cells, are originally produced in the bone marrow and then migrate to the thymus, were they continue their development until matured. In the thymus the TCR is assembled and expressed on the surface. Due to the high diversity, and to avoid cells recognizing self from leaving the thymus and entering the peripheral immune system, with the potential of devastating results, the precursor cells are undergoing a selection process for the ability to distinguish self from non-self (reviewed in Miller, 1999). In principal, only cells that are not activated by self-antigens are allowed to mature and leave the thymus. After leaving the thymus, T cells will circulate in the blood and the lymphatic system, entering the secondary lymphoid organs "searching" for evidence of foreign or abnormal cells, such as bacteria, infected cells or cancer cells. Similarly, NKT cells are dependent on the thymus for development and shares the earlier developmental stages with the mainstream T cells.

The T cell receptor

The TCR is in its basic construction strikingly similar to the antigen receptor expressed by B cells. Still, there are some major differences between T cell and B cell antigen recognition. The B cell receptor (BCR) recognize soluble antigen while the TCR only recognize antigen on the surface of a cell and in the context of MHC. The TCR is a heterodimer composed of an α and β chain ($\alpha\beta$TCR), or a γ and δ chain ($\gamma\delta$TCR). The $\alpha\beta$ receptor was first identified in 1982 (Allison et al., 1982) and soon thereafter the TCRβ chain was cloned (Hedrick et al., 1984; Yanagi et al., 1984). The $\gamma\delta$ receptor was found in 1986 (Brenner et al., 1986). Just as the BCR, the TCR consists of a variable and a constant domain.

Several separate gene segments, containing a variable number of sequences, encode each chain. The β chain is composed of a variable (V), diversity (D), joining (J), and constant (C) region, while the α chain consists of a V, J, and C region. To complete and transcribe the TCR, a sequence is randomly chosen for the V, D and J region and brought together by somatic rearrangements, carried out by recombination activating gene (RAG) proteins. These random events generate a high diversity in the specificity

of the TCR, which can be further increased by addition or removal of nucleotides in the joining sites (Leiden and Strominger, 1986).

Both the $\alpha\beta$ and $\gamma\delta$ TCR associates with the five-chain complex called CD3 (Samelson et al., 1985), which is involved in the TCR signal transduction. The TCR/CD3 complex is composed of a TCR, non-covalently associated pairs of CD3ε-CD3δ and CD3ε-CD3γ and disulfide-linked CD3ζ-CD3ζ homodimers or sometimes CD3ζ-CD3η heterodimers (reviewed in Clevers et al., 1988). In TCRβ^+NK1.1$^+$ cells the ζ-ζ/ζ-η dimers can be replaced by ζ-FcεRIγ heterodimers. The FcεRIγ chain is a component of the Fc receptor CD16. However, the FcεRIγ is not essential for the development of TCRβ^+NK1.1$^+$ cells as these cells develop normally in FcεRIγ deficient mice (Arase et al., 1995).

Development

T cells originate from hemapoetic stem cells, called the common lymphoid progenitor, which can differentiate into T cells, B cells, or NK cells. These progenitor cells migrate to the thymus, which was shown by Miller to be involved in the generation of T cells already in 1961 (discussed in Miller, 1999). After entering the thymus, the progenitors migrate through different thymic regions where the TCR selection occurs, to finally leave the thymus as naïve mature T cells.

T cell lineage commitment

As lymphoid progenitor cells enter the thymus, they go through several differentiation stages defined by the surface expression of CD25 and CD44, and further on by CD4 and CD8. Progenitor cells enter the thymus at the cortico-medullary junction, and migrate to the outer cortex. The progenitor cell is directed into T cell differentiation upon the binding of Notch-1 ligand (Pui et al., 1999; Radtke et al., 1999; Harman et al., 2003) expressed in the thymus (Felli et al., 1999; Anderson et al., 2001). The cell starts out as a CD4$^-$CD8$^-$ double negative (DN) thymocyte. During the DN stage four different surface phenotypes can be distinguished, CD44$^+$CD25$^-$ (DN1), CD44$^+$CD25$^+$ (DN2), CD44$^-$CD25$^+$ (DN3), and CD44$^-$CD25$^-$ (DN4) (Godfrey et al., 1993). During DN1 and DN2, thymocytes go through a proliferative stage (Kawamoto et al., 2003), after which RAG protein expression occurs, at stage DN2 and DN3. Simultaneously, TCRβ, γ, and δ chains are rearranged. A correctly assembled receptor is required for differentiation signals and further survival. For $\gamma\delta$ commitment, this means a complete $\gamma\delta$TCR/CD3 complex

(Bonneville et al. 1989). For αβ commitment, this means a TCRβ together with a invariant pre-TCRα chain, called pre-TCR, and the CD3 complex (Fehling et al., 1995). When a pre-TCR is expressed, further β chain rearrangement is arrested, leading to allelic exclusion (Uematsu et al., 1988). The precise mechanism for commitment to γδ or αβ expression is still debated. There are two suggested models, either the cells are precommitted to become γδ or αβ T cells (the selective model), or the expression of a functional γδ TCR or pre-TCR is sufficient to direct the cells to adopt a γδ or αβ cell fate (the instructive model). Upon pre-TCR signaling, thymocytes enter the DN4 stage, were a new proliferation phase precedes the expression of CD4 and CD8 coreceptors.

NKT cell lineage commitment
NKT cells use an αβTCR and are also dependent on pre-TCR expression for development (Eberl et al., 1999a), but instead of interacting with MHC to get survival signals, they interact with CD1d molecules. If some thymocytes are precommitted to become NKT cells or if it is an instructive pathway is under debate. Transgenic expression of the Vα14 chain or an α and β chain from a non-Vα14 NKT cell TCR was sufficient to direct cells into Vα14 and non-Vα14 NKT cell differentiation (Bendelac et al., 1996; Sköld et al., 2000; Paper III).

NKT cells differ from conventional T cells in that they are selected on CD1d instead of classical MHC class I and II, but also depended on other factors not necessary for conventional T cell development. The development of TCRβ$^+$NK1.1$^+$ cells in mice deficient for Fyn, a protein tyrosine kinase, was severely decreased while they had normal levels of conventional T cells (Eberl et al., 1999b; Gadue et al., 1999). The adaptor protein SAP, known to associate with and activate Fyn, has also been shown to be required for efficient TCRβ$^+$NK1.1$^+$ and Vα14 NKT cell development (Pasquier et al., 2005; Nichols et al., 2005). Mice lacking the expression of lymphotoxin had relatively normal conventional T and B cell numbers but showed a dramatic reduction in TCRβ$^+$NK1.1$^+$ cell numbers. The lack of lymphotoxin had a strong effect on differentiation of TCRβ$^+$NK1.1$^+$ cells early in ontogeny (Elewaut et al., 2000). In the absence of RelB, a transcription factor, and functional nuclear factor κB inducing kinase (NIK), Vα14 NKT cell differentiation was blocked at a late stage, after TCR acquisition, while conventional T cells and B cells differentiated with no major abnormalities (Elewaut et al., 2003a; Sivakumar et al., 2003).

TCR expression

The proliferation at the DN4 stage generates a pool of CD4$^+$CD8$^+$ double positive (DP) thymocytes with a productive TCRβ gene rearrangement. A second wave of RAG expression drives the DP thymocytes into TCRα chain rearrangement (Wilson et al., 1994). A successful gene rearrangement results in a TCRα chain that combines with the already existing TCRβ chain to form a αβTCR. The complete αβTCR/CD3 complex is then expressed on the surface of the DP thymocytes, where the selection process is initiated. At this stage, these DP thymocytes still have the possibility to be committed to the NKT cell lineage (Gapin et al., 2001). Rearrangement of the α chain continues until a αβTCR that can recognize an antigen-presenting molecule is expressed on the surface (Turka et al, 1991; Brandle et al., 1992). As a result, any given T cell only expresses one specific TCR. However, as a consequence of the late termination of α chain rearrangement, some T cells may express two different α chains (Padovan et al., 1993).

TCR selection

Of all the different TCR specificities expressed by the thymocytes, only a small number have a structure suitable for MHC/peptide engagement. In the thymus, cortical epithelial cells express MHC class I and class II loaded with self-peptides, and play a key role in the TCR selection of conventional T cells, a process referred to as positive selection.

In the positive selection, thymocytes expressing a TCR that cannot recognize any MHC/antigen complex, will not receive a survival signal and therefore enter into programmed cell death, apoptosis. Consequently, only cells that express a TCR that have the ability to communicate with antigen presenting molecules will survive (Wilkinson et al., 1995).

The selection of TCR expression on NKT cells seems to be under high pressure, as the TCR usage on at least a subset of mature NKT cells are biased toward specific α and β chains. Importantly, human Vα24 NKT cells are not as dominant among human NKT cells as Vα14 NKT cells in mice. The β chain expression on these cells is more diverse but predominantly uses Vβ8 (>50%), Vβ7, or Vβ2 in mice, and Vβ11 in humans. Even though the predominant use of the Vα14 chain among NKT cells in mice implicates a possible directed rearrangement, the TCR rearrangement in NKT cells is likely to be a random event, as shown by Shimamura et al. (1997).

NKT cells are selected on CD1d and can develop in mice lacking the expression of MHC class II (Cardell et al., 1995), MHC class I (Xu et al., 2003) or in the absence of TAP (Adachi et al., 1995). Cortical epithelial cells involved in the positive selection of conventional T cells do not mediate positive selection of NKT cells. Instead, NKT cells are selected on CD1d expressing DP thymocytes (Bix et al., 1993; Coles and Raulet, 1994; Bendelac et al., 1995a; Coles and Raulet, 2000). Apparently, CD1d interaction is not the only signal required for NKT cell development, as CD1d expression by thymic epithelial cells under the control of a MHC class II promoter in CD1d deficient mice does not support NKT cell development (Forestier et al., 2003).

Even though TCR signaling is essential for survival and differentiation of developing thymocytes, as described above, not all TCR ligation results in survival. Self-peptides must not lead to activation of the mature T cell. On the contrary, if the signaling is too strong, with the potential to induce autoimmunity, the T cells will be negatively selected, a process called clonal deletion (Kappler et al., 1987).

Several studies suggest that NKT cells are also undergoing negative selection. Adding α-GalCer, a potent glycolipid ligand agonist for Vα14 NKT cells, to fetal thymic organ culture blocked the development of the cells (Pellicci et al., 2003). Restoring CD1d knockout mice with CD1d expression under the control of a MHC class I promoter only partially reconstituted the Vα14 NKT cell compartment (Xu et al., 2003). This was suggested to be due to a higher level of CD1d expression on cortical thymocytes in the transgenic mice, leading to an increased negative selection of Vα14 NKT cells. Similarly, increasing the level of CD1d expression caused a reduction in the number of Vα14 NKT cells and reduced Vβ8.2 chain usage (Chun et al., 2003). In line with this, the Vα14/Vβ8.2 TCR has been reported to have a higher avidity binding to α−GalCer loaded on CD1d, compared to Vα14/Vβ7 TCR (Schümann et al., 2003).

CD1d trafficking is important for the presentation of selecting ligands. In mice deficient for cathepsin L, a lysosomal cysteine protease involved in CD1d trafficking, Vα14 NKT cells are essentially absent while a non-Vα14 NKT cell population is detected in normal numbers (Honey et al., 2002). The adaptor protein AP-3, involved in the localization of membrane proteins to lysosomes, is required for Vα14 NKT cell development (Elewaut et al., 2003b). Together, these findings suggest that Vα14 NKT cells are selected by an antigen generated in the lysosomal compartment, while some non-Vα14 NKT cells are not.

CD4/CD8 lineage commitment in conventional T cells

Similar to $\gamma\delta$ and $\alpha\beta$ TCR commitment, there are two models of CD4/CD8 lineage commitment in conventional T cells, the instructive and the selective model. In the instructive model, the TCR signaling together with the signaling of the CD4 or CD8 coreceptors, CD4 if the TCR recognizes MHC class II and CD8 if the TCR recognizes MHC class I, is thought to be crucial for development of a CD4$^+$ or CD8$^+$ single positive (SP) mature T cell. It is suggested that the signal from the TCR, together with the CD4 signal is relatively strong, while TCR plus CD8 generates a weaker signal. The strong signal will induce CD4 commitment and terminate CD8 gene transcription, just as a weak signal will induce CD8 commitment and terminate CD4 gene transcription (Itano et al., 1996; Itano and Robey, 2000). The result is that DP thymocytes differentiate into CD4 or CD8 SP T cells with the correct TCR and coreceptor MHC specificity. Thus, in this model all cells with a MHC interacting TCR have the possibility to become a mature T cell. In the selective model the TCR/coreceptor signal is not important for lineage commitment. Instead, the TCR signal induces a random termination of one of the two coreceptor genes. In this short-lived stage only thymocytes with matching TCR and coreceptor MHC-specificity will receive a survival signal. Thymocytes expressing mismatching TCR and coreceptor MHC-specificity do not receive a survival signal and will undergo apoptosis (Davis et al., 1993; Corbella, et al., 1994). After becoming either SP CD4$^+$ or CD8$^+$ the cells will leave the thymus to circulate as mature T cells.

NKT cell surface phenotype

In mice, mature NKT cells are mainly CD4$^+$ or CD4$^-$CD8$^-$ DN, while they can also be CD8$^+$ in humans. The lack of CD8 expressing NKT cells in mice may be due to the enhancement of the signal by TCR and CD8 together to CD1d, as forced expression of CD8 is suggested to result in negative selection (Bendelac et al., 1994). The relevance of CD4 expression is quite mysterious, as it is a coreceptor binding to MHC class II molecules and does not bind to CD1d. However, studies have shown functional differences in human CD4$^+$ and CD4$^-$ Vα24 NKT cells (Gumperz et al., 2001).

Just as $\alpha\beta$ T cells, NKT cells are suggested to be differentiating via a CD4$^+$CD8$^+$ DP precursor (Gapin et al., 2001; Egawa et al., 2005). Thymic NKT cells are described to go through 3 stages of development, from CD44^{low}NK1.1$^-$ (stage 1) to

CD44^{high}NK1.1$^-$ (stage 2) to CD44^{high}NK1.1$^+$ (stage 3). Upregulation of other NK cell receptors, such as Ly49, NKG2D, Ly6 and 2B4, and CD122 occurs at the latest stage (Figure 1). Recently, HSA^{high} cells representing the earliest stages in Vα14 NKT cell development were characterized. These cells were shown to go through a CD4$^+$CD8$^+$ DP^{low} and CD4$^+$ stage, and displayed a CD44^{low}NK1.1$^-$ phenotype (Bendelac et al., 2005).
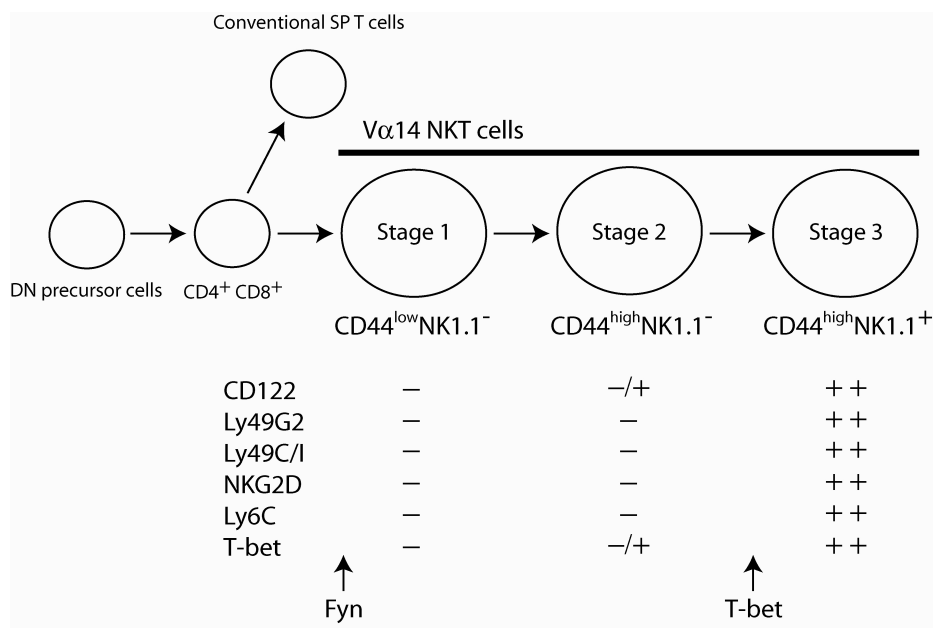
Figure 1. Phenotypic changes during stage 1-3 in Vα14 NKT cell maturation (adapted from Matsuda and Gapin, 2005).

NKT cells have an activated/memory surface phenotype, including high CD44 expression, CD69 expression, and low CD62L expression. High affinity interaction between TCR and CD1d during the development of NKT cells may be the reason of activation marker expression. CD69 is a very early activation marker upregulated on T cells, B cells, and NK cells shortly after stimulation. However, as shown in Paper I, not all NKT cells express CD69. CD44 is a late activation marker also found on memory cells, and CD62L, is an adhesion molecule involved in T cell homing to lymph nodes (LN) and known to be down regulated upon activation. As shown in humans and mice, Vα14/Vα24 NKT cells also have a chemokine receptor profile

similar to activated T cells, with the capacity to home to inflammatory sites (Thomas et al., 2003; Johnston et al., 2003).

NKT cells also share expression of some receptors commonly associated with NK cells, such as NK1.1, Ly49 receptors, and CD49b.

NK1.1 expression

NK1.1 upregulation is occurring late in the development of NKT cells. Some Vα14 NKT cells leave thymus as NK1.1⁻ cells and upregulate NK1.1 expression in the periphery (Pellicci et al., 2002). The NK1.1 receptor was originally thought to be a pan-NK cell marker, only expressed by NK cells, and can act as an activating receptor on both NK cells and NKT cells (Arase et al., 1996). The NK1.1 gene, NKR-P1C, is located in the mouse NK gene complex on chromosome 6 (Ryan et al., 1992). Four different haplotypes of the NKR-P1 gene complex are known in the mouse (Yokoyama et al., 1991). Two of these haplotypes are found in mouse strains that do not express the NK1.1 marker at all, or is at least not detectable by the NK1.1 antibody (Ab), such as BALB/c and NOD mice. The other two express NK1.1 on NK cells, and only one of them, which is the genotype of the C57Bl/6 (B6) mouse strain, expresses NK1.1 on both NK and NKT cells. This fact, that only a few mouse strains express the NK1.1 receptor (Giorda et al., 1992), has made comparative studies on TCRβ⁺NK1.1⁺ cells between different mouse strains rather limited.

To be able to compare the TCRβ⁺NK1.1⁺ population in B6 and BALB/c mice, we created a congenic BALB/c mouse that expresses the NK1.1 gene from the B6 background (Paper II). Another NK1.1 congenic BALB/c mouse strain, originally selected for cytomegalovirus resistance (Scalzo et al., 1995), as well as a NK1.1 congenic NOD mouse strain, was used in another comparative study published during our work, to evaluate the TCRβ⁺NK1.1⁺ cell population in these mice (Poulton et al., 2001). It was shown that TCRβ⁺NK1.1⁺ cells could be found in all three strains, but at the same time they questioned the NK1.1 receptor as an NKT cell marker. Part of the Vα14 NKT population, mainly CD4⁺ cells, did not express NK1.1, a phenomenon that was most pronounced in the NK1.1 congenic BALB.NK1.1 mouse (Hammond et al., 2001).

The development of CD1d-tetramers (tet) loaded with α-GalCer has made the identification of Vα14 NKT cells possible also in NK1.1 negative strains. Even in NK1.1 expressing strains, not all NKT cells express NK1.1 (Chen and Paul, 1998).

Through this technique it is also possible to study NK1.1$^-$ Vα14 NKT cells (Benlagha et al., 2000; Matsuda et al., 2000). α-GalCer loaded CD1d-tet specifically stains Vα14 NKT cells, leaving other CD1d-restricted NKT cells undetected. This limits the use of α-GalCer loaded CD1d-tet in the study of other NKT cell subsets. However, sulfatide loaded CD1d-tet have recently been shown to stain a non-Vα14 NKT cell population (Jahng et al., 2004).

Ly49 receptor expression
Just as NK1.1, Ly49 expression appears late in NKT cell development (Benlagha et al., 2002). Ly49 receptors are type II integral membrane proteins expressed by NK cells, subsets of NKT cells (Lantz et al., 1997; Ortaldo et al., 1998; Sköld and Cardell, 2000), memory CD8 T cells (Coles et al., 2000), and NK1.1$^+$TCR$\gamma\delta^+$ T cells (Emoto et al., 2000).

At present, 23 different Ly49 receptors are known (Ly49A-W), and they are either activating or inhibitory receptors, depending on the presence of immuno receptor tyrosine based activating or inhibitory motifs (ITAM/ITIM) in their intracellular tail. Different forms of Ly49 receptors have distinct specificities and bind selectively to certain allelic forms of MHC class I molecules (reviewed in Dimasi and Biassoni, 2005). Cell surface levels of Ly49 receptors on both NK and NKT cells are down-regulated in the presence of a cognate MHC class I ligand (Karlhofer et al., 1994; Held et al., 1996; Sköld et al., 2003).

The majority of the Ly49 receptors is inhibitory and work in concert with activating NK signals to control NK cell activation. Only inhibitory Ly49 receptors are expressed by NKT cells and may be a way to regulate potentially CD1d-autoreactive NKT cells. Engagement of the Ly49 receptors on CD3$^+$NK1.1$^+$ cells has been shown to influence the cytokine production and cytotoxic activity of (Ortaldo et al., 1998). As shown by Sköld and Cardell (2000), Ly49 expression is modulated by the presence of the MHC class I ligand, and engagement can inhibit the proliferation of TCRβ^+NK1.1$^+$ cells. Further, they showed that Ly49 receptor expression is organ-specific and differently expressed on CD4$^+$ and DN TCRβ^+NK1.1$^+$ cells. In line with this, Ly49 expression also seems to correlate to the TCR used by the NKT cell (Sköld et al., 2003), as Ly49 expression on Vα14 NKT cells markedly differs from Ly49 expression on non-Vα14 NKT cells. Further, as demonstrated in the same paper, a high selective Ly49C/I expression found on thymic TCRβ^+NK1.1$^+$ cells seems independent of MHC class I expression.

Transgenic expression of an inhibitory Ly49 receptor, in the presence of the recognized MHC class I molecule, has been shown to affect the TCR selection during the development of TCRβ$^+$NK1.1$^+$ cells (MacDonald et al., 1998). Transgenic expression of an activating Ly49 receptor also leads to a defect in NKT cell selection, which partially can be overcome by simultaneous expression of an engaged inhibitory receptor (Voyle et al., 2003).

Functional Ly49 receptor expression is not found in humans, instead human NK cells express killer immunoglobulin-like receptors (KIR) carrying out the same function as mouse Ly49 receptors (Moretta and Moretta, 2004).

CD49b expression
Another pan-NK cell marker expressed by a small subset of T cells is recognized by the DX5 monoclonal Ab. The TCRβ$^+$DX5$^+$ population is partially overlapping with TCRβ$^+$NK1.1$^+$ cells, and has been used as an alternative marker for TCRβ$^+$NK1.1$^+$ cells in NK1.1 negative strains. DX5 was shown to recognize CD49b, α_2-integrin (Arase et al., 2001). α_2-integrin forms together with β_1-integrin the very late antigen (VLA)-2, a receptor for collagen and laminin. CD49b is expressed at high levels by a subset of TCRβ$^+$NK1.1$^+$ cells, mainly consisting of DN TCRβ$^+$NK1.1$^+$ cells with high IFN-γ and low IL-4 production (Paper I).

The role of CD49b expression, other than as an adhesion receptor for collagen, on NKT cells and NK cells is not known. However, in one report sorted CD49b$^-$ NK cells were shown to have decreased cytotoxicity compared to CD49b$^+$ cells (Arase et al., 2001). Notably, NK cytotoxicity was not blocked by addition of DX5 mAb.

NKT cell function

NKT cell subsets

Vα14 and non-Vα14 NKT cells

NKT cells are often divided into invariant and diverse, referring to their TCR. Invariant NKT cells, also called Vα14 NKT or classical NKT cells, are referred to as such because of their use of a TCR consisting of a invariant Vα14 chain (Vα24 chain in humans) predominantly joined with Vβ8, Vβ7, or Vβ2 chains (Vβ11 in humans). This population of NKT cells can be readily identified by α-GalCer loaded CD1d-tet (Sidobre and Kronenberg, 2002). All non-Vα14 NKT cells are commonly referred to as having a diverse TCR, or as non-classical NKT cells. This may be misleading, as this population of NKT cells may contain subpopulations with different invariant TCR than the Vα14 (Park et al., 2001).

Non-Vα14 NKT cells are not activated by α-GalCer (Gumperz et al., 2000; Makowska et al., 2000), which constitutes a problem in how to specifically identify this population. In mice, the majority of NKT cells carry the Vα14 TCR. Importantly, it has been demonstrated that T cells expressing non-Vα14 TCR can recognize CD1d and develop to become cells with similar characteristics as Vα14 NKT cells (Sköld et al., 2000). Interestingly, the non-Vα24 population is more common among NKT cells in humans than non-Vα14 NKT cells in mice. Stimulation of mouse splenic cells with CD1d-expressing transfectants results in a panel of CD1d reactive T cells (Behar et al., 1999). Several of the clones expressed a Vα14 TCR, but the majority of the T cell clones used a non-Vα14 TCR repertoire. These cells were shown to have cytolytic capacity and to be potent IL-10 and IFN-γ producers, when activated. The CD1d recognition by different NKT TCR has been shown to be tissue specific, depending on the CD1d presenting cell (Brossay et al., 1998; Park et al., 1998).

To be able to specifically investigate non-Vα14 NKT cells, we have constructed a transgenic B6 mouse strain with a non-Vα14 TCR, consisting of a Vα3.2 and a Vβ9 chain, from a CD1d-reactive T cell hybridoma (Cardell et al., 1995). The transgenic mice were shown to have elevated numbers of non-Vα14 NKT cells with the capacity to rapidly produce cytokines (Sköld et al., 2000; Paper I). We have also established a transgenic nonobese diabetic (NOD) mouse expressing the same non-

Vα14 TCR to ask whether these cells can prevent or delay diabetes development (Paper III).

Functional NKT cell subsets
The evidence of NKT cell involvement in various immunological responses and diseases is intriguing. As TCRβ⁺NK1.1⁺ cells are stimulated, they can rapidly produce a wide range of cytokines, including IL-4 and IFN-γ (Hayakawa et al., 1992; Arase et al., 1993; Yoshimoto and Paul, 1994). One explanation may be that the NKT cell population consists of functional subsets.

Early on it was suggested that the cytokines produced by NKT cells differed depending on CD4 expression. Upon *in vitro* stimulation with a CD3-specific antibody, the CD4⁺ fraction of TCRβ⁺NK1.1⁺ cells produced higher levels of IL-4 than the DN counterpart (Hammond et al., 1999). Deletion of CD4⁺ cells, including all CD4⁺ NKT cells, in a tumor model was shown to be sufficient to prevent the CD1d-dependent suppression of tumor rejection, supporting the hypothesis that CD4⁺ and DN NKT cells seem to serve different functions (Terabe et al., 2000). The majority of CD4⁺ TCRβ⁺NK1.1⁺ cells has the Vα14 TCR, while non-Vα14 TCR are more common among DN TCRβ⁺NK1.1⁺ cells, which in the light of our results (Paper I) may explain the correlation between CD4 expression and functional difference.

Transgenic expression of an non-Vα14 TCR, consisting of rearranged TCR Vα3 and Vβ9 chain (called 24αβ TCR), leads to the development of mainly DN NKT cells, both on B6 and NOD background (Sköld et al., 2000; Paper III), while transgenic expression of the Vα14 chain promotes development of CD4⁺ NKT cells (Bendelac et al., 1996). Upon in vitro stimulation, 24αβ TCR NKT cells produced high levels of IFN-γ and low IL-4 (Paper I; Paper III), and Vα14 NKT cells produced both IL-4 and IFN-γ at high levels, both on B6 and NOD background (Paper I; Lehuen et al., 1998), supporting the notion of functionally distinct NKT subsets.

NKT cell activation
NKT cells can be activated through TCR interaction with CD1d associated with lipid or glycolipid. Because all leukocytes, as well as several other cells express CD1d, many candidates for ligand presentation and activation of NKT cells exist. Recently, DCs were shown to be critical for efficient α-GalCer stimulation of NKT cells in

vivo, while B cells only induce low IL-4 production (Bezbradica et al., 2005; Schmieg et al., 2005).

NKT cells can also be activated through the NK1.1 receptor. Similar to NK cells, *in vitro* cross-linking of NK1.1 on TCRβ⁺NK1.1⁺ cells results in IFN-γ production (Arase et al., 1996).

In 1997, the first ligand for NKT cells was identified by Kawano et al., who showed that synthetic α-GalCer, originally identified in the marine sponge *Agelas mauritianus*, could activate mouse Vα14 NKT cells in a CD1d-restricted manner. Later it was also shown that α-GalCer could stimulate the corresponding human cells, Vα24 NKT cells, in the same way (Spada et al., 1998; Nieda et al., 1999). The major question has been the relevance of this ligand, as it does not occur naturally in human or mouse potential pathogens. Further, it is not clear if it mimics an endogenous or exogenous derived antigen. However, there is no doubt that it is a potent Vα14 NKT cell ligand, which has been of great use both in the identification and activation of Vα14/Vα24 NKT cells in different systems.

Much effort has been put into identifying natural NKT cell ligands. The autoreactive nature of NKT cells suggested that the ligand of these cells were of endogenous origin. However, during the last years several ligands have been identified and suggested to be natural NKT cell ligands, both of microbial origin and of self-origin.

Exogenous ligands
A subset of mouse Vα14 and human Vα24 NKT cells binds and reacts to soluble CD1d loaded with material purified from mycobacterial cell walls enriched for phosphatidylinositol mannoside (Fisher at al., 2004). Other reports have identified cell wall glycosphingolipids (GSL) such as α-glucuronosylceramide (α-GlcUCer), expressed by *Sphingomonas paucimobilis*, and synthetic GSL-1 and –2 to be targets for mouse and human Vα14/Vα24 NKT cells (Sriram et al., 2005; Kinjo et al., 2005; Wu et al, 2005). Interestingly, GSL is expressed by gram-negative bacteria, which do not express lipopolysaccharide (LPS). This suggests that the Vα14 NKT TCR may act as a pattern recognition molecule, similar to a toll-receptor, identifying gram-negative bacteria in an innate-like way.

Endogenous ligands

When testing a panel of CD1d-reactive T cell hybridomas, including both Vα14 and non-Vα14 cells, it was shown that some of these hybridomas were stimulated by purified phospholipids (Gumperz et al., 2000). Interestingly, phospholipid-reactivity could be detected both among α-GalCer reactive Vα14 T cell hybridomas as well as among non-Vα14 T cells hybridomas. The tumor-derived ganglioside GD3, expressed on human melanoma, has also been shown to activate a subpopulation of Vα14 NKT cells in a CD1d-dependent manner (Wu et al., 2003). In a recent report, another endogenous ligand, isoglobotrihexosylceramide (iGb3), was detected. This ligand is an endogenous lysosomal GSL (Zhou et al., 2004b), that is presented by LPS-activated dendritic cells and needed for induction of IFN-γ production by NKT cells in a *Salmonella* infection (Mattner et al., 2005).

A self-glycolipid, sulfatide, has been shown to activate a population of CD1d-reactive non-Vα14 NKT cells in an autoimmune model (Jahng et al., 2004). Interestingly, this population is distinct form the α-GalCer reactive Vα14 NKT cells. A sulfatide reactive non-Vα14 T cell hybridoma (Cardell et al., 1995) was found to bind to sulfatide but not α-GalCer loaded CD1d-tet. An increase of IFN-γ-secreting sulfatide reactive T cells was detected during EAE (Jahng et al., 2004), showing the importance and ability of non-Vα14 NKT cells in immune regulation. Taken together, NKT cells can be stimulated by both exogenous and endogenous antigens.

NKT cells involved in diverse immunological responses

The ability of NKT cells to rapidly produce both Th1 and Th2 cytokines, such as IFN-γ, IL-4, IL-10, and IL-13, makes it a very interesting lymphocyte population with a suggested immunoregulatory role. It is clear from several studies, mostly done in CD1d knockout mice or investigating the effect of Vα14 NKT cells, that NKT cells are potent regulatory T cells that have the capacity to either initiate or shut down a wide variety of immune responses.

Tolerance

Anterior chamber-associated immune deviation (ACAID) is thought to represent a mechanism responsible for the immune-privileged status of the eye. Studies in CD1d-deficient mice have revealed that this is an NKT cell-dependent phenomenon. ACAID in these mice can be restored by transfer of splenic TCRβ$^+$NK1.1$^+$ cells (Sonoda et al., 1999) and was shown to be dependent on IL-10 production by Vα14

NKT cells (Sonoda et al., 2001). The antigen specific suppression in this model is mediated by CD8⁺ regulatory T cells suggested to be induced by CD4⁺ NKT cells (Nakamura et al., 2003).

Vα14 NKT cells have been shown to participate in cardiac allograft tolerance (Seino et al., 2001; Higuchi et al., 2002). Blockade of CD28/B7 interactions could inhibit cardiac allograft rejection in wildtype mice but not in Vα14 NKT cell-deficient mice. Similarly, CD4⁺ Vα14 NKT cells have been shown to be necessary to promote survival of rat xenogeneic pancreatic islet grafts (Ikehara et al., 2000). Bone marrow derived TCRβ⁺NK1.1⁺ cells have also been shown to inhibit graft versus host disease (GVHD) in mice (Zeng et al., 1999b). The inhibition was dependent on IL-4 production by TCRβ⁺NK1.1⁺ cells, suggesting that the IL-4 production dominates the response in this model.

Allergy
Vα14 NKT cells have been indicated to be required for the induction of airway hypersensitivity (Lisbonne et al., 2003). Transfer of NKT cells into Vα14 NKT cell-deficient mice were shown to restore the susceptibility. Also, either IL-4 or IL-13 knockout NKT cells could do this, while NKT cells deficient for both cytokines could not (Akbari et al., 2003). Recently, intranasal administration of α-GalCer was shown to inhibit the symptoms of asthma in sensitized and challenged mice. The treatment induced Vα14 NKT cell accumulation in the lungs, and a shift in their cytokine profile from pro-asthmatic IL-4 to a protective IFN-γ production (Hachem et al., 2005).

Tumor rejection
The earliest documented effect of α-GalCer as an immunotherapeutic agent is its ability to promote NKT cell-dependent rejection of experimental tumor lines (Cui et al., 1997). It appears as if NKT cell-derived IFN-γ production is critical for protection through α-GalCer treatment, and that other potentially tumoricidal products of NKT cells, such as TNF, Fas ligand, and perforin, seem less important (Hayakawa et al., 2001; Smyth et al., 2002). NKT cells are suggested not to directly kill the tumor cells but instead recruit and promote a response by effector cells in an IFN-γ-dependent manner. α-GalCer treated NKT cells may enhance NK and cytotoxic T cell activity, which both are effector cells that have been implicated in α-GalCer-induced tumor rejection. NKT cells may also enhance tumor immunity via CD40 ligand upregulation promoting APC activation and IL-12 production (Kitamura et al., 1999;

Fujii et al., 2003). Sarcoma cell lines have been shown to preferentially grow in NKT cell deficient mice (Smyth et al., 2000; Crowe et al., 2002). Just as in α-GalCer-induced antitumor immunity, IFN-γ production by NKT cells was critical for rejection.

In other models, NKT cells have been shown to play a immuno-suppressive role. In two tumor models, using the experimental tumors 15-12RM and 4T1, tumors were rejected in CD1d deficient mice, but grew in wildtype mice (Terabe et al., 2000; Ostrand-Rosenberg et al., 2002). The suppressive effect was shown to be IL-13-dependent and IL-4 independent in the 15-12RM model, while the suppression of 4T1 tumor rejection was IL-13-independent. These results show the existence of multiple mechanisms by which NKT cells can inhibit tumor rejection.

Infections

It has been found that NKT cells are activated and participate in protection from a variety of bacteria, viruses and parasites (reviewed in Van Kaer and Joyce, 2005; Van Dommelen and Degli-Esposti, 2004; Sköld and Behar, 2003). Some of these results have been shown to depend on the genetic background. For example the, effect of NKT cells on the immune response to respiratory syncytical virus and cerebral malaria differs between B6 and BALB/c mice (Johnson et al., 2002; Hansen et al., 2003).

NKT cells can operate in either a stimulating or suppressive fashion. Clearance of herpes simplex virus type 1 is reduced in NKT cell deficient mice (Grubor-Bauk et al., 2003), while NKT cells suppress the response to lymphocytic choriomeningitis virus (Roberts et al., 2004). In a model of *Salmonella* infection, both TCRβ[+]NK1.1^{+} cells and Vα14 NKT cells were shown to be activated and induced to produce IFN-γ, but not IL-4, in response to the infection (Brigl et al, 2003; Berntman et al., 2005). Additionally, DCs were shown to upregulate CD1d upon *in vitro* infection with *Salmonella*, suggesting that they may be involved in the activation of NKT cells in this model.

Clearly, NKT cells can play a role in infection. The possibility to enhance the immunity via NKT cells, directly or indirectly, to microbial organisms emphasizes the importance of research in this field.

Autoimmunity

In the absence of microbial or anti-tumor triggering, the role of NKT cells may be to regulate the adaptive immune system from harmful autoimmunity. In mice, functional defects and reduction in NKT cell numbers have been shown in several disease models, such as experimental allergic encephalomyelitis (EAE) (Yoshimoto et al., 1995), and autoimmune diabetes (Gombert et al., 1996). The majority of investigations on NKT cells in autoimmunity have been done in autoimmune diabetes, which will be discussed in the next chapter.

Experimental allergic EAE is an animal model of multiple sclerosis (MS). α-GalCer treatment has in some studies been shown to prevent the disease (Singh et al., 2001; Furlan et al., 2003), but in others to have no effect or even accelerate disease (Pal et al., 2001; Miyamoto et al., 2001; Jahng et al., 2001). The preventive effect is thought to be mediated by a shift in the balance of a pathogenic Th1 towards a Th2 response. In contrast, in Vα14 transgenic mice, the increase in Vα14 NKT cells led to impaired EAE induction, protection was associated with a reduction in IFN-γ production by autoantigen-specific T cells rather than a Th2 shift in their response (Mars et al., 2002). Interestingly, IL-4 was shown not to be required. In an animal model of systemic lupus erythematosis (SLE), CD1d deficiency in mice was associated with an exacerbated disease (Yang et al., 2003; Yang et al., 2004). Vα14 NKT cells were shown in one report to suppress SLE (Mieza et al., 1996), a finding that was later on contradicted in another paper (Zeng et al., 2003). The suppression and promotion of this disease was recently shown to be dependent on the mouse strain used (Singh et al., 2005).

In humans a decrease of Vα24 NKT cells has been suggested to be associated with a variety of autoimmune diseases (van der Vliet et al., 2001), such as systemic sclerosis (Sumida et al., 1995), rheumatoid arthritis (Yanagihara et al., 1999), multiple sclerosis (Illes et al., 2000), and autoimmune diabetes (Wilson et al., 1998).

Evidently, NKT cells may influence the outcome of autoimmunity. It is therefore of great importance to determine the mechanisms leading to the NKT cell activation and which factors that are involved in the suppression or enhancement of the autoimmune response.

NKT cells in autoimmune diabetes

Autoimmunity

The immune system is built up to respond to non-self. The extensive diversity of the adaptive immune system, generated through random antigen-specific receptor rearrangement discussed above, will unavoidably generate some lymphocytes that can recognize self-antigens, with the potential to mount an autoimmune response, sometimes leading to serious complications. To avoid this from happening the immune system has developed mechanisms ensuring the establishment and maintenance of lymphocyte self-tolerance. Lymphocyte tolerance mechanisms are divided into central and peripheral tolerance. In the description below I have chosen to focus on the generation of T cell self-tolerance.

Central tolerance

The mechanisms of positive and negative selection in the thymus, described above, are instruments in shaping the self-tolerant T cell repertoire. Originally, thymic selection was considered an effective tolerogenic mechanism only for widely expressed self-molecules. However, proteins previously thought of as tissue-restricted were shown to be expressed in the thymus by medullary epithelial cells (MEC) (Derbinski et al., 2001). The protein encoded by the *aire* gene (*AIRE* in humans) is a transcription factor mainly expressed in MEC. Analysis of *aire*-deficient mice revealed that the normal control of autoimmunity requires MEC expression of *aire*, and *aire*-negative MECs have diminished expression of genes encoding otherwise tissue-restricted proteins (Anderson et al., 2002; Derbinski et al., 2005).

Peripheral tolerance

The negative selection in the thymus is not 100% efficient. In reality, autoreactive cells are circulating the system even in healthy individuals. Then, why do not all individuals develop autoimmunity?

To prevent autoimmunity the immune system has developed mechanisms to control autoreactive lymphocytes that escape negative selection. In fact, there are several mechanisms, such as ignorance, anergy, apoptosis and suppression (reviewed in Walker and Abbas, 2002).

In ignorance, cells that are quite capable of making a response are not triggered by the presence of their autoantigen. This can be due to that the antigen may simply be present in too low concentration. Since all lymphocytes have a threshold for the receptor signalling that is required to trigger a response a very low concentration of antigen will not be sensed. A second possibility is that some antigens are sequestered from the immune system in locations that are not freely exposed to surveillance. These are termed immunologically privileged sites. Anergy is when a T cell recognition of a self-antigen leads to functional inactivation, either through lack of costimulation or signalling through alternative receptors. One such important signal has been shown to be the ligation of cytotoxic T lymphocyte associated antigen 4 (CTLA-4), required for anergy in vivo. Another way to prevent autoimmunity is to delete the self-reactive T cell clone by activation-induced cell death (AICD). One key mechanism involved in AICD is the ligation of the Fas death receptor by its ligand.

Auto-reactive T cells can also be suppressed by regulatory T cell populations. NKT cells are suggested to be one such population, but here I will mention a couple of other T cell populations with regulatory capacities. The most studied regulatory T cell population is natural $CD4^+CD25^+$ regulatory T cells. The $CD4^+CD25^+$ regulatory T cells suppress other T cell populations by direct cell contact with effector cells, but also by secreting suppressive cytokines such as TGF-β and IL-10, interfering with T cell activation (reviewed in von Boehmer, 2005). Other cell populations that have been implicated in immunoregulation are the $\gamma\delta$T cells localized to the epidermis called dendritic epidermal T cells (DETC) and the $CD8\alpha\alpha^+$ $TCR\beta^+$ intestinal intraepithelial lymphocytes (IEL).

Autoimmune diabetes

Autoimmune diabetes, also called type 1 diabetes (T1D) or insulin dependent diabetes mellitus (IDDM), is one of the most common autoimmune endocrine disorders in humans. T1D is usually diagnosed in children and young adults, and was previously known as juvenile diabetes. T1D is a life-long disease for which there is not yet a cure. The cause of type 1 diabetes is unknown.

This autoimmune disease results from the failure to produce insulin, the hormone that allows glucose to enter cells to provide fuel. It is the result of an autoimmune process in which the immune system attacks and destroys the insulin producing β-cells of the

pancreas. The incidence of T1D is increasing in the western world and constitutes 5-10% of the total diabetes cases.

One of the most important discoveries in the research on autoimmune diabetes is the discovery of the nonobese diabetic (NOD) mouse (Makino et al., 1980). Research on NOD mice has greatly expanded our knowledge of the pathogenesis of autoimmune diabetes. This mouse model spontaneously develops autoimmune diabetes that shares many pathological features with human T1D. The disease has a strong genetic link (Todd et al., 1987), and is manifested by the destruction of the insulin producing β-cells in the islets of Langerhans in the pancreas, leading to insulin deficiency and hyperglycemia.

In the early phase of the disease, already at 3 weeks of age in NOD mice, mononuclear cells, consisting of APCs such as macrophages and DCs, infiltrate the pancreas, and are located around the islet without entering it. This is followed by infiltration of CD4^{+} T cells, CD8^{+} T cells, and B cells (Jansen et al., 1994). This infiltration process is called insulitis, and the early nondestructive phase is called peri-insulitis. In humans the peri-insulitis phase does not appear, or may appear for a very short period of time. As the disease progresses, the infiltrate invades the islets and becomes destructive insulitis. When less than 10% of the β-cell mass of the pancreas is left, irreversible clinical diabetes occurs (Sreenan et al., 1999).

Macrophages and DCs are the first cells to infiltrate the pancreas during the progression of diabetes. Macrophages are thought to play an important role in creating a suitable microenvironment, as T cells cannot differentiate into β-cell cytotoxic cells in a macrophage-depleted environment (Jun et al., 1999). B cells may play a critical role in diabetes progression, as B cell deficient NOD mice have a dramatically reduced pathology (Akashi et al., 1997). However, the production of auto-antibodies does not appear to be directly pathogenic to β-cells (Serreze et al., 1998), suggesting that the role of B cells in the pathogenesis of diabetes is as APC. In line with this, B cell-mediated MHC class II antigen presentation has been shown to regulate the development of CD4^{+} auto-reactive T cell development in NOD mice (Greeley et al., 2001). NOD mice have also been reported to have an enlarged marginal zone (MZ) B cell population (Rolf et al., 2005). The MZ B cell population expresses high levels of CD1d, which implicates a possible interactive role with NKT cells.

NOD mice lacking CD4 T cells, either depleted by anti-CD4 antibodies or genetically deficient, are protected from diabetes (Shizuru and Fathman, 1993), and CD8$^+$ T cells and MHC class I expression is required for diabetes development (Katz et al., 1993; Serreze et al., 1997).

Many studies investigating regulation of the development of autoimmune diabetes has focused on the possibility to switch from a destructive Th1 immune response to a more protective Th2 response. Destructive lesions have been characterized by an abundance of IFN-γ producing T cells, while nondestructive lesions have more T cells expressing IL-4 and fewer IFN-γ producing cells (Shehadeh et al., 1993). In support of this, treatment of NOD mice with IL-4 or IL-10 can protect from diabetes development (Rapoport et al., 1993; Pennline et al., 1994), while administration of Th1 cytokine IL-12 precipitates disease (Trembleau et al., 1995). On the other hand, IL-4 knockout mice do not have an accelerated diabetes development (Wang et al., 1998), and INF-γ deficient mice can develop diabetes (Hultgren et al., 1996), while IFN-γ treatment reduces insulitis (Campbell et al., 1991). These results show the complexity of cytokine interactions in the immune system, and how many different features that may be involved in the development of autoimmune diabetes.

NKT cells in the regulation of autoimmune diabetes

The first suggestion on the involvement of NKT cells in autoimmune diabetes showed that NOD mice have a decreased population of TCRβ$^+$CD4$^-$CD8$^-$ cells, as well as a functional deficiency in IL-4 production (Gombert et al., 1996; Baxter et al., 1997; Godfrey et al., 1997; Poulton et al., 2001). Recently, this defect was shown to map to major diabetes and lupus susceptibility loci (Esteban et al., 2003), and was suggested to be a defect in a lineage fate decision that elicits a deficiency in fetal thymic NKT cells (Wagner et al., 2005). Introduction of the NK locus from B6 mice into NOD mice reduced disease incidence and improved NK and α-GalCer reactive NKT cell performance (Carnaud et al., 2001). Human studies, including studies on identical twins, showed that T1D patients have fewer Vα24 NKT cells (Wilson et al., 1998), which also had a functional defect in their IL-4 producing capacity.

Cell transfer of TCRβ$^+$CD4$^-$CD8$^-$ thymocytes, including some NKT cells, into NOD mice prevented diabetes (Baxter et al., 1997). This supported that NKT cells could play a role in the regulation of autoimmune diabetes. The precise mechanisms involved in the regulation are still not determined. IL-4 and/or IL-10 production by

TCRβ^+CD4$^-$CD8$^-$ thymocytes were suggested to skew the Th1/Th2 balance to a more non-destructive Th2 response (Hammond et al., 1998). Others have suggested that a functional defect in the production of IFN-γ by NOD CD3$^+$Ly49A$^+$CD122$^+$ cells, including some NKT cells, is responsible for the inability to protect from disease (Falcone et al., 1999).

An overexpression of Vα14 NKT cells in NOD mice could also decrease the incidence of diabetes (Lehuen et al., 1998), again suggested to be an IL-4 mediated process (Laloux et al., 2001). In accordance with this, administration of α-GalCer to NOD mice, inducing IL-4 production in Vα14 NKT cells, prevented the onset of diabetes (Sharif et al., 2001; Hong et al., 2001). The overexpression of Vα14 NKT cells did not completely protect against diabetes development, implying that also the development of transgenic Vα14 NKT in the NOD mouse may suffer from a functional defect.

The role of NKT cells were even further supported when it was shown that NOD mice lacking CD1d expression, and thereby all NKT cells, have an accelerated diabetes development (Wang et al., 2001; Shi et al., 2001). More specific investigations of the role and capacity of non-Vα14 NKT cells to regulate autoimmune diabetes have, so far, only been addressed by our group (Paper III). In this model we show that a non-Vα14 NKT cell population, that are strong IFN-γ and weak IL-4 producers, can prevent or delay the onset of diabetes.

More recent discoveries concerning the role of NKT cells in regulation of autoimmune diabetes will be discussed in "Discussion of this thesis" below.

Aim

The aim of these studies has been to increase the knowledge of the non-Vα14 NKT cell subset, and the ability of NKT cells that do not express the Vα14 TCR to prevent and regulate autoimmune diabetes.

– *Can functionally different NKT cell subsets be distinguished by surface marker expression and TCR type?*
NKT cells have been suggested to be involved in a wide range of diverse immunological situations, due to their capability to rapidly produce the immunoregulatory cytokines IFN-γ and IL-4, known to be involved in Th1 and Th2 skewing in an immune response. Our previous studies had indicated that these cytokines could be differently expressed by NKT cells. We asked whether the same NKT cell produce these cytokines or if there is a functional difference among NKT cells. And if so, can this functional difference be distinguished by surface marker expression and TCR type?

– *Does the composition of NKT cell subsets differ between mouse strains?*
NKT cells were first defined as TCRβ[+]NK1.1^{+} cells. However, the NK1.1 receptor is only expressed in a few mouse strains, such as the B6 mouse, which has led to that most of our knowledge of the entire NKT cell population is based on the NKT cells found in B6 mice. To be able to study TCRβ[+]NK1.1^{+} cells in a different mouse strain than B6, we created a congenic mouse, which express the NK1.1 receptor from the B6 mouse on the BALB/c genetic background, called BALB.B6-*NK1.1[b]*.

– *Can non-classical NKT cells protect against autoimmune diabetes?*
Vα14 NKT cells have been shown to be able to regulate autoimmune diabetes, both by transgenic expression of Vα14 TCR and in cell transfer experiments. To determine if non-classical (non-Vα14) NKT cells share the capacity to prevent autoimmune diabetes, we created a TCR transgenic NOD mouse, expressing the transgenic non-Vα14 TCR previously investigated on the B6 background (Sköld et al., 2000). After the publication of this paper we have conducted further analysis on the characterization of and the cells involved in this process, aiming to determine possible mechanisms of regulation, which will be discussed at the end of this chapter.

Summary of original papers

I. **Surface receptors identify mouse NK1.1⁺ T cell subsets distinguished by**
function and T cell receptor type.
Stenström, M., Sköld, M., Ericsson, A., Beaudoin, L., Sidobre, S., Kronenberg,
M., Lehuen, A. and Cardell S. *Eur. J. Immunol.* 2004. 34:56-65.

When this study was initiated two types of CD1d-restricted T cells were known that
could be distinguished by the TCR they carried. The major population carried a
restricted TCR comprised of the canonical Vα14-Jα18 gene rearrangement combined
with Vβ8, Vβ2 or Vβ7. The other CD1d-restricted T cells expressed a variable TCR
repertoire different from the Vα14 TCR. Activation of TCRβ⁺NK1.1⁺ cells is known
to result in rapid production of the regulatory cytokines IFN-γ and IL-4, and many
different immunological roles has been assigned to NKT cells. However, some
seemed contradictory as they involve IFN- γ in some cases, and IL-4 in others. This
suggested that the NKT cell population could consist of functionally different
subsets. We wanted to determine if functional subsets of NKT cells could be
distinguished by surface marker expression and TCR usage. We analyzed the surface
phenotype and cytokine secretion profile of TCRβ⁺NK1.1⁺ and α-GalCer loaded
CD1d-tet⁺ cells in B6 mice.

We showed that TCRβ⁺NK1.1⁺ cells could be divided into two functional subsets,
which we called NKT1 and NKT2 cells. NKT1 cells expressed high levels of the
CD49b molecule, were potent producers of IFN- γ but poor IL-4 producers. In
contrast, NKT2 cells were CD69⁺, with no or low expression of the CD49b molecule,
and potent producers of IL-4 as well as IFN- γ.

To specifically analyze CD1d-restricted cells we used two TCR transgenic B6 mice,
representing Vα14 and non-Vα14 NKT cells. The results showed that the TCR type
differed between the NKT1 and NKT2 subsets. Transgenic Vα14 NKT cells
belonged to NKT2 the cell subset, while the transgenic non-Vα14 NKT cells
belonged to the NKT1 subset.

II.   Natural killer T-cell populations in C57BL/6 and NK1.1 congenic BALB.NK mice – a novel thymic subset defined in BALB.NK mice.
Stenström, M., Sköld, M., Andersson, Å. and Cardell L., S. *Immunology* 2005. 114:336-345.

At the time when this study was initiated NKT cells were defined as TCRβ[+]NK1.1^{+} T cells. As only a few mouse strains, such as the C57BL/6, express the NK1.1 receptor, the knowledge of the entire NKT cell population was to a large extent based on TCRβ[+]NK1.1^{+} T cells found in C57BL/6 mice. To be able to study TCRβ[+]NK1.1^{+} T cells in another mouse strain, we created a congenic mouse, which expressed the NK1.1 antigen from the C57BL/6 mouse on the BALB/c genetic background (BALB.B6-*NK1.1[b]*, referred to below as BALB.NK mice).

We analyzed and compared the TCRβ[+]NK1.1[+] and CD1d-tet[+] populations in the BALB.NK and B6 mouse and could demonstrate a dominance of NKT2 cells in the BALB.NK spleen. Further, we detected a novel TCRβ[+]NK1.1[+] cell subset in the BALB.NK thymus. These cells expressed unusually high levels of TCRβ and low levels of NK1.1, as well as poor display of NK cell markers suggesting that they were not completely differentiated.

III.   Prevention of diabetes in nonobese diabetic mice mediated by CD1d-restricted nonclassical NKT cells.
Duarte, N., Stenström, M., Campino, S., Bergman, M-L., Lundholm, M., Holmberg, D. and Cardell L., S. *J. Immunol*. 2004. 173:3112-3118.

At the start of this project NKT cells were implied in the regulation of autoimmune diseases in both human and mice. Both NOD mice and human diabetes patients were known to have a small and functionally defective NKT cell-like population. These findings in association with disease development, suggested a deficiency in the regulatory function of NKT cells in autoimmune diabetes. Further, NOD mice lacking CD1d and NKT cells were later shown to display an accelerated onset and increased incidence of diabetes. The capacity of Vα14 NKT cells to prevent diabetes had been shown using TCR-transgenic mice. However, the role in autoimmune diabetes of non-Vα14 NKT cells had not been addressed. In this study we investigated the potential of non-Vα14 NKT cells to regulate autoimmune diabetes, using a TCR-transgenic mouse model with an increased population of non-Vα14

NKT cells (the 24αβNOD mouse). Diabetes development was efficiently suppressed in the TCR-transgenic mice. Transgene expressing NKT cells could prevent diabetes in an adoptive transfer experiment, using T and B cell deficient NOD*scid* mice as recipients. This demonstrated for the first time a role for non-Vα14 NKT cells in the regulation of autoimmune diabetes.

Discussion of this thesis

A novel thymic NKT cell population (Paper II)

When analyzing the BALB.NK thymus we detected a TCRβ$^+$NK1.1$^+$ population that had unusual characteristics. B6 and the congenic BALB.NK mice had similar frequencies of TCRβ$^+$NK1.1$^+$ cells in the thymus. However, in the BALB.NK thymus the cells could be divided into two populations with regard to the level of TCRβ and NK1.1 expression. One population overlapped with the thymic TCRβ$^+$NK1.1$^+$ population in B6 mice, with intermediate expression of TCRβ and high NK1.1 expression, here referred to as TCRβ^{int} cells. The second population had high levels of TCRβ and intermediate levels of NK1.1, referred to as TCRβ^{high} cells (Paper II, figure 1). A similar population is also present in another NK1.1 congenic BALB/c strain, but was not further investigated (Hammond et al., 2001). It is difficult to say if the congenic expression of NK1.1 influences the selection of NKT cells in these mice. Such interference may be responsible for the unusual TCRβ$^+$NK1.1$^+$ phenotype. However, in a report investigating the expression of the H4 T cell activation marker on thymocytes, an H4^{high} TCR^{high} expressing population suggested to be NKT cells was identified in the BALB/c thymus (Yagi et al., 1999). H4^{high} TCR^{high} cells was also present in the B6 thymus, but in lower numbers and within the NK1.1$^-$ population.

The TCRβ^{int} population in B6 and BALB.NK thymus shares the same expression pattern, while the TCRβ^{high} population had a lower expression of NK-cell associated markers (Paper II, figure 2). Using the αGalCer loaded CD1d-tetramer we found a equal division of the CD1d-tet$^+$ population into CD1d-tet^{int} and CD1d-tet^{high} cells, sharing the difference in surface phenotype that was seen between TCRβ^{int} and TCRβ^{high} cells (Paper II, figure 3). The CD1d-tet^{high} population was essentially negative for NK markers, with the exception of NK1.1, which was expressed by 40% of the CD1d-tet^{high} subset. NK cell markers are upregulated late in the NKT cell development, which suggested that these cells could represent a precursor stage to NKT cells. These cells were not immature thymocytes, as shown by the expression of CD5 and absence of HSA on both the intermediate and high population. However, several other NK cell marker are up-regulated during the differentiation of NKT cells. After the DP stage Vα14 NKT cell development can be divided into three stages (Figure 1); CD44^{low}NK1.1$^-$ (stage 1), CD44^{high}NK1.1$^-$ (stage 2), and CD44^{high}NK1.1$^+$

(stage 3). NK cell associated markers are not expressed until stage 3 (reviewed in Matsuda and Gapin, 2005).

BALB.NK TCR^{high} cells did not express CD122, a shared cytokine receptor chain used by the IL-2 and IL-15 receptor that is upregulated during the transition between stage 2 and 3. Of these two cytokines, IL-15 has been shown to be the one involved in TCRβ⁺NK1.1⁺ cell development, as TCRβ⁺NK1.1⁺ cells develop normally in IL-2 deficient mice (Ohteki et al., 1997), while IL-15 deficient mice have a reduction in the number of thymic CD44^{high}NK1.1⁺ Vα14 NKT cells (Matsuda et al., 2002). In line with this, in mice lacking the common γ-chain, a critical component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, the TCRβ^{int} thymocytes do not express NK1.1 or Ly49C and have reduced levels of CD122 (Lantz et al., 1997). These cells are not exported to the periphery. In T-bet deficient mice the Vα14 NKT cell number are reduced. The development of Vα14 NKT cells in these mice seems to be blocked between stage 2 and 3, as they express CD44 but not NK1.1, Ly49C/I, and CD122 (Townsend et al., 2004). This phenotype resembles the CD1d-tet^{high} population in BALB.NK mice. Taken together, IL-15 signaling is important for the complete differentiation of NKT cells. IL-15 is also thought to be important for the expression of Ly49 receptors on NK cells (Kawamura et al., 2003), and has been described to be important in NK cell development (Suzuki et al., 1997; Lodolce et al., 1998; Kennedy et al., 2000). In BALB.NK mice we only detected TCR^{high} or CD1d-tet^{high} cells in the thymus. Additionally, BALB.NK mice had a smaller TCRβ⁺NK1.1⁺ splenic population compared to B6 mice, suggesting a block in the NKT cell differentiation or a defect in the export of NKT cells from the thymus. Thus, the deficient upregulation of CD122 expression and inhibitory Ly49 receptor expression could block further differentiation and/or export of TCR^{high} and CD1d^{high} cells.

We observed a bias towards a higher frequency of Vβ8.2 expressing cells within the TCR^{high} population compared to the TCR^{int} population (Paper II, figure 2). This implies that Vα14 NKT cells using the Vβ8.2 chain dominate the novel thymic population. This could mean that TCR^{high} and TCR^{int} cells display TCR repertoires that differ in avidity to the thymic CD1d-complex during differentiation.

Just as in B6 thymus the majority or TCRβ^{int} cells in BALB.NK expressed Ly49C/I (Paper II, figure 2; Sköld et al., 2003; Sköld and Cardell, 2000), a receptor expression almost absent in the TCRβ^{high} population. Similarly, in the thymus of 3 weeks old B6 mice only a minority of TCRβ⁺NK1.1⁺ cells expressed Ly49C/I (Sköld et al., 2003).

The frequency of TCRβ$^+$NK1.1$^+$ expressing Ly49C/I increased until 16 weeks of age. Perhaps the TCR^{high} population in BALB.NK mice resembles the NKT cell population dominating in young mice.

NKT1 and NKT2, defining two functional NKT subsets (paper I-III)

When activated, NKT cells can rapidly and prominently produce the regulatory cytokines IFN-γ and IL-4. These cytokines are also produced by conventional CD4$^+$ T cells involved in the skewing of an immune response into a Th1 or Th2 response, respectively. The rapid cytokine production by NKT cells suggests that they may also be involved in the early regulation of an immune response. The finding of non-Vα14 NKT cells in MHC class II knockout mice proved the existence of CD1d-restricted NKT cells expressing a different TCR than the Vα14 (Cardell et al., 1995). Our question was if surface marker expression and TCR type could distinguish functionally different NKT subsets. Further investigation of the surface marker expression and functional phenotype, in MHC class II knockout mice and in the TCR transgenic 24αβB6 mouse (Sköld et al., 1999; Sköld et al., 2000), showed that non-Vα14 NKT cells seemed to be functionally different from Vα14 NKT cells. Our hypothesis was that NKT cells with a non-Vα14 TCR are functionally different and has a surface phenotype distinct from that of Vα14 NKT cells. Our main interest was to further characterize non-Vα14 NKT cells, and to include as many NKT cells as possible in the analysis we investigated the TCRβ$^+$NK1.1$^+$ population in B6 and BALB.NK mice.

In a previous report, Ly49 receptors were expressed by a high proportion of DN TCRβ$^+$NK1.1$^+$ cells in spleen, liver, and to some extent bone marrow, while Ly49 expression within the CD4$^+$ subsets in spleen and liver was almost absent (Sköld and Cardell, 2000). This suggested that distinct subsets perhaps could be divided by CD4 expression.

In paper I, high IL-4 production was mainly found in CD4$^+$Vβ8.2$^+$ TCRβ$^+$NK1.1$^+$ cells in B6 spleen, thymus and bone marrow, while the DN Vβ8.2$^-$TCRβ$^+$NK1.1$^+$ cells in spleen and bone marrow, but not in thymus, showed lower IL-4 production (Paper I, table 2). These results suggested that the different subsets rather correlated with TCR type than with CD4 expression.

Splenic TCRβ⁺NK1.1⁺ cells could be divided into two essentially non-overlapping populations, distinguished by CD69 and CD49b expression (Paper I, figure 3; Paper II, figure 6). The CD69 and CD49b surface phenotype could be correlated to the IL-4 and IFN-γ production, respectively, by these subsets. IL-4 producing cells appeared almost exclusively confined to the CD69^{+}CD49b$^{-/low}$ subset, named NKT2 cells. Both subsets produced IFN-γ, but in the spleen the CD69^{-}CD49b^{high} cell subset, named NKT1 cells, contained a higher frequency IFN-γ-producing cells than the CD69^{+}CD49b$^{-/low}$ subset (Paper I, figure 3). It is difficult to explain the lack of CD69 expression on NKT1 cells. This may be due to a weaker TCR/CD1d complex interaction during selection, compared to Vα14 TCR/CD1d complex interaction. Recently it was shown that CD1d-tet⁻ CD49b⁺ cells, containing both non-Vα14 NKT cells and CD49b⁺ CD1d-independent T cells, are strong IFN-γ and weak IL-4 producers (Pellecci et al., 2005), similar to our observations. The lack of reliable markers for non-Vα14 NKT cells makes it difficult to determine if the IFN-γ producing cells are CD1d-dependent or -independent. The results in paper I and III, showing that the CD1d-restricted 24αβ transgenic cells express CD49b, both on B6 and NOD background (Paper I, figure 5; Paper III, figure 2), argue that it is very likely that there are CD1d-restricted NKT cells among the CD49b⁺ T cells.

We also used α-GalCer loaded CD1d-tet or -dimers to specifically detect Vα14 NKT cells. Splenic CD1d-tet⁺ cells in B6, BALB.NK and NOD mice, had the NKT2 surface phenotype, and produced both IL-4 and IFN-γ (Paper I, figure 4; Paper II, figure 7; Paper III, figure 2 & 4). DN CD1d-tet⁻ CD3⁺NK1.1⁺ cells in B6 spleen had NKT1 surface phenotype (Paper 1, figure 4). To be able to specifically compare Vα14 NKT cells with non-Vα14 CD1d-restricted T cells, we used two TCR transgenic models; Vα14-Jα18 transgenic B6 mouse (Lehuen et al, 1998), representing Vα14 NKT cells, and 24αβ transgenic B6 mouse (Sköld et al., 2000), representing non-Vα14 CD1d-restricted T cells. The 24αβ transgenic NKT cells use a CD1d-restricted Vα3.2/Vβ9 TCR, isolated from a CD1d-reactive T cell hybridoma (Cardell et al., 1995). CD49b was expressed at intermediate to high levels by non-Vα14 NKT cells (Paper I, figure 5). CD49b expression was also detected on transgenic non-Vα14 NKT cells in the 24αβ transgenic NOD mouse (Paper III, figure 2). Transgenic Vα14 NKT cells expressed CD69, but were negative or low for CD49b (Paper I, figure 5). The IL-4 production by transgenic non-Vα14 NKT cells was clearly lower than among transgenic Vα14 NKT cells, in both B6 and NOD mice (Paper I, figure 5; Paper III, figure 4). This further demonstrated the correlation of the

NKT1 surface phenotype and cytokine profile with non-Vα14 NKT cells, and of the NKT2 surface phenotype and cytokine profile with Vα14 NKT cells.

To summarize all data, we presented a model for functional division of NKT cells into NKT1 and NKT2 cells (Paper I, figure 6). However, we could also determine that non-Vα14 NKT cells up regulate CD69 and down regulate CD49b during activation (Paper I, figure 7). Consequently, through this change in surface phenotype upon stimulation, the surface phenotype of non-Vα14 NKT cells become more similar to Vα14 NKT cells.

Vα24 NKT cells from human peripheral blood samples can be divided into two functional subsets (Gumperz et al., 2002; Lee et al., 2002). The CD4^{+} Vα24 NKT cells produce both Th1 and Th2 cytokines, while the DN Vα24 NKT cells only produce Th1 cytokines. Additionally, NK cell receptors are mainly expressed by the DN population. These subsets resemble the NKT1 and NKT2 functional subsets in mice, except for the TCR specificity. *In vivo* stimulation of Vα14 NKT cells in mice with α-GalCer does not show the same pattern (Matsuda et al., 2000). In both liver and spleen, CD4 and DN Vα14 NKT cells produced both cytokines, supporting our model that the functional differences, at least in mice, may be more dependent on TCR specificity than CD4 expression.

In line with this, both the CD4^{+} and DN CD1d-tet^{+} population in BALB.NK mice had the NKT2 surface phenotype and cytokine profile of NKT2 (Paper II, figure 7). In BALB.NK, also the DN CD1d-tet[-] TCRβ[+]NK1.1[+] population displayed the surface phenotype of cytokine profile of NKT2 cells (Paper II, figure 4 & 5), indicating that the correlation between TCR and functional subset is not absolute. DN non-Vα14 NKT cells among TCRβ[+]NK1.1^{+} cells in BALB.NK could belong to the same functional subset as Vα14 NKT cells. However, the correlation between surface phenotype and functional subset that we had found in B6 mice were confirmed in BALB.NK mice.

Non-Vα14 NKT cells, reactive to sulfatide and not α-GalCer, are involved in the protection from EAE in mice (Jahng et al., 2004). Most of these cells expressed CD4 and were weak IL-4 and strong IFN-γ producers upon *in vitro* stimulation. A population of non-Vα14 NK1.1[+] CD1d-dependent T cells were involved in the induction of acute hepatitis (Baron et al., 2002). Surprisingly, the vast majority of NK1.1[+]TCRβ^{+} cells eluted from the liver was not α-GalCer reactive. Similar to the

DN population BALB.NK mice, these cells were DN and produced both IL-4 and IFN-γ. Again showing that some non-Vα14 NKT cells belong to the NKT2 subset. A higher frequency TCRβ⁺NK1.1⁺ cells from PaLN in NOD mice produce IL-4 alone, rather than both IL-4 and IFN-γ or IFN-γ alone, compared to TCRβ⁺NK1.1⁺ cells from the spleen (Laloux et al., 2002). Perhaps this is a third subset of NKT cells producing IL-4 and no IFN-γ. Another explanation could be the antigen that activated these cells. The length of the glycolipid chains on α-GalCer can influence the cytokine profile of mouse Vα14 NKT cells and human Vα24 NKT (Miyamoto et al., 2001; Goff et al., 2004). Either a shorter phytosphingosine chain or a shorter acyl chain was shown to increase the amount of IL-4 released relative to IFN-γ.

In conclusion, functionally different NKT cell subsets exist, and differ in surface phenotype. There is a preference for Vα14 TCR usage among NKT2 cells and non-Vα14 TCR usage among NKT1 cells, but this correlation is not absolute. The tissue distribution of these functional subsets may vary between organs.

Interestingly, the non-Vα24 NKT population seems to be much more common in humans (Behar et al., 2001; Exley et al., 2002; Fuss et al., 2004) than non-Vα14 NKT cells in mice. This underlines the importance of further research on the non-Vα14 NKT cells in mice.

The influence of genetic background on NKT cells (paper II)
To determine if the genetic background could influence the composition of NKT cell subsets in a mouse strain, we wanted to compare the NKT cell population in BALB/c mice with B6 mice. To analyze TCRβ⁺NK1.1⁺ cells on a BALB/c background we backcrossed a genetic fragment containing *Nkrp1-c* from the B6 onto the BALB/c genetic background. We named this NK1.1 congenic BALB/c strain BALB.NK.

The TCRβ⁺NK1.1⁺ population in BALB.NK spleen was about one-third the size compared to B6 mice (Paper II, figure 4). 80% of the TCRβ⁺NK1.1⁺ cells in BALB.NK spleen expressed CD4, compared to 65% in B6 spleen. Surface phenotype and cytokine profile of splenic BALB.NK TCRβ⁺NK1.1⁺ cells showed a strong dominance of NKT2 cells in this strain. In comparison, the splenic CD4⁺ TCRβ⁺NK1.1⁺ population was very similar in B6 and BALB.NK mice, while the DN TCRβ⁺NK1.1⁺ population, shown to contain mostly NKT1 cells in the B6 mice, was more NKT2-like in the BALB.NK mice (Paper II, figure 4 & 5). However, we could

confirm the association the CD69$^+$ CD49b$^-$ phenotype and IL-4 production, and absence of IL-4 production in CD49b$^+$ NKT cells (Paper II, figure 6).

As only one third of the BALB.NK DN splenic TCRβ$^+$NK1.1$^+$ population was CD1d-tet$^+$, this suggested a higher proportion of NKT2-like cells among non-Vα14 DN NKT cells in the BALB.NK mice. The DN TCRβ$^+$NK1.1$^+$ cells in BALB.NK spleen were not only more NKT2-like but also less frequent. Still, one has to take into account that the CD1d-restricted T cell population in BALB.NK mice may comprise NK1.1$^-$ non-Vα14 NKT cells not included in the analysis. The BALB/c genetic background clearly influenced the ratio of TCRβ$^+$NK1.1$^+$ cell subsets. This would suggest that the NKT cell population in BALB.NK mice have a more limited range of functional repertoires, a feature that may have a strong impact in the very early stages of an immune response.

The composition of NKT1 and NKT2 cells in different mouse strains may show a dramatic variation. NKT cells have been shown to be able to both suppress and augment SLE. The suppression and promotion of this disease was recently shown to be dependent on the mouse strain used in the study (Singh et al., 2005). α-GalCer treatment in pristine-induced lupus prevented lupus in BALB/c mice but augmented disease in SJL mice. In SJL mice, the Vα14 NKT cell population was reduced, while BALB/c mice had similar numbers of Vα14 NKT cells as B6 mice.

In conclusion, different mouse strains clearly have different compositions of NKT cell subsets that may influence the outcome of an immune response. These differences can for example affect the susceptibility to some pathogens or the propensity to develop autoimmune diseases.

Regulation of autoimmune diabetes by non-Vα14 NKT cells (Paper III)

Involvement of NKT cells and TCRβ$^+$NK1.1$^+$ cells in the regulation of autoimmune diabetes has by now been shown in several studies, however none of these have specifically focused on the non-Vα14 NKT cell population. An over expression of Vα14 NKT cells in NOD mice has been shown to decrease the incidence of disease (Lehuen et al, 1998). We wondered if an over expression of non-Vα14 NKT cells, belonging to the NKT1 subset, would lead to a similar result, assigning a regulatory capacity and importance of non-Vα14 NKT cells. We created a TCR transgenic NOD

mouse, expressing the transgenic non-Vα14 TCR previously shown to promote development of non-Vα14 NKT cells with an NKT1-like phenotype in B6 mice (Paper I; Sköld et al., 2000). In the surface phenotype analysis we could determine that the 24$\alpha\beta$NOD mouse had an increased population of T cells with NKT1 cell function and marker expression (Paper III, figure 1, 2 & 4), similar to what we showed in 24$\alpha\beta$B6 mice (Paper I).

The transgenic expression of the non-Vα14 TCR on the NOD background had a strong impact on the diabetes incidence (Paper III, figure 5). Mice having the transgenic non-Vα14 NKT population were almost completely protected against diabetes development. Further analysis revealed that while these mice were protected from disease onset, they still had immune cells infiltrating the pancreas, which suggested that these mice developed autoaggressive cells and were able to initiate an autoimmune response. This result was consistent with our hypothesis that non-Vα14 NKT cells belonging to the NKT1 subset could prevent the autoaggressive cells from full destruction of the insulin producing β-cells.

To see if these cells could actively prevent diabetes we set up cell transfer experiments where we used NOD$scid$ mice, lacking T cells and B cells, as recipients. We could transfer the disease to these mice by injecting 2×10^6 diabetogenic spleen cells taken from diabetic NOD mice. Disease developed within 5 to 11 weeks after the injection. In contrast, coinjection of transgenic non-Vα14 NKT cells together with the diabetogenic cells could prevent or delay the onset of diabetes. This determined that non-Vα14 NKT cells, just as Vα14 NKT, harbor a regulatory capacity in autoimmune diabetes.

A population of TCRβ^+ CD49b expressing cells was shown to be involved in controlling the aggressiveness of the insulitis and β-cell destruction caused by BDC2.5 transgenic cells, expressing the rearranged TCR α and β chain from a diabetogenic β-cell specific CD4$^+$ T cell clone isolated from a diabetic NOD mouse (Gonzalez et al., 2001; Katz et al., 1993). Using NK1.1 expressing congenic NOD mice, the TCRβ^+CD49b$^+$ population was argued not to be NKT cells based on the lack of NK1.1 expression on these cells, though they may belong to a NK1.1$^-$ CD1d-restricted T cell subset. Our finding that NKT1 cells express CD49b and can protect against diabetes in a similar way indicates that these cells could belong to the same subset. Additionally, transgenic expression of BDC2.5 cells in CD1d-deficient NOD

mice increased the diabetes incidence compared to BDC2.5 expression in normal NOD (Shi et al., 2001).

Studies on IL-4 and IL-10 knockout NOD mice demonstrated that the regulatory effect of α-GalCer stimulated Vα14 NKT cells was IL-4 but not IL-10 dependent (Mi et al., 2004). Treatment with OCH, a synthetic glycolipid shown to induce a higher ratio of IL-4 producing Vα14 NKT cells than α-GalCer (Miyamoto et al., 2001), results in an even more profound inhibition of insulitis than α-GalCer treatment (Mizuno et al., 2004). Thus, IL-4 seemed critical for the regulation of autoimmune diabetes by NKT cells. Interestingly, the transgenic non-Vα14 NKT cells in our model were strong IFN-γ and low IL-4 producers (Paper III, figure 4). Theoretically, this would promote a Th1 response that could accelerate the destruction of insulin producing β-cells in the pancreas. Instead these cells were shown to have a protective influence. A recent report showed that the Vα14 NKT cell regulation of T cell differentiation *in vitro* was cell contact dependent and cytokines, including IL-4, only played a marginal role (Novak et al., 2005). This questions the relevance of these cytokines in the regulatory mechanisms involved in autoimmune diabetes.

In support of our finding that a non-Vα14 NKT cell population can regulate autoimmune diabetes, a population of sulfatide reactive non-Vα14 NKT cells has been shown to protect mice from developing EAE (Jahng et al., 2004). Interestingly, sulfatide is expressed in the islets of Langerhans, and treatment of NOD mice with sulfatide reduced the incidence of diabetes (Buschard et al., 2001) suggesting that a similar non-Vα14 NKT cells may be involved in this model.

In conclusion, both NKT1 and NKT2 cells have the capability to protect NOD mice against the development of autoimmune diabetes. If this is achieved by a shared mechanism, and if the two cell types target different stages and/or immune cells involved in the development of disease is not known. The difference in cytokine profile between NKT1 and NKT2 cells urges the importance of identifying other ways of regulation by NKT cells.

Putative NKT cell mechanisms in the regulation of autoimmune diabetes

Research focused on the mechanisms in NKT cell regulation of autoimmune diabetes is suggesting a direct regulation of the auto-aggressive cells or the induction of tolerogenic DCs.
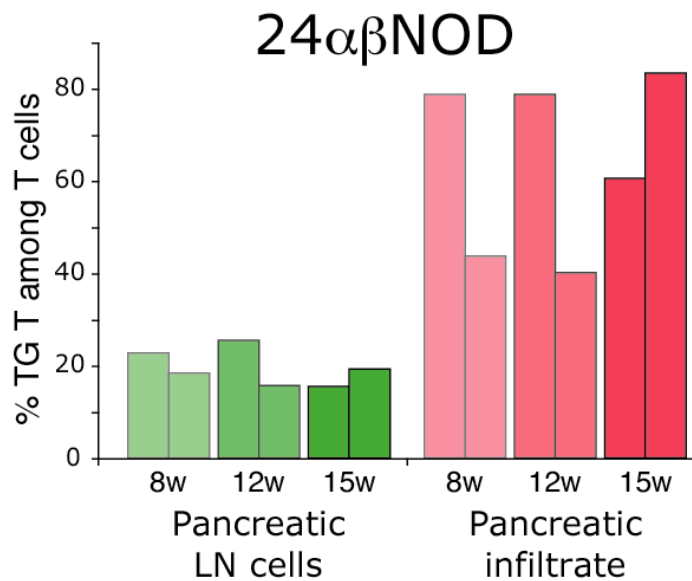

Figure 2. Transgenic T cells accumulate in the pancreas of 24αβNOD mice. Frequency of transgenic (TG) T cells among T cells in the pancreatic infiltrates compared to pancreatic lymph nodes (LN), in 8, 12 and 15 weeks old 24αβNOD mice . Each bar represents one mouse.

Investigating the presence of transgenic cells in the draining LNs and in the pancreatic lesions showed a large population of transgene expressing T cells both in PaLNs and in the pancreas (Figure 2). There was a higher degree of activation among transgenic NKT cells in the pancreas than in the PaLN, as shown by CD69 and CD44 expression (Figure 3). This suggests that the protective effect of the transgenic non-Vα14 NKT cells may be carried out locally in the pancreas, permitting self-reactive activation and invasion of the pancreas, similar to what was seen for another regulating CD49b expressing T cell population (Gonzalez et al., 2001), but preventing the destruction of β-cells.
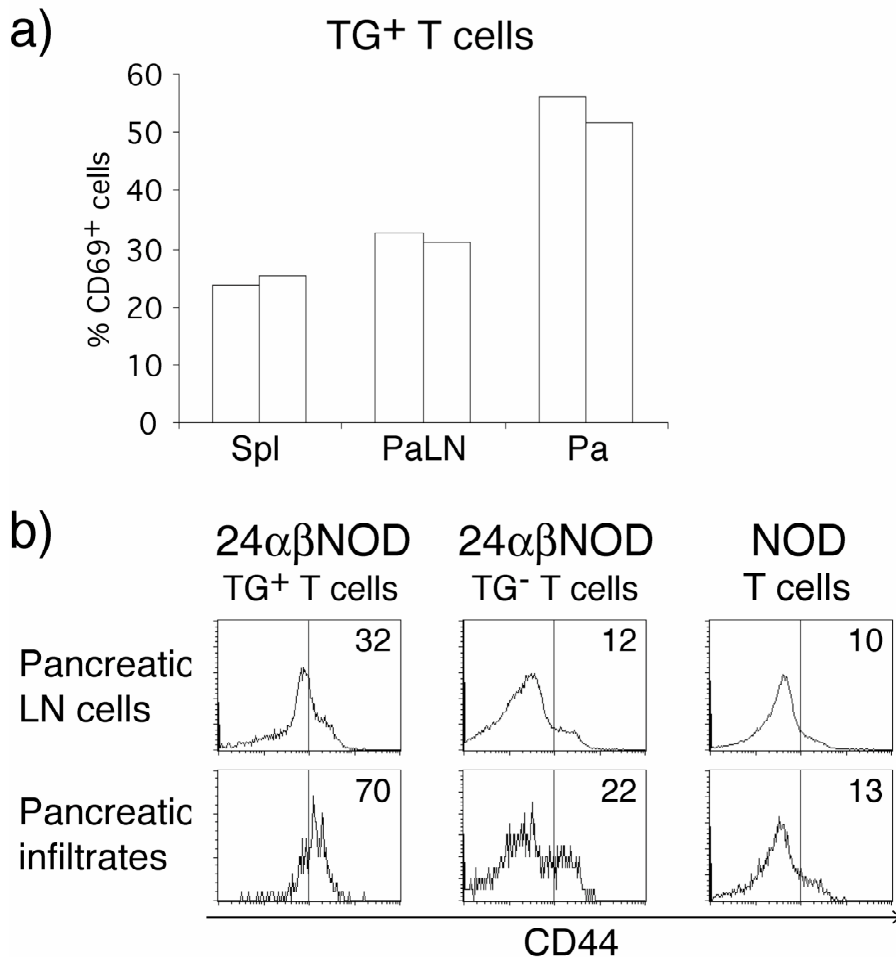
Figure 3. Higher frequency of activated transgenic (TG+) cells in the pancreatic infiltrates (Pa) compared to pancreatic lymph nodes (PaLN) and spleen (Spl). **a)** Frequency of $CD69^+$ cells among TG^+ cells in spleen, PaLN and pancreatic infiltrates, in 12 weeks old 24αβNOD mice . Each bar represents one mouse. **b)** CD44 expression in PaLN and pancreatic infiltrates on TG^+ and TG^- cells from 12 weeks old 24abNOD mice, and T cells from 12 weeks old NOD mice. Numbers represent percentage of cells above the vertical line.

Because we found infiltrating cells in the transgenic mice, without disease development, we investigated the infiltrates by immunohistology. Transgenic mice showed a decrease in the number of islets with invasive infiltration, as well as an increase of infiltrated areas where no islet could be detected (Figure 4). This

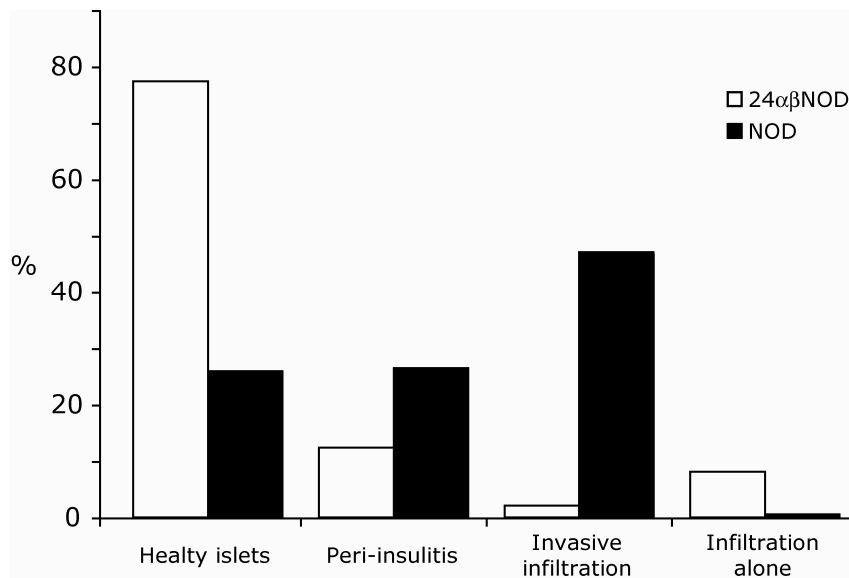suggested that the transgenic infiltrates were less aggressive, possibly regulated by transgenic NKT cells.

Figure 4. Charaterization of the infiltration of 24αβNOD and NOD pancreas. Pancreatic sections from transgenic and wildtype NOD mice were analyzed for infiltration, divided into four different groups. 1. Healthy islet, no visible infiltration; 2. Peri-insulitis, infiltrating cells but no invasive insullitis; 3. Invasive infiltration, cells inside the islet; 4. Infiltration with no visible islet.

In a model using the BDC2.5 transgenic cells, Vα14 NKT cells inhibited part of the expansion and the differentiation of BDC2.5 cells into IFN-γ producing cells in PaLN (Beaudoin et al., 2002). Protection from diabetes was not seen if pre-activated BDC2.5 transgenic cells were transferred. This observation suggests that the protective effect of Vα14 NKT cells was not carried out locally in the pancreas but during the activation and/or differentiation stage. Islet reactive naïve T cells were shown to be activated in PaLN before migrating to the islets (Höglund et al., 1999). Even if diabetogenic cells also could be found in gut-associated LNs in the early stages of disease (Jaakkola et al., 2003), PaLN seems to be important for the initiation of the autoimmune response, as removal of PaLN protected mice against insulitis and diabetes development (Gagnerault et al., 2002). This demonstrated the importance of PaLN in the priming of auto aggressive T cells and a possible site of regulation.

In accordance with this, α-GalCer treatment of mice resulted in the accumulation of Vα14 NKT cells and myeloid DCs in PaLNs, suggesting that the immunoregulatory role of Vα14 NKT cells is manifested by the recruitment of tolerogenic DCs to the PaLN, resulting in the inhibition of an ongoing autoimmune inflammation. Transfer of these myeloid DC into NOD mice completely prevented diabetes development (Naumov et al., 2001). Repeated injection of α-GalCer into mice has recently been shown to result in splenic DCs acquiring properties of regulatory DCs (Kojo et al., 2005).

Most of the studies on NKT cell regulation of autoimmune diabetes have been focused on the regulation of CD4$^+$ T cells. A system involving CD8$^+$ diabetogenic T cells has recently shown that Vα14 NKT cells provided help to CD8$^+$ diabetogenic T cells resulting in an exacerbated development of diabetes (Griseri et al., 2005). Hypothetically, other subsets of NKT cells may have the ability to regulate autoaggressive CD8$^+$ T cells.

Further studies on cells present in the pancreatic infiltrates and PaLNs of transgenic mice can hopefully reveal the mechanisms involved in transgenic non-Vα14 NKT cell interactions with APCs and/or autoaggressive cells in this model. Understanding the differences and similarities in the regulation of autoimmune diabetes by NKT1 and NKT2 cells will extend the possibilities to use this knowledge to control the disease.

Concluding remarks

The interest in NKT cells has increased enormously in the last decade. Indeed, it is an intriguing T cell population that is clearly different from conventional T cells in several ways. Regulation of the immune system has become a large and very interesting field in immunology research, and NKT cells most certainly play a part in immunoregulation. The capacity to rapidly secrete large amounts of immunoregulatory cytokines, such as IL-4 and IFN-γ, has suggested their importance in early skewing of an immune response. But that is not all, NKT cells have been shown to be involved in an enormous range of immunological processes and disease models. In this thesis we suggest that this high diversity of NKT cell function is carried out by different functional subsets, which can be identified by TCR specificity and surface marker expression. We also show that the composition of these subsets is different depending on the genetic background, which may contribute to why different mouse strains in some situations are more prone to mount a Th1 or a Th2 response. In the diabetes model we show that, although NKT1 and NKT2 cells may differ in some functional abilities, such as cytokine production, other immunological roles can be shared by both subsets, such as regulation of autoimmunity. Taken together, this shows the importance of not neglecting the non-Vα14 NKT cell population. Even though a lot of progress has been made regarding NKT cell biology, there is still a lot more to be understood concerning their function and how, and by which means, they can influence an immune response. This is especially true for non-Vα14 NKT cells. Trying to find out which mechanisms that are shared by all NKT cells and which are not, will broaden our knowledge on NKT cell biology even further, and increase our possibility to control the immune system in a way that may prevent diseases and autoimmunity.

Populärvetenskaplig sammanfattning

Immunsystemet består av ett interaktivt nätverk av olika celler som tillsammans utgör vårt försvar mot bakterier och virus, men också mot celler som blir onormala som t.ex. cancerceller. Immunsystemet är involverat i nästan alla delar av kroppen, vilket gör det till ett mycket intressant och komplext system med många olika funktioner. Immunsystemet kan delas in i medfött och adaptivt immunförsvar. Det medfödda försvaret består av celler som kan känna igen typiska patogena strukturer. Det medfödda försvaret är det som är först att attackera en ny okänd patogen och fungerar som ett första försvar under tiden som det adaptiva försvaret byggs upp. Det adaptiva immunförsvarets viktigaste celler är T- och B-lymfocyter. B-lymfocyter är de celler som producerar antikroppar som binder till bakterier och virus vilket leder till att de kan oskadliggöras av andra celler. T-cellerna delas in i cytotoxiska T-celler och hjälpar T-celler. De cytotoxiska cellerna kan attackera och döda en cell som t.ex. blivit infekterad, medan hjälpar-cellerna fungerar som hjälp för andra celler i immunförsvaret. Utöver dessa T-celler finns det flera små populationer som har specifika uppgifter i immunsystemet. En sådan okonventionell T-cellspopulation är de naturliga mördar, eller natural killer (NK), T-celler som den här avhandlingen handlar om.

NKT-celler skiljer sig markant från vanliga T-celler genom att de är tidigt involverade i ett immunförsvar. De är snabba på att producera cytokiner, kemiska signalsubstanser, som kan styra hur ett immunsvar utvecklas. Dessutom känner de igen och aktiveras av lipider, istället för peptider som vanliga T-celler. Dessa lipider presenteras på en antigenpresenterande molekyl som heter CD1d. NKT-celler har visats vara inblandade i en mängd olika immunologiska situationer, som bekämpning av bakterier och parasiter, reglering av autoimmunitet och cancertumörer. En anledning till att de kan ha så varierande egenskaper kan förklaras med att NKT-celler består av flera funktionella populationer.

I vårt arbete har vi identifierat ytmarkörer som gör det möjligt av känna igen olika funktionella NKT-celler, som vi kallar NKT1- och NKT2-celler. Vidare så har vi jämfört dessa populationer i två olika musstammar, och sett att fördelningen av NKT1- och NKT2-celler kan variera beroende på musstam. I det tredje arbetet använde vi oss av en musmodell, kallad NOD, som spontant utvecklar autoimmun diabetes. En population av NKT-celler har tidigare visats kunna reglera

diabetesutvecklingen i denna musstam. Dessa NKT-celler tillhör den funktionella NKT2-populationen. Vi ville veta om även NKT-celler som tillhör NKT1-populationen kan reglera diabetes i NOD-möss. Genom att transgent uttrycka en NKT1 T-cellreceptor, och därmed öka antalet NKT1-celler, utvecklade mössen inte diabetes. Genom att överföra NKT1-celler tillsammans med diabetesinducerande celler kunde vi visa att även NKT1-celler har förmågan att reglera autoaggressiva celler i autoimmun diabetes.

Genom att identifiera de mekanismer som är involverade i NKT-cellers reglering av autoimmunitet och hur olika funktionella populationer fungerar, kommer vi att öka möjligheten att i framtiden kunna styra och reglera vårt immunförvar på ett för oss fördelaktigt sätt, som att t.ex. kunna motverka en autoimmunsjukdom.

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