ORIGINAL PAPERS

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

I. Julia Rolf[*], Vinicius Motta[*], Nadia Duarte, Marie Lundholm, Emma Berntman, Marie-Louise Bergman, Lydia Sorokin, Susanna L. Cardell[§] and Dan Holmberg[§]. (2005). The enlarged population of marginal zone/CD1d(high) B lymphocytes in nonobese diabetic mice maps to diabetes susceptibility region Idd11. J Immunol. *174*, 4821-7.

II. Julia Rolf, Emma Berntman, Martin Stenström, Emma Smith, Robert Månsson, Hanna Stenstad, Tetsuya Yamagata, William Agace, Mikael Sigvardsson and Susanna L. Cardell. (2007). Molecular profiling reveals distinct functional attributes of CD1d-restricted natural killer (NK) T cell subsets. Submitted manuscript.

III. Emma Berntman, Julia Rolf, Cecilia Johansson, Per Andersson and Susanna L. Cardell. (2005). The role of CD1d-restricted NK T lymphocytes in the immune response to oral infection with *Salmonella typhimurium*. Eur J Immunol. *35*, 2100-9.

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ABBREVIATIONS

ACAID	anterior chamber-associated immune deviation
AFC	antibody forming cell
AICD	activation induced cell death
αGalCer	alpha-galactosylceramide
APC	antigen-presenting cell
BAFF	B cell-activating factor of the tumour necrosis factor family
BCR	B cell receptor
CD	cluster of differentiation
CDR	complementarity determining region
cM	centiMorgan
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DN	double negative ($CD4^-CD8^-$)
dNKT	diverse natural killer T cell
DP	double-positive ($CD4^+CD8^+$)
dsDNA	double-stranded DNA
EAE	experimental allergic encephalomyelitis
ER	endoplasmatic reticulum
FDC	follicular dendritic cell
FO B	Follicular B cell
GC	germinal center
ICAM	intercellular adhesion molecule
Idd	insulin dependent diabetes
IEL	intraepithelial lymphocyte
IFN	interferon
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IL	interleukin
iNKT	invariant natural killer T cell
J	joining
LFA-1	lymphocyte function associated antigen 1
LOD	logarithm of odds
LPS	lipopolysaccharide
Ly49	lymphocyte antigen 49 complex
MAdCAM	mucosal vascular addressin cell adhesion molecule 1
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MZ	marginal zone
NF B	Newly Formed B cell
NK	natural killer cell
NKT	natural killer T cell
NOD	nonobese diabetic
NZB/NZW	New Zealand black/white
PaLN	pancreatic lymph node
PC	phosphorylcholine
p.i.	post infection
PP	Peyer's patches
SLE	systemic lupus erythematosus
T1/T2	transitional 1/ transitional 2
T1D	type 1 diabetes
TCR	T cell receptor
TD	thymus dependent
Th	T helper cell
TI	thymus independent
TLR	Toll like receptor
TNFSF	tumour necrosis factor super family
UC	ulcerative colitis
V	variable
VCAM	vascular cell adhesion molecule

GENERAL INTRODUCTION

The immune system poises the human body for the ongoing battle against invading microbes. Through the constant exposure to harmful pathogens, the immune system has been shaped under high evolutionary selective pressure. The most primitive mechanisms of immune protection are mediated through recognition of certain microbial molecules that do not exist in multi-cellular organisms. The branch of the immune system that immediately identifies and becomes activated by microbes is called the innate immune system. Mechanical barriers and chemical barriers together with innate immunity are forming the first line of defence against invading pathogens. If the barriers are breached by pathogens, the innate immune system will promote inflammation that is aimed at attracting immune cells and to neutralize the invader. The recognition of typically foreign molecules is pivotal for induction of innate immunity, but microbes have developed mechanisms to counteract the innate immune system. The more complex branch of the immune system called adaptive immunity is superior in adjusting to the evasion mechanisms used by the pathogens. Adaptive immunity is incredible flexible due to the process of rearrangement of genetic segments that create extremely specific antigen receptors. The antigen-specific repertoire of lymphocytes is capable of almost infinite diversity. However, the adaptive immune system is time-consuming to mobilize, since the activation phase is very complex. In recent years there has been an increasing focus on lymphocytes that possess innate-like properties, such as rapid activation. The innate-like lymphocytes are in a naturally activated state and can directly exert their functions, similarly to the immediate effects of innate immunity. The innate-like lymphocytes share their effector mechanisms with conventional lymphocytes, including antibody-production and secretion of immunomodulatory substances. The innate-like T lymphocytes include natural killer T (NKT) cells that are activated by endogenous as well as foreign glycolipids. The NKT cells are a specialized lineage of T cells that respond very rapidly and potently to stimulation. In this thesis, the global gene expression profile of NKT cells and the response of NKT cells to bacterial infection were studied. Another innate-like lymphocyte population is the marginal zone (MZ) B cells that are important for early responses against microbes in the blood. MZ B cells are capable of recognizing many types of antigens and have been implied in the activation of conventional lymphocytes. In this thesis, the MZ B cells in the nonobese diabetic (NOD) mouse model of autoimmune type 1 diabetes were analyzed. Both NKT cells and MZ B cells play unique roles in the immune system and participate in combating infections, self-destructive disease such as type 1 diabetes and in maintaining tolerance. In summary, this thesis aspires to deepen the understanding of the two innate-like lymphocyte populations NKT cells and MZ B cells in different immunological settings.

INNATE-LIKE LYMPHOCYTES

The specialized innate-like lymphocytes with limited diversity of antigen receptors may represent the primordial repertoire of lymphocytes. The innate-like lymphocyte antigen receptors can identify molecules derived from pathogenic microbes but also self-antigens. In many cases these molecules are carbohydrates or glycolipids that represent another set of antigens than the proteins or peptides recognized by conventional lymphocytes. The innate-like lymphocytes include Marginal Zone (MZ) B cells, B1 B cells, Natural Killer T cells (NKT), γδ T cells and CD8αα TCRαβ$^+$ T cells. CD8αα-expressing TCRαβ$^+$ T cells and CD8αα or DN γδ T cells constituted type b intraepithelial lymphocytes (IELs) in the gut. Specialized γδ T cells called dendritic epidermal T cells (DETC) form a cellular network in the dermis of the skin.

The innate-like lymphocytes are localized to areas of high antigenic exposure in the epithelial layer of the gut or in specialized niches within organs such as the marginal zone in the spleen. Innate-like lymphocytes exist in a naturally activated state. This naturally activated phenotype facilitates rapid activation upon antigen recognition and innate-like lymphocytes may perform their effector functions within hours after antigenic challenge (reviewed by (Bendelac et al., 2001)).

The gene expression profile of innate-like lymphocytes

Microarray analysis provides a powerful tool to expand the knowledge of the innate-like lymphocyte profile. Many functionally important genes are shared by the innate-like T lymphocyte populations NKT, CD8αα TCRαβ$^+$ and γδ T cells (Denning et al., 2007; Paper II Yamagata et al., 2004). The activation status alone does not confer the typical gene expression associated with innate-like lymphocytes to conventional effector/memory lymphocytes (Yamagata et al., 2006). Therefore, the innate-like lymphocytes can be considered as a separate entity within the immune system. Since the functions and characteristics of innate-like lymphocytes were previously relatively unknown, microarray analysis has provided profound new knowledge of the features of these cell-types and their functional capacities.

The selection and development of innate-like lymphocytes

During the development of lymphocytes there are strict mechanisms that mediate purging of too strongly self-reactive lymphocytes. However, there is an increasing understanding that the dogma of deletion of self-reactive lymphocytes has exceptions. The key feature of the development of innate-like lymphocytes is that there is a window of positive selection by self-antigen recognition. The requirement for self-reactivity during selection has been studied during the formation of CD8αα TCRαβ$^+$ cells and B1 B cells. Positive selection in the thymus of CD8αα TCRαβ$^+$ cells was crucially dependent on the presence of the self-antigen recognized by their TCR (Leishman et al., 2002). Similarly to CD8αα TCRαβ$^+$, development of B1 B cells required the expression of self-antigen in order for positive selection to occur (Hayakawa et al.,

1999). Taken together, development of innate-like lymphocytes may in some cases depend on specific recognition of self-antigens.

Rapid activation of innate-like lymphocytes

The innate-like lymphocyte subsets are generally rapidly responding and may initiate their effector functions immediately upon activation. The "activated yet resting" state of type b IELs is reflected in their gene expression profile that contains many cytolytic effector molecules such as granzymes and the apoptosis-inducing molecule Fas (reviewed by (Hayday et al., 2001)). NKT cells also constitutively express mRNA for the immunomodulatory cytokines IL-4 and IFN-γ (Stetson et al., 2003). Despite the mRNA expression of effector molecules enhancing immunity, all the innate-like T cell populations are also strongly associated with protection against autoimmunity. The type b IELs are known to be involved in maintaining epithelial cell integrity and wound healing (reviewed by (Cheroutre, 2005)). The overall interpretation of innate-like T lymphocyte effector functions is that they do mediate potent immunological responses but that they are maintained in an "activated yet resting" state.

The main function of B1 B cells and MZ B cells is to respond to encapsulated bacteria and rapidly secrete protective IgM (reviewed by (Martin and Kearney, 2000a)). The B1 B cell population consists of two sub-populations: B1a and B1b B cells. The B1a B cells express the negative regulator of BCR signalling CD5 and are the major contributors to natural IgM that can bind to extracellular bacteria (Haas et al., 2005). These low-affinity IgM antibodies are poly-reactive and therefore likely to target any antigen present in the systemic circulation. B1b B cells on the other hand respond specifically to antigens by producing IgM and can mount memory-responses (Alugupalli et al., 2004; Haas et al., 2005). B1 B cells also play an important role by producing IgA that is secreted into the lumen of the intestine (reviewed by (Fagarasan and Honjo, 2003)). The innate-like B lymphocytes are able to mount efficient antibody-responses that neutralize pathogens and also to secrete protective antibodies in the absence of T cell help. The B1 B cells and MZ B cells are functionally united in mediating humoral immunity against extracellular bacteria during the early phase of an infection.

MARGINAL ZONE B CELLS

MZ B cells reside in the spleen

The spleen of a mouse contains about 100 million lymphocytes and has an extraordinary capacity to initiate immune response to infectious agents spreading through the blood stream. The spleen is an organ with densely packed lymphocytes residing primarily in the white pulp that is divided into the B cell follicle and the T cell area (Figure 1). The red pulp surrounds the white pulp areas and consists of blood vessel networks and contains macrophages that remove aged erythrocytes. Histological studies of the murine spleen demonstrated a specialized region sandwiched between the white pulp and the red pulp, dubbed the marginal zone (MZ) (MacNeal, 1929). Further analysis revealed that the MZ region contained a large fraction of immunoglobulin (Ig)-positive cells, termed marginal zone B cells.

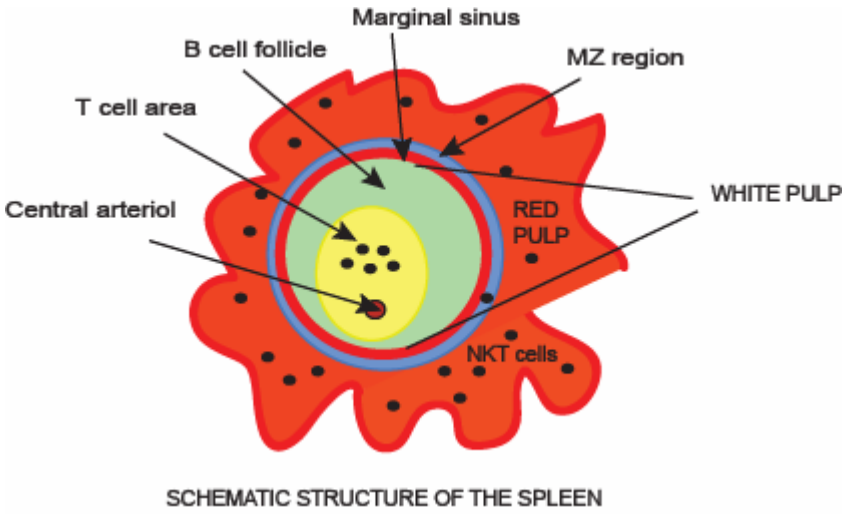

Figure 1. The structure of the spleen. The white pulp consists of the T cell area surrounded by the B cell follicle. The MZ region encircles the white pulp and is localized between the marginal sinus and the red pulp. The NKT cells seen as black dots are dispersed in the T cell area and the red pulp.

The MZ B cell population in rats was extensively characterized by MacLennan's research group during the 1980ies. The MZ B cells were found to be larger in size than Follicular (FO) B cells, sessile in the MZ region, $IgM^{+}IgD^{-}$ and expressed the complement receptor CD21/35 and low levels of the IgE Fc-receptor II CD23 (Gray et al., 1982; Gray et al., 1984; Kumararatne et al., 1981; Waldschmidt et al., 1991). The functions of MZ B cells include binding to complement-coated antigens and also to mount antibody responses to thymus independent 2 (TI-2) antigens that are repetitive polysaccharide structures that can be found in bacterial capsules (Gray et al., 1984; Lane et al., 1986). The MZ region was also found to be the preferred residence for memory B cells derived from thymus dependent (TD) immune responses (Liu et al., 1988; Shih et al., 2002). Stimulation with LPS of MZ B cells resulted in rapid proliferation, IgM and IgG_3 antibody secretion and up-regulation of the co-stimulatory molecule B7-2 (CD86) (Oliver et al.,

1997; Oliver et al., 1999). The phenotype of MZ B cells is distinct and defined as $CD21^{hi}CD23^{lo}IgM^{hi}IgD^{lo}$, in contrast to FO B cells that are determined as $CD21^{lo}CD23^{hi}IgM^{lo}IgD^{hi}$ B cells. In B6 mice, MZ B cells represent 5-10 % and FO B cells 80% of the splenic B cells. In terms of localization the MZ B cells are always found in the MZ region during homeostasis, whereas the FO B cells are continuously re-circulating through the body. Another important feature of MZ B cells is their high levels of CD1d that presents glycolipid antigens to NKT cells, implying that MZ B cells may be capable to act as antigen-presenting cells (APCs) to NKT cells (Amano et al., 1998; Makowska et al., 1999; Roark et al., 1998). The development of the MZ/$CD1d^{hi}$ B cell population occurs late during ontogeny and this population is fully formed at approximately 7 weeks of age in mice (Makowska et al., 1999). The reason for the delay in MZ B cell development compared to other B cell subsets is not known. The features of MZ B cells, such as surface phenotype, localization, antibody-responses and development demonstrate that MZ B cells represent a unique B cell lineage.

MZ B cell localization

Arteriolar branches terminate in the marginal sinus and the blood that is emptied into the marginal sinus percolates into the MZ region. The blood seeps slowly through the MZ niche, resulting in direct encounter between the APCs and antigens present in the blood. The MZ APC-populations include MZ macrophages, marginal metallophilic macrophages, DCs and the MZ B cells (reviewed by (Mebius and Kraal, 2005)). The MZ B cell localization is intimately linked to their major role in the immune system, namely to rapidly respond to blood-borne antigens (reviewed by (Martin and Kearney, 2002)). The MZ B cell localization is dependent on the integrins $\alpha_L\beta_2$ and $\alpha_4\beta_1$ binding to ICAM-1 and VCAM-1 on stromal cells, respectively (Figure 2). *In vivo* blocking of these integrin pairs results in dislodgement of the MZ B cells from the MZ (Lu and Cyster, 2002). Data from our group in collaboration Prof. Lydia Sorokin's group has shown that the MZ region contained a basement membrane-like extracellular matrix structure and that the MZ B cells expressed integrin pairs that bind specifically to matrix proteins. MZ B cells were capable of attaching to extracellular matrix molecules such as laminin *in vitro* (Lokmic, Z. and Rolf, J. et al., unpublished observations). The MZ B cell localization seems to be mediated by integrin-binding to cell-adhesion molecules and extracellular matrix.

The retention of MZ B cells is a balance between sphingosine-1-phosphate receptor 1 (S_1P_1) that counteracts the CXCR5 signalling in the MZ B cell. The chemokine CXCL13 binding to CXCR5 will attract MZ B cells into the B cell follicle after *in vivo* activation, whereas S_1P_1 promotes MZ B cell retention in the MZ region during homeostasis (Cinamon et al., 2004). Several components down-stream of G-protein coupled receptor (GPCR) signalling and signalling molecules that regulate integrin binding are required for keeping the MZ B cells in the MZ region and the absence of these components results in the loss of MZ B cells.

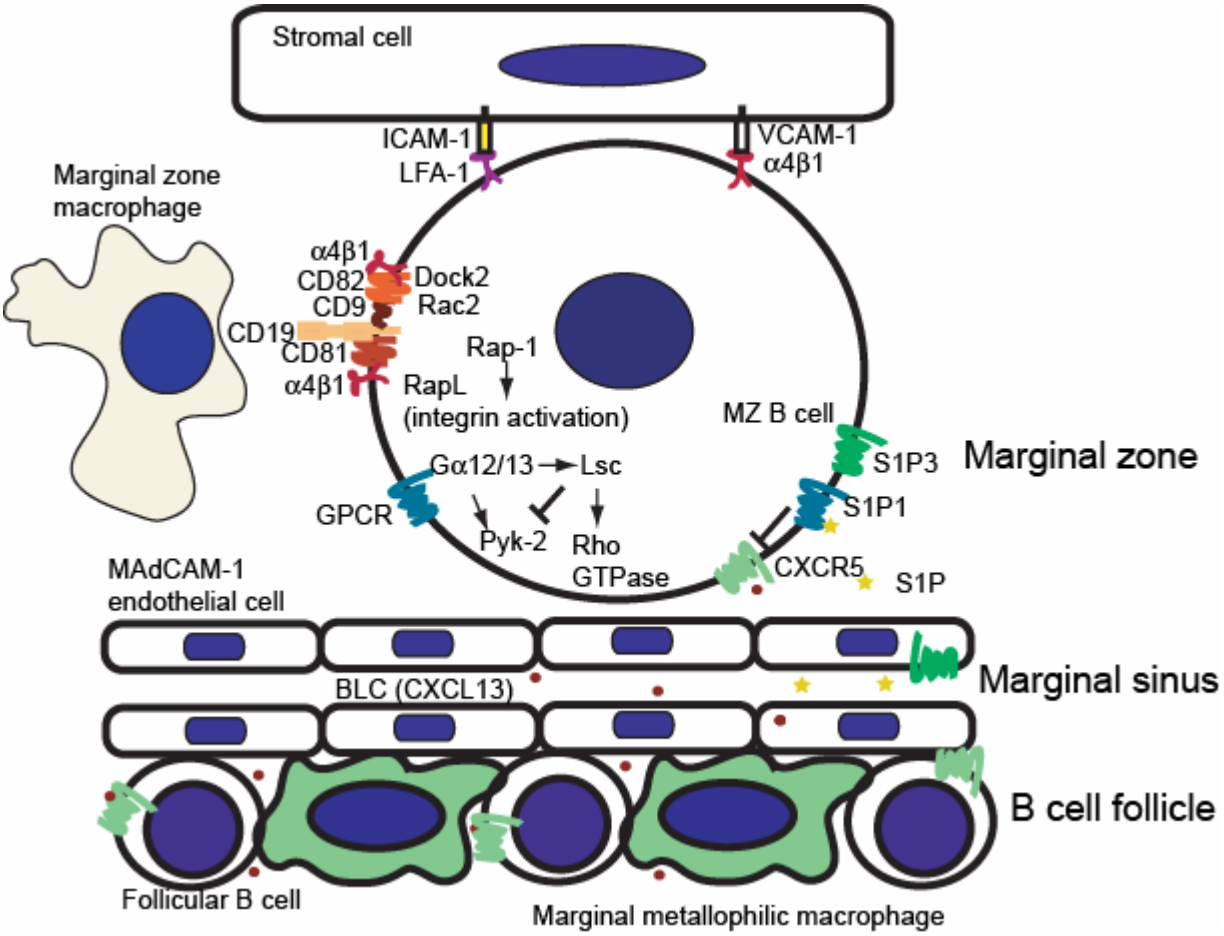

Figure 2. The MZ B cells are retained in the MZ region. The MZ B cell localization in the MZ region is an active process that requires GPCR signalling, integrin activation and binding to stromal cells and MZ macrophages found in the MZ region. Absence of the molecules regulating MZ B cell localization results in loss of the entire MZ B cell population.

The B cell receptor repertoire of MZ B cells

Although the selection of MZ B cells is not completely understood, there are indications that multi-reactive BCR specificities are enriched within the MZ B cell pool, presumably by positive selection (Martin and Kearney, 2000b). The lack of nontemplate (N) nucleotide insertions into the sequence of IgH chain genes has been suggested to give multi-reactive antibody-specificities that are enriched in the B1 B cell compartment (Benedict and Kearney 1999). Just like the multi-reactive B1 BCR specificities, the MZ B cell BCR repertoire was highly enriched for IgH chains that lack N-nucleotide inserts (Dammers et al., 2000; Kretschmer et al., 2003). Moreover, the IgH complementarity determining region CDR3 that makes contacts with the antigen was on average two amino acids shorter among MZ B cells than among FO B or the immature Newly Formed (NF) B cells and this feature may be linked to multi-reactivity (Dammers et al., 2000).

Taken together, the multi-reactive MZ B cell BCR specificities may result from the lack of N-nucleotide insertions and short CDR3 regions (Chen et al., 1997b; Oliver et al., 1999).

Human MZ B cells

In humans, it is thought that the lack of MZ B cells during infancy is correlated to the high sensitivity to infections caused by encapsulated bacteria such as *Streptococcus pneumoniae* in young children. The *S. pneumoniae* are encapsulated gram-positive bacteria that cause serious upper respiratory tract infections in infants, elderly and in asplenic individuals. In fact, the MZ in humans was not fully populated by $CD21^{+}$ cells until 2 years of age, which coincided with development of efficient antibody-responses to TI-2 antigens present in bacterial capsules (Timens et al., 1989). The role of MZ B cells in *S. pneumoniae* infections suggests that in terms of function, human and rodent MZ B cells are similar. However, there are also several important differences between the human and rodent MZ B cell populations and MZ structures. In humans, most of the MZ B cells had mutated IgH chains, which strongly suggested that they had undergone germinal center reactions and therefore represented antigen-experienced memory B cells (Dunn-Walters et al., 1995). Unlike rodents, human MZ B cells express CD27, which is correlated to memory and they are also capable of continuously re-circulating throughout the body. The anatomical structure of the human MZ is different from rodents. Humans lack the blood vessel network called the marginal sinus that separates the MZ from the B cell follicle in rodents. The human MZ region is directly overlying the follicle and the outer border of the human MZ is surrounded by a specialized region of the red pulp called the perifollicular zone (reviewed by (Steiniger et al., 2006)). The impact of these anatomical differences on the MZ niche in humans compared to rodents remains to be further elucidated. Despite the phenotypical and anatomical differences, the functional role of MZ B cells seems comparable between humans and rodents.

MZ B CELL DEVELOPMENT

Central and peripheral B cell development

The development from a haematopoietic stem cell into an immature $IgM^{+}IgD^{-}$ B cell occurs in the bone marrow (Figure 3). Only a few percentages of the total immature B cells generated in the bone marrow complete the entire maturation process. The majority of immature B cells undergo apoptosis as a result of unsuccessful Ig rearrangement, clonal deletion or anergy due to auto-reactivity. It is estimated that 75 % of human early immature B cells in the bone marrow display some degree of auto-reactivity, but that the number of auto-reactive B cells is reduced during development (Wardemann et al., 2003). In the bone marrow, auto-reactive immature B cells can perform receptor editing to obtain a new light chain and thus gain different antigen-specificity. The immature B cells that have successfully completed Ig rearrangement and central selection emigrate from the bone marrow and undergo peripheral maturation in the spleen. The immature B cells in the spleen are called Transitional 1 (T1) or NF B cells. However, receptor editing is not supported in the spleen, thus the inappropriately self-reactive T1 B cells that persist after central selection become anergic (Sandel and Monroe, 1999). The state of anergy represents unresponsiveness to stimulation via BCR, caused by alterations of the downstream signalling pathway and also exclusion from pro-survival signals (reviewed by (Cambier et al., 2007)). Interestingly, the T1 B cell anergization is defect in nonobese diabetic mice and the dysfunctional peripheral B cell development is associated with autoimmunity (Quinn et al., 2006; Silveira et al., 2006). Taken together, both central B cell development in the bone marrow and peripheral B cell development in the spleen promote maturation of self-tolerant B cells.

B cell receptor signalling strength determines peripheral B cell maturation

The T1 B cells that are not anergized will develop into the T2 stage in the spleen (Figure 3). The BCR signalling strength is central for maturation of T2 B cells into the MZ B cell compartment or into the FO B cell pool (reviewed by (Su et al., 2004)). However, the progress of development from T2 B cells into the MZ B cell or FO B cell populations are currently described by two contradictory models. One model suggests the MZ B cell versus FO B cell lineage choice is determined by the antigen-specificity of the BCR and that there is a general tendency of multi-reactive BCR-specificities to accumulate into the MZ B cell pool. The selection by weak self-antigen recognition is supported by work showing that transgenic B cells that are multi-reactive are enriched within the MZ B cell population, whereas FO B cells typically recognize foreign protein antigens (Martin and Kearney, 2000b). The other model of MZ B cell versus FO B cell development describes MZ B cell selection to be promoted by weak BCR signals whereas FO B cells are selected by stronger BCR signalling (reviewed by (Pillai et al., 2005)). This hypothesis is supported by different genetically modified mice that have lower level of BCR signalling, which promotes MZ B cell development. Conversely, in genetically modified mice with high BCR signalling level, the FO B cell development is enhanced (Cariappa et al., 2001).

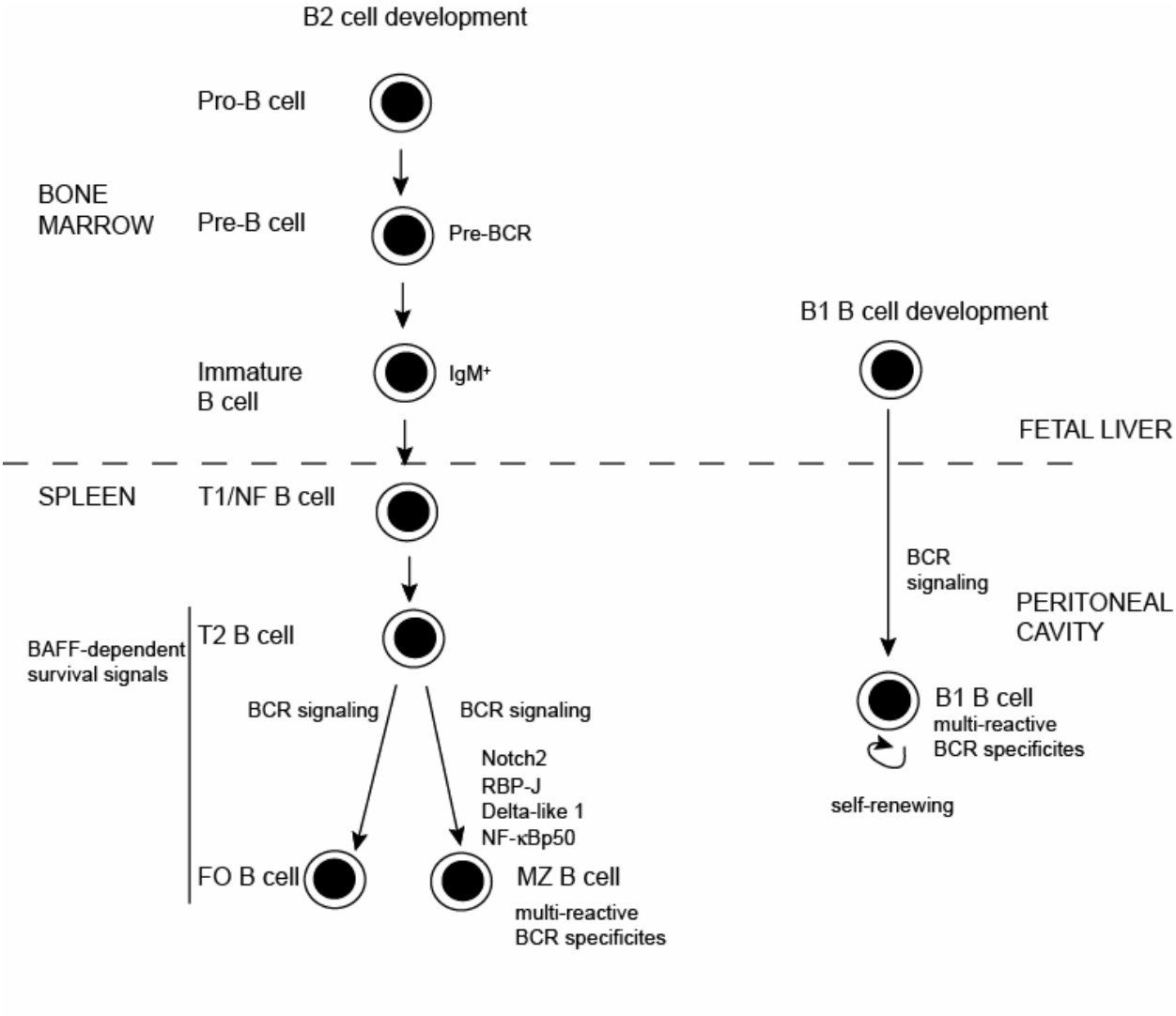

Figure 3. The B cell development. The central B cell development takes place in the bone marrow. The immature B cells that have completed the maturation process are exported to the periphery where they mature further in the spleen. The transitional B cells that are tolerant to self-antigens will develop either into FO B cell or MZ B cell compartments depending on BCR signalling and lineage determining factors, such as Notch2. The mature B cells are responsive to pro-survival signals provided by BAFF. The B1 B cells develop from precursors in the fetal liver and are enriched in the peritoneal cavity where they contribute to the natural IgM production. Both MZ B cells and B1 B cells contain clones with multi-reactive BCR-specificities. Adapted from (Pillai et al., 2005).

The common denominator between these two models is that the BCR signalling is crucial. However, it is unclear how multi-reactive MZ B cells would be selected based on low levels of BCR signalling when in fact the MZ B cells weakly recognize self-antigen and FO B cells typically are ignorant towards self-antigens. One critical issue to understand is the nature of the antigens that determines B cell development. The level of self-antigen directly influenced B cell development and revealed differences between the three B cell lineages: B1 B cells, MZ B cells and FO B cells. B1 B cells were shown to be positively selected based on auto-reactive

specificity of their transgenic BCR towards a carbohydrate antigen on the Thy-1 molecule normally expressed on T cells (Hayakawa et al., 1999). Reduced levels of the Thy-1 antigen led to the formation of MZ B cells, rather than B1 B or FO B cells. In the complete absence of Thy-1, FO B cells were the only B cell population that completed maturation, indicating that FO B cells did not need to recognize the Thy-1 antigen to develop (Wen et al., 2005). One possible model of B cell development is that the FO B cells represents the default pathway to B cell maturation, but that certain ligands that induce a rather weak BCR signalling level will promote the MZ B cell fate. In addition to BCR signalling strength, the expression of Notch2 together with NF-κBp50 (Cariappa et al., 2000; Moran et al., 2007; Saito et al., 2003), the Notch ligand Delta-like 1 (Hozumi et al., 2004) and the Notch down-stream signalling component RBP-J (Tanigaki et al., 2002) were crucial for MZ B cell formation. Also, the balance between the transcription factors E2A and Id3 regulated MZ B cell fate (Quong et al., 2004). In summary, the MZ B cell development depends on ligand-recognition followed by BCR signalling but also on lineage determining factors.

MZ B CELL EFFECTOR FUNCTIONS

The immune cells in the MZ provide first line of defence against blood-borne pathogens

Dissemination of microbes in the blood poses a life-threatening condition by causing septic shock that may lead to collapse of the blood pressure and multi-organ failure. Therefore, the APCs in the MZ region in the spleen play a crucial role by capturing antigens or entire micro-organisms present in the blood via scavenger receptors and complement receptors. The marginal zone macrophages (MZM) are efficiently trapping bacteria such as *Listeria monocytogenes* present in the blood and thereby prevent the systemic spread of pathogens (Aichele et al., 2003). The scavenger receptors MARCO and scavenger-receptor A were found to be important for sequestering of antigens by MZM. The scavenging by MZM was also suggested to enhance IgG3 antibody response to TI-2 antigens such as *S. pneumoniae* polysaccharides (Chen et al., 2005a). The close interaction between MZM and MZ B cells is important for the early induction of immunity against micro-organisms that are present in the systemic circulation. The most important physiological role of MZ B cells is to produce protective antibodies against extracellular bacteria such as *S. pneumoniae*. The MZ B cells are associated with TI-antibody production to polysaccharide antigens and to phosphorylcholine (PC) derived from *S. pneumoniae* (Martin et al., 2001). The formation of MZ B cell plasmablasts that produce antibodies against *S. pneumoniae* is extremely rapid (Balazs et al., 2002). In summary, the MZM and MZ B cells are vital for the protection against microbes in the blood and efficiently trap antigens or organisms present in the systemic circulation.

MZ B cells capture complement-coated antigens

Activation of MZ B cells against *S. pneumoniae* bacteria involves complement-recognition. Natural IgM binding to *S. pneumoniae* capsule polysaccharides elicit the classical complement pathway that results in the targeted bacteria being coated by complement. The expression of the complement receptor CD21 that binds complement component C3d(g) by MZ B cells, was crucial for mounting an efficient immune response against purified Group B *Streptococcus* polysaccharide (Pozdnyakova et al., 2003). CD21-expression on MZ B cells allows them to capture complement-coated IgM-immune complexes and then transport these complexes to the follicular dendritic cells (FDCs) in the B cell follicle (Ferguson et al., 2004). FDCs are a specialized cell-population that forms a network in the B cell follicles and its main function is to promote germinal center (GC) reaction. The GC reaction is a process of selection of optimal BCR specificities through proliferation, somatic hypermutation, affinity maturation and isotype switching of B cells. The GC reaction requires cognate T cell help. The capacity of MZ B cells to capture complement-coated antigens and transfer them to FDC, suggests that MZ B cells provide a functional link between the innate immune system represented by complement and natural IgM antibodies and the adaptive immune system GC reaction. The complement receptor CD21 is associated with CD19 forming the BCR co-receptor complex, which positively regulates B cell activation. The close connection between innate immunity exemplified by complement receptor

expression and adaptive immunity such as BCR specificities contribute to the rapid antibody-producing capacity of MZ B cells compared to FO B cells.

MZ B cell antibody-responses

MZ B cells have been suggested to primarily be important for induction of TI IgM-production rather than GC-dependent TD-response (Phan et al., 2005). The importance of the MZ B cell population in the humoral immune response was first demonstrated in the tyrosine kinase Pyk-2 deficient mice that have a selective reduction in the MZ B cell population. The Pyk2-/- mice had reduced IgM titers to all antigens, reduced IgG2a antibody titers to TI-1 antigen and TI-2 antigen and reduced IgG3 antibody titers to TI-2 antigen. In addition to the well-characterized role of MZ B cells in TI-immune responses, the MZ B cells also contributed to high levels of IgM against protein-antigens and rapidly became extrafollicular plasma cells (Guinamard et al., 2000). Similar results were obtained after immunization with haptenated-proteins. The MZ B cells differentiated into antibody-forming cells (AFCs) in the red pulp and secreted high levels of IgM and IgG during the first week after challenge. In contrast, FO B cells responded slower and tended to undergo GC reactions that gave rise to somatically hypermutated, high affinity IgG-antibodies. MZ B cells are also capable of forming GCs, although they seem to be more important during the first week after antigenic challenge when the IgG derived from MZ B cell AFCs had higher affinity to the antigen than IgG produced by FO B cells (Song and Cerny, 2003). These results were corroborated in a study showing that MZ B cells participated both in the TI and TD antibody responses against viral particles at earlier time-points than FO B cells (Gatto et al., 2004). Taken together, MZ B cells are most important in the early phase of an immune response by capturing antigens and producing IgM. The IgM antibodies can neutralize the invading pathogen before high-affinity IgG antibodies derived from FO B cells undergoing GC-reactions are formed.

MZ B cell production of auto-antibodies

Autoimmune diseases are complex and depend on many factors that lead to the break-down of tolerance to self-molecules. In general, T and B lymphocytes are the main culprits in autoimmune diseases, since they express antigen-receptors with the potential to recognize self-molecules. The selection of lymphocytes is crucial for avoidance of autoimmunity, but in the case of MZ B cells the multi-reactive specificities are allowed to persist. This leads to the question if the MZ B cells may initiate autoimmunity when recognizing self-molecules.

B cell auto-reactivity is manifested in system lupus erythematosus (SLE) characterized by high levels of circulating anti-nuclear antibodies directed against double-stranded (ds) DNA, histones and nuclear proteins. Immune-complexes form as a result of the auto-antibodies binding to self-antigen and these complexes accumulate in the kidney with age. There are several factors implying MZ B cells in auto-antibody mediate autoimmunity such as multi-reactive IgM, low threshold of activation, anatomical localization that provides easy accessibility to antigens, the

ability to trap complement-coated antigens via CD21, the capacity to transfer immune complexes to FDCs and also the potent ability to prime naïve $CD4^+$ T cells. Analysis of the MZ B cell population has been done in several common SLE models in order to determine if they are involved in autoimmunity. The MZ B cell population was observed to be expanded in the SLE model (NZBxNZW) F1 mice (Wither et al., 2000a; Wither et al., 2000b). However, the auto-antibody production in (NZBxNZW)F1 mice was a genetically separate trait from the increased MZ B cell population. This indicates that the expansion of the MZ B cell population does not directly contribute to autoimmunity in (NZBxNZW)F1 mice (Atencio et al., 2004). In another well established SLE model, the Yaa mutation causes lupus predominantly in males. In the Yaa male mice, the MZ B cell population was strongly diminished due to B cell-intrinsic factors and therefore not likely to contribute to lupus pathology (Amano et al., 2003). In contrast, estrogen-treatment enhanced the proportion of MZ B cells in mice expressing a low-affinity dsDNA-binding BCR-transgene and promoted the MZ B cell mediated anti-DNA antibody-secretion (Grimaldi et al., 2001). Several studies have shown that weakly auto-reactive B cells are enriched in the MZ B cell compartment. Partially auto-reactive anti-dsDNA specific B cells are permitted to exist in the MZ B cell pool (Li et al., 2002). However, the actual contribution of these low-affinity self-recognizing antibodies to autoimmunity is still unclear. There are differences in MZ B cell and FO B cell contribution to autoimmunity. Weakly auto-reactive MZ B cells in mice transgenic for glucose-6-phosphate-isomeras (GPI) spontaneously secreted IgM antibodies reactive to GPI. In contrast, the re-circulating FO B cell population did not display signs of auto-reactivity unless provided with cognate $CD4^+$ T cell help (Mandik-Nayak et al., 2006). In conclusion, the weakly auto-reactive nature of MZ B cells may promote the initial phase of antibody-mediated autoimmunity by spontaneous IgM-secretion in the absence of T cell help, but the relative importance of MZ B cells in autoimmunity seems to be highly dependent on the nature of the autoimmune model.

The survival factor BAFF promotes MZ B cell-mediated autoimmunity

The survival signals mediated by B cell activating factor of the tumour necrosis factor family (BAFF) binding to the BAFF-R, was shown to be absolutely required for normal mature B cell homeostasis (Schiemann et al., 2001; Thompson et al., 2001). However, BAFF has been implied in autoimmunity, since excess of the survival factor BAFF allowed the maturation of auto-reactive B cells into the FO B or MZ B cell populations (Thien et al., 2004). Analysis of BAFF-transgenic mice revealed that they had an expanded MZ B cell population and developed SLE-like symptoms and also Sjögren's syndrome, caused by autoimmune destruction of the salivary glands (Mackay et al., 1999; Groom et al., 2002). BAFF also played an important role in positive regulation of integrin expression on B cells, thereby promoting efficient localization of MZ B cells to the MZ region. Transfer of MZ B cells into BAFF transgenic mice, resulted in higher levels of anti-dsDNA auto-antibodies compared to transfer of FO B cells. In addition, MZ B cells produced pathogenic anti-dsDNA IgG autoantibodies in BAFF-transgenic mice lacking T cells. Thus, MZ B cells are potent producers of auto-antibodies causing SLE when provided with

survival signals from BAFF and can then circumvent the requirement of cognate T cell help (Enzler et al., 2006).

MZ B cells efficiently activate naïve CD4$^+$ T cells

Although the MZ B cells respond to TI antigens, they also become activated by protein antigens and mount TD antibody-responses. Interestingly, the MZ B cells themselves were shown to be efficient antigen-presenting cells (APCs) to naïve CD4$^+$ T cells. MZ B cells constitutively expressed high levels of the co-stimulatory molecule CD86 (B7-2) and this feature is most likely a key component in their efficient activation of T cells. The MZ B cells were more potent than FO B cells as APCs to naïve CD4$^+$ T cells in terms of inducing T cell proliferation and secretion of cytokines. The MZ B cells also became activated by the cognate interaction with T cells and rapidly differentiated into antibody-secreting cells. Taken together, MZ B cells gave rise to a faster and stronger immune response than FO B cell-mediated activation of T cells (Attanavanich and Kearney, 2004). The role of MZ B cells in autoimmunity may be linked to their multi-reactive antibody production and also their potent capacity to activate naïve CD4$^+$ Th cells.

CD1: THE THIRD WAY OF ANTIGEN-PRESENTATION TO T CELLS

The basis for T cell immune responses is the specific recognition of antigen presented on specialized molecules. The major histocompatibilty complex (MHC) exists in two forms: MHC class I presenting 8-10 amino acid long peptides to $CD8^+$ T cells and MHC class II presenting exogenously derived 13-25 amino acid long peptides to $CD4^+$ T cells. The TCR-repertoire is enormously diverse and selected to be able to recognize different MHC molecules presenting peptides. However, there are T cell subsets that are not MHC-restricted. In addition to MHC class I and II, there is another family of antigen-presenting molecules: CD1. The CD1 molecules are highly conserved throughout evolution, predating even the separation between mammals and avian species approximately 300 million years ago (Miller et al., 2005; Salomonsen et al., 2005). The CD1 family of antigen-presenting molecules contains the five members CD1a-e in humans but only CD1d in mice. CD1d belongs to group 2 of CD1 molecules and in mice the highly homologues CD1d1 and CD1d2 exist, although CD1d2 is not of functional importance (Chen et al., 1999). The antigens commonly presented on the CD1d-molecule are glycolipids. The CD1d molecule shares properties both with MHC class I and MHC class II, although the CD1d-molecule represents a third way of antigen-presentation by being adapted to present non-peptide antigens (reviewed (Brigl and Brenner, 2004)). Structurally, the CD1d molecule is most similar to MHC class I and has association to β2-microglobulin in common with MHC class I. The CD1d antigen binding groove is deep, narrow, composed of hydrophobic amino acids and closed at both ends. There are two pockets in the antigen-binding groove designated A' and F' and access to the groove is through a narrow opening. The overall structure of CD1d suggests that it presents highly hydrophobic antigens that are "hidden" in the deep binding groove (Zeng et al., 1997). The hydrophobic antigens are now identified as glycolipids that may be endogenous or exogenously derived. The CD1d+glycolipid antigen complexes are recognized by the T cell receptor (TCR) of NKT cells (Bendelac et al., 1995). The concept of CD1d as the antigen-presenting molecule critical for NKT cell thymic selection and functions sparked studies of the molecular structure of the ligands presented on CD1d and the nature of the antigen-presenting cells expressing CD1d.

The CD1d-antigen presenting cells

CD1d has one important feature in common with MHC class II, namely the preferential expression on professional APCs, in contrast to MHC class I that is expressed on nucleated cells. The mouse CD1d molecule is expressed on haematopoietic cells at varying levels and CD1d-reactive T cell hybridomas are generally auto-reactive towards CD1d-expressing cells derived from the same tissue as the hybridomas themselves (Brossay et al., 1998; Cardell et al., 1995; Park et al., 1998). The strongest CD1d-expression is found on professional APCs such as dendritic cells (DCs), macrophages and B cells and also on double-positive thymocytes that are required for CD1d-mediated selection of NKT cells during development (Park et al., 1998; Roark et al., 1998). Among the B cells, the CD1d-levels are several-fold higher on MZ B cells

and T2-like B cells than on the FO B cell population (Amano et al., 1998; Makowska et al., 1999; Roark et al., 1998). The genetic regulation of CD1d mRNA expression is mediated by the Ets-family of transcription factors. The transcription factor Elf-1 regulates the level of CD1d on B cells, whereas PU.1 is a negative regulator of CD1d in myeloid cells (Geng et al., 2005). The transcriptional regulation of CD1d may be one means of increasing CD1d-levels, which has been shown to occur during bacterial infections and in the presence of pro-inflammatory cytokines (Skold et al., 2005).

The three different populations of professional APCs: DCs, macrophages and B cells have varying capacity to activate the innate-like lymphocyte population Vα14 invariant NKT cells. The Vα14 iNKT cell TCR specifically recognizes the exogenous ligand alpha-galactosylceramide (αGalCer) presented on CD1d (Kawano et al., 1997). The lymphoid $CD11c^{+}CD8\alpha^{+}$ DC subset residing in the white pulp area of the spleen were critical mediators of the αGalCer activation. Myeloid $CD11c^{+}CD8\alpha^{-}$ DCs or MZ B cells only made minor contributions to iNKT cell-derived cytokine-production upon αGalCer challenge. In contrast, in the liver the specialized population of CD1d-expressing macrophages called Kupffer cells were vital for activation of liver iNKT cells (Schmieg et al., 2005). Surprisingly, in mice lacking B cells, the injection of αGalCer gave higher cytokine-production by iNKT cells than in normal mice. *In vitro* co-culture experiments revealed that B cells inhibited the DC activation of iNKT cells through cell-cell contact dependent mechanisms (Bezbradica et al., 2005). The iNKT cell activation after administration of low doses of αGalCer presented on DCs was potentiated by endogenous ligands. The synergistic effect of low-dose exogenous antigen+endogenous antigen presented on CD1d, rapidly induced the iNKT cell cytokine storm after αGalCer injection *in vivo* (Cheng et al., 2007). Taken together, the DC population is by far the most potent APC to iNKT cells and the combined effect of exogenous and endogenous ligands presented on CD1d enhances the rapid activation of iNKT cells.

The pathways of antigen processing and presentation on CD1d

The antigens displayed on the CD1d-molecule may either be endogenously derived or stem from exogenous sources. After assembly in the endoplasmatic reticulum (ER), the newly produced CD1d molecules are loaded with lipids on the way from the ER to the cell-surface. The CD1d-molecule is constantly recycling between the plasma-membrane and the endosomal pathway (Figure 4). The loading of foreign glycolipids as well as some self-lipids occurs in the late endosome/lysosome and the lipid exchange molecules called saposins actively transfer lipid-ligands onto the CD1d-molecule (Zhou et al., 2004a). The normal lipid loading onto the CD1d-molecule is highly dependent on the endosomal targeting motif. This motif allows the CD1d-molecule to recycle between the cell-surface and the endosome/lysosome compartments. The CD1d-molecules that are loaded with lipid in the late endosome/lysosome will then present the lipid antigens on CD1d at the cell-surface for recognition by NKT cells (Jayawardena-Wolf et al., 2001). In mice with tail-truncated CD1d molecules lacking the endosomal pathway targeting,

the Vα14 iNKT cell population is reduced and dysfunctional. However, diverse NKT cells that recognize CD1d+antigen but do not express Vα14 TCR are auto-reactive to CD1d molecules that do not undergo endosomal trafficking. The endogenous lipids required for Vα14 iNKT cell and dNKT cell activation are different and dNKT cell ligands can be loaded independently of the endosomal pathway (Brossay et al., 1998; Chiu et al., 1999; Chiu et al., 2002).

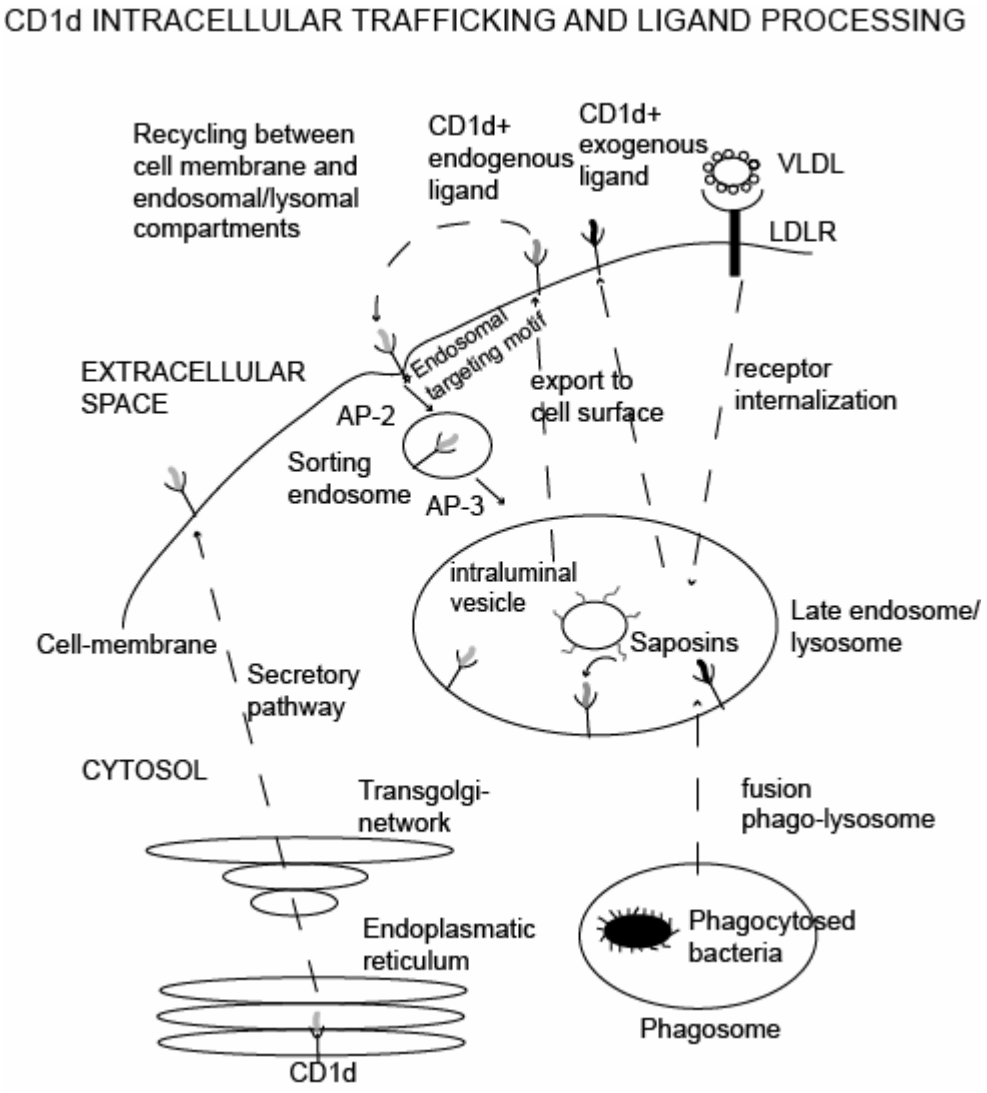

Figure 4. The CD1d intracellular trafficking and ligand-processing. CD1d-molecules are loaded with endogenous lipids in the ER and transported via the secretory pathway to the cell-surface. The CD1d-molecule is then recycled from the surface to the late endosome/lysosome through the endosomal pathway. Endogenous or exogenous lipids are loaded onto the CD1d molecule by saposins and the CD1d molecule is once more transported to the surface for antigen-presentation to NKT cells. VLDL-very low density lipoprotein, AP-adaptor protein. Adapted from (Bendelac et al., 2007).

Exogenous CD1d-ligands

Studies have shown that the ligands presented on CD1d have the fatty acids "hidden" in the hydrophobic pockets and the polar head of the glycolipid protruding towards the TCR of the NKT cell (Zajonc et al., 2005). The crystal structure of the human CD1d-αGalCer complex bound by Vα24-Jα18 TCR, corresponding to the mouse Vα14 iNKT cell TCR, of the human iNKT cells has been resolved. The binding of the iNKT TCR to CD1d+αGalCer complex was fundamentally different from typical TCR interactions with MHC+peptide. One important feature distinguishing the NKT-CD1d-αGalCer binding was that the TCR docks at the end of the CD1d molecule. Also, the TCR did not alter its conformation after binding the CD1d-αGalCer complex and this rigid "lock and key" interaction was mediated by the invariant TCRα chain contacts with the CD1d-αGalCer complex (Borg et al., 2007). The finding of the synthetic ligand αGalCer derived from the marine sponge *Agelas mauritianus* as an anti-metastatic drug has led to numerous studies on iNKT cell functions (Kawano et al., 1997). The α-anomeric sugar moiety of αGalCer is thought to identify the αGalCer molecule as exogenous, since in mammals glycosphinoglipids are only found in β-anomeric conformation (Zajonc et al., 2005). Naturally occurring ligands that are immunologically relevant have been identified from the LPS-negative Gram-negative bacterial species *Sphingomonas* (α-branched galactosylceramide) and *Borrelia burgdorferi* (α-galactosyldiacylglycerols) (Kinjo et al., 2005; Kinjo et al., 2006; Mattner et al., 2005). Phosphatidylinositol mannoside (PIM_4) derived from *Mycobacterium* has also been suggested to be a CD1d-ligand that activates NKT cells (Fischer et al., 2004). The protozoa parasite *Leishmania donovani* lipophosphoglycans bind to CD1d and stimulated NKT cells (Amprey et al., 2004). In summary, the exogenous ligands presented on CD1d can be derived from several different pathogens and direct recognition of exogenous ligands presented on CD1d induces NKT cell activation.

Endogenous CD1d-ligands

The endogenous antigens presented on CD1d, such as glycosphingolipids, are loaded onto CD1d-molecules both directly after synthesis and also upon recycling to the lysosome. The nature of the endogenous ligands presented on CD1d that activates iNKT cells and dNKT cells are different, but both cell-types recognized lipid extracts from tumour cell lines added to CD1d (Gumperz et al., 2000; Makowska et al., 2000). The first identified endogenous ligand, disialoganglioside (GD3) derived from human melanoma cells, was weakly stimulatory for a subset of iNKT cells (Wu et al., 2003). The ligand sulfatide activated a specific sub-population of dNKT cells that were not reactive to αGalCer (Jahng et al., 2004). The quest for the endogenous ligands took a new turn when the iNKT cell population was shown to be diminished in absence of the enzyme β-hexosaminidase B and the enzymatic product iGb3 was shown to directly activate Vα14 iNKT cells (Zhou et al., 2004b). The topic of the endogenous ligands presented on the CD1d-molecule is controversial but it is clear that glycolipid processing is absolutely crucial and that iGb3 is one potential self-ligand but its relative importance is

questioned (reviewed by (Godfrey et al., 2006)). There seems to be a general connection between lysosomal storage diseases and iNKT cell deficiency. Lysosomal storage diseases are characterized by mutations in proteins that mediate processing of glycolipids in the lysosomes and thus the loading of glycolipids onto CD1d-molecules is disturbed (Gadola et al., 2006). The nature of several endogenous ligands presented on CD1d has been described, although there are probably additional, yet unidentified, ligands that activate NKT cells.

NATURAL KILLER T CELLS

Definition of NKT cells

Natural Killer T (NKT) cells are a specialized lineage of TCR$\alpha\beta^+$ cells that also co-express the NK1.1 marker and their unique features have been unravelled since they were first described in the 1980:ies and early 1990:ies (reviewed in (Godfrey et al., 2004)). Most NK1.1$^+$ TCRβ^+ cells are dependent on the MHC class-I like molecule CD1d for selection in the thymus and therefore the term NKT cells has been adopted for CD1d-restricted cells (Bendelac et al., 1995). The NK1.1$^+$TCR$\alpha\beta^+$ cell definition is hampered by the lack of NK1.1 expression in many mouse strains (Hammond et al., 2001). In addition, NK1.1 is absent on immature NKT cells (Benlagha et al., 2002; Gadue and Stein, 2002; Pellicci et al., 2002) and is down-regulated after strong activation (Chen et al., 1997a; Crowe et al., 2003; Wilson et al., 2003). In CD1d-/- mice, NK1.1$^+$TCRβ^+ cells are fewer, but are still present (Chen et al., 1997c; Mendiratta et al., 1997) and NK1.1 may be expressed by conventional T cells activated during viral infections (Slifka et al., 2000). Therefore, the NK1.1 expression is not sufficient or reliable for identification of CD1d-restricted NKT cells. In this thesis the following definitions are used (Figure 5):

NKT cell: CD1d-restricted TCR$\alpha\beta^+$ cell
Invariant NKT (iNKT) cell: CD1d-restricted, Vα14-Jα18 TCRα chain rearrangement
Diverse NKT (dNKT) cell: CD1d-restricted, non-Vα14-Jα18 TCRα chain rearrangement
NK1.1$^+$ TCRβ^+ cell: T cell expressing the NK1.1 marker

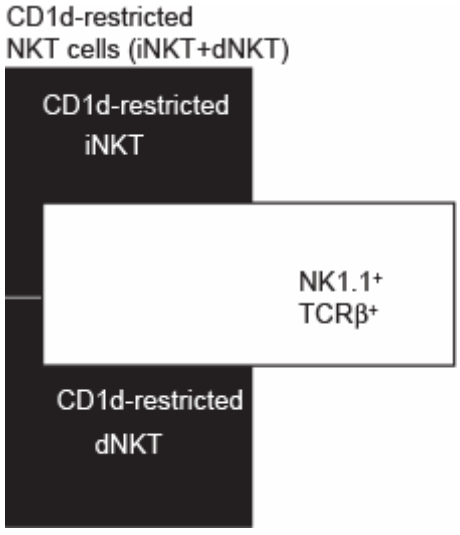

Figure 5. The definition of NKT cells. The iNKT cells and dNKT cells are CD1d-restricted, whereas NK1.1$^+$ TCRβ^+ cell population contains a mixture of T cells that are CD1d-restricted and conventional MHC-restricted T cells that have up-regulated the activating NK cell receptor NK1.1 on their cell-surface. Adapted from (Cardell, 2006).

THE NATURAL KILLER T CELL SUBSETS

The two CD1d-restricted NKT cell subsets are defined based on their TCR usage: iNKT cells express Vα14-Jα18 TCRα chain and dNKT cells contain populations with different TCR chains. The two subsets express NK-receptors belonging to the NKR-P or Ly49 family of genes. They also share many phenotypic properties, such as an activated/memory-like phenotype that is $CD44^{hi}CD122^{hi}$(reviewed by (Behar and Cardell, 2000)). In terms of cytokine secretion after *in vitro* stimulation, Vα14 iNKT cells potently produced both IL-4 and IFN-γ whereas dNKT cells are biased towards IFN-γ (Stenstrom et al., 2004). There are also differences in preferential localization of iNKT cells to thymus and liver whereas non-Vα14 $NK1.1^{+}TCR\beta^{+}$ cells are more abundant in the spleen and bone marrow (Apostolou et al., 2000). CD1d-αGalCer loaded tetramers identifying iNKT cells bind approximately 75% of $NK1.1^{+}TCR\beta^{+}$ cells in the thymus and liver but only 35 % of the T cells expressing NK1.1 in the spleen (Matsuda et al., 2000). The direct identification of CD1d-restricted Vα14 iNKT cells among $NK1.1^{+}$ $TCR\beta^{+}$ cells was accomplished by utilizing CD1d-αGalCer loaded tetramers (Benlagha et al., 2000; Matsuda et al., 2000). However, the obstacle of defining CD1d-restricted non-Vα14 diverse NKT (dNKT) cells remains, since the $NK1.1^{+}$ $TCR\beta^{+}$ population is always contaminated by cell populations that express NK1.1 but are not reactive to CD1d.

Table I. Overview of the properties of iNKT cells and dNKT cells.

Property	iNKT cells	dNKT cells
TCR chain usage	Vα14-Jα18 Vβ8, 7, 2	Diverse repertoire
Phenotypic markers	Naturally activated NK cell receptors $CD69^{hi}$ $CD49b^{lo}$	Naturally activated NK cell receptors $CD69^{lo}CD49b^{hi}$
Preferential localization	Thymus, liver	Spleen, bone marrow
Frequency	30% in liver, 2-3 % in the spleen and around 0.5 % in lymph nodes, thymus and blood among T cells	The total frequency of dNKT cells is unknown (sulfatide-reactive dNKT cells constitute 0.3% of splenocytes)
Activating glycolipid-antigens presented on CD1d	αGalCer, microbial glycosphingolipids GD3, iGb3 unknown	Sulfatide unknown
Cytokine-profile	IL-4+IFN-γ	IFN-γ, IL-4, IL-13

Vα14 iNKT cells

The NK1.1$^+$ TCRβ$^+$ cells have a strong skewing towards Vβ8, Vβ7 and Vβ2 TCR chain usage and were later shown to contain cells that expressed the Vα14-Jα18 rearrangement (Arase et al., 1992; Bendelac et al., 1994; Budd et al., 1987; Fowlkes et al., 1987; Lantz and Bendelac, 1994). The relative abundance of the Vα14-Jα281 rearrangement (later termed Jα18) with the conserved expression of the amino acid glycine followed by an aspartate in the V-J junctional region implied that thymocytes expressing this particular TCRα gene segment are positively selected (Koseki et al., 1991; Lantz and Bendelac, 1994). The Vα14-Jα18 TCR is crucial for binding to the artificial ligand αGalCer (Kawano et al., 1997; Matsuda et al., 2001), although the Vβ chain may make a minor contribution in the recognition of CD1d (Schumann et al., 2003). The interaction of CD1d-αGalCer tetramer with TCR has a low K_D (dissociation-coefficient) due to an extremely slow off-rate of the binding between TCR and the CD1d-αGalCer complex (Sidobre et al., 2002). In humans, CD4$^-$CD8$^-$ (DN) T cells in blood are enriched for expression of TCR chain segments Vα24-JαQ (also termed Jα18) and Vβ8, 2, 11 and 13 that correspond to the mouse Vα14 iNKT cells (Porcelli et al., 1993). Thus, studies of mouse Vα14 iNKT cells are relevant for understanding the role of human iNKT cells.

Diverse NKT cells

The dNKT cell subset was discovered by generation of hybridomas from CD4$^+$ T cells in the MHC II-/- mice. Several of the hybridomas were auto-reactive to CD1d but did not carry the Vα14-Jα18 TCR chain (Cardell et al., 1995). The presence of dNKT cells is particularly prominent in the spleen and bone marrow, although due to contamination by NK1.1$^+$TCRαβ$^+$ that do not recognize CD1d, the estimates of the size of the CD1d-restricted dNKT cell population varies considerably (Eberl et al., 1999). In addition, it seems likely that dNKT cells that are NK1.1$^-$ also exist, although these cells are difficult to distinguish due to the lack of specific markers. The most direct way of studying dNKT cells is by generating hybridomas or clone lines of CD1d-reactive T cells that are not using the Vα14-Jα18 TCR rearrangement and analyze their CD1d-restriction/auto-reactivity (Behar et al., 1999; Cardell et al., 1995; Park et al., 2001). In humans, the majority of CD1d-reactive T cells are non-Vα24 and hence the human NKT cell population is dominated by dNKT cells (Exley et al., 2001b). The dNKT cell population contains many different TCR specificities. The usage of the Vα8 TCRα chain is frequent among dNKT cells. It seems that the dNKT cell population contains cells expressing the Vα3.2Vβ8 TCR chains which may represent a subset of dNKT cells (Park et al., 2001). Two TCR-transgenic models that represent dNKT cells have been created: Vα4.4Vβ9 (Cheng et al., 1996; Zeng et al., 1998) and the Vα3.2Vβ9 TCR that is expressed in the 24αβ transgenic mouse model of dNKT cells (Skold et al., 2000). A distinct population of dNKT cells that recognize the glycolipid sulfatide presented on CD1d represent about 0.3% of splenocytes and the sulfatide-reactive dNKT cells use the TCR chains Vβ8.1/2 or Vβ6 (Jahng et al., 2004). There are many similarities between non-Vα14 (CD1d-αGalCer-tetramer negative) NK1.1$^+$TCRβ$^+$ cells from

wild-type B6 mice and the dNKT cell transgenic model 24αβB6. Non-Vα14 NK1.1^{+}TCRβ^{+} splenic cells in B6 mice expressed higher levels of the α2-integrin CD49b detected by the Dx5 antibody similarly to 24αβ dNKT cells. *In vitro* stimulation induced non-Vα14 DN NK1.1^{+}TCRβ^{+} cells from B6 mice and NK1.1^{+}TCRβ^{+} transgenic 24αβ cells to secrete large amounts of IFN-γ and IL-2, but low levels of IL-4 and no IL-10, suggesting that dNKT cells are Th1-tilted (Stenstrom et al., 2004). Taken together, the dNKT cell subset comprises several sub-populations that have different TCRs. The dNKT cell antigen-recognition and effector functions upon activation may vary. Functionally, the dNKT cells play significant roles in infection models, autoimmunity and tumour surveillance. Just as is the case for iNKT cells, the multifaceted roles of dNKT cells implies that they act as immunomodulatory cells with capacity to direct the responses of other immune cell populations.

NKT CELL DEVELOPMENT

The process of rearrangement of the TCR locus on the genetic level and expression of a functional TCR on the surface of thymocytes is a prerequisite for development of thymocytes into conventional T cells. However, the diversity of the rearranged TCRs results in the formation of thymocytes that either do not recognize endogenous MHC+self-peptide complexes at all or become activated by self-antigen. In order to prevent these two types of T cell specificities to persist in the mature T cell pool, thymocytes are positively selected based on a moderate TCR-binding capacity to MHC+self-peptide complexes on the surface of thymic epithelial cells. The process of positive selection provides survival signals to developing thymocytes whose TCR bind weakly to MHC+self-peptide complexes. Thymocytes that bind strongly to MHC+self-peptide complexes undergo negative selection that eliminates auto-reactive thymocytes. Thymocytes undergo a complex maturation process, described by their expression pattern of the co-stimulatory molecules CD4 and CD8. The developmental program of NKT cells is different from that of conventional $CD4^+$ or $CD8^+$ T cells in many respects, although they originate from the same precursor population (Figure 6). The developmental program of iNKT cells has been characterized, and most likely represents the general maturation process of both NKT cell subsets. In contrast to conventional T cells that are selected by weak binding to MHC+self-peptide presented by thymic epithelial cells, iNKT have been shown to be positively selected by CD1d+self-ligands expressed on double-positive thymocytes (Bendelac et al., 1995; Coles and Raulet, 2000). CD1d-/- mice or mice that have defects in CD1d-antigen processing or presentation on the surface, lack mature iNKT cells (reviewed by (Godfrey and Berzins, 2007)). In a bone marrow chimera model where CD1d is only expressed by $CD4^+$ $CD8^+$ double-positive (DP) thymocytes, the iNKT cells still develop in the thymus. However, the iNKT cells in this mouse model were functionally altered, which may suggest that CD1d-expression on APCs contribute to certain features of iNKT cell development (Wei et al., 2005). In addition to being positively selected by recognition of CD1d+endogenous glycolipids, iNKT cells can undergo negative selection when CD1d is over-expressed. Negative selection by high levels of CD1d skewed the Vβ TCRβ-chain usage from the high-avidity Vβ8 and Vβ7 to Vβ2 and the iNKT cells with the skewed Vβ-repertoire were less responsive to stimulation (Chun et al., 2003). Professional APCs, especially thymic DCs cannot mediate positive selection and rather enhance negative selection, in contrast to DP thymocytes that mediate both positive and to a lesser extent negative selection (Schumann et al., 2005).

The iNKT cells derive from double-positive DP thymocytes (Egawa et al., 2005). The rearrangement of the iNKT Vα14-Jα18 chain paired with Vβ8, Vβ7 or Vβ2 is a stochastic non-directed event (Shimamura et al., 1997). Therefore, extremely few of the newly rearranged DP^{low} CD1d-αGalCer $tetramer^+$ iNKT cells, that are the first precursors of iNKT cells, can be identified (Benlagha et al., 2002; Gapin et al., 2001; Pellicci et al., 2002). The cells that successfully have rearranged the Vα14-Jα18 invariant TCRα chain begin to undergo the process of thymic

selection during the transition from DP to stage 1 of the iNKT cell maturation (Benlagha et al., 2005; Gadue and Stein, 2002). The developmental stages of iNKT cells include $CD44^{-}NK1.1^{-}$ (stage 1) cells that develop into $CD44^{+}NK1.1^{-}$ (stage 2) cells. The stage 2 iNKT cells up-regulate NK1.1 ($CD44^{+}NK1.1^{+}$ stage 3) either in the thymus or after export to the periphery where they complete the maturation process (reviewed by (Matsuda and Gapin, 2005)). The normal thymic development and functional maturation of NKT cells require a unique set of expression of signalling molecules and transcription factors in addition to recognition of CD1d+endogenous glycolipids.

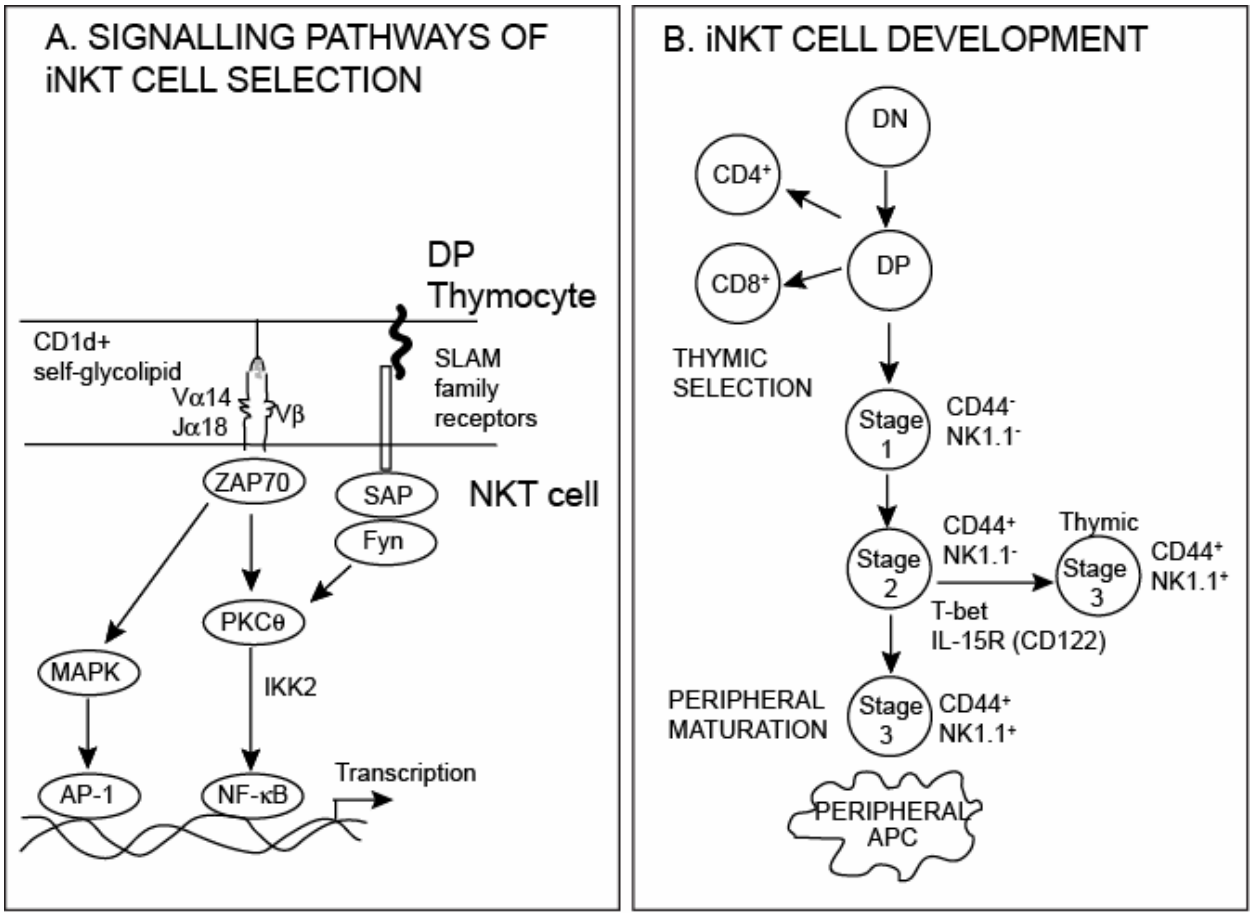

Figure 6. The development of iNKT cells. The thymic development of iNKT cells is different from the development of conventional T cells after the DP stage when the Vα14-Jα18 TCRa chain is being expressed on the iNKT cell surface. Components of intracellular signalling pathways such as Fyn and NF-κB, receptors of the SLAM-family, transcription factors such as T-bet and also the antigen-presenting molecule CD1d and components of glycolipid antigen loading are required for iNKT cell development but can be dispensible for normal T cell development. The overview of the developmental stages are shown in panel B.

NKT CELL FUNCTIONS

NKT cells become activated in many types of immunological settings and possess versatile effector functions. The NKT cells have the capacity to secrete both IL-4 and IFN-γ upon activation. It seems likely that the cytokine-profile may depend on the features of NKT cells, such as expression of CD4 and other surface markers and the organ that they originate from. The cytokine-profile also depends on the mode of activation, exemplified by IL-12 secretion from APCs that promoted IFN-γ and abolished IL-4 secretion by NKT cells. The role of iNKT cells after administration of αGalCer in different immunological responses has been extensively studied and often serves as a tool to explore the potential effector functions of iNKT cells. Another approach to analyzing the role of NKT cells is to use the Jα18-/- mouse model lacking all iNKT cells or CD1d-/- mice without both iNKT cells and dNKT cells. By using Jα18-/- or CD1d-/- mice, the natural roles of iNKT cells and/or dNKT cells in the immune system can be studied.

The effector functions of iNKT cells activated by αGalCer

The artificial ligand αGalCer presented on CD1d specifically activates the iNKT cells via their canonical TCR and the effect of αGalCer-administration is absolutely dependent on the presence of iNKT cells (Kawano et al., 1997). Although αGalCer is not a physiological stimulus, the specific activation of iNKT cells by αGalCer has facilitated the understanding of the functional capacities of iNKT cells (reviewed by (Kronenberg, 2005)). The propensity of iNKT cells to simultaneously secrete IL-4 and IFN-γ results in a broad immunomodulatory panorama of functions and αGalCer-activation of iNKT cells has been shown to affect many different immunological processes (Matsuda et al., 2003; Stetson et al., 2003). In situations that require strong Th1-immunity such as infections with intracellular pathogens and anti-tumour responses the iNKT cell IFN-γ production is beneficial. For example, αGalCer-stimulated iNKT cells enhanced $CD8^+$ T cell and $CD4^+$ T cell responses against malaria (Gonzalez-Aseguinolaza et al., 2000; Gonzalez-Aseguinolaza et al., 2002). Also, IFN-γ derived from NKT cells directly activated NK cells, which promote anti-tumour immunity (Carnaud et al., 1999; Eberl and MacDonald, 2000; Smyth et al., 2002). Injection of αGalCer-loaded DCs rather than free αGalCer enhanced the long-term IFN-γ production by iNKT cells (Fujii et al., 2002). The interaction between iNKT cells producing IFN-γ and DCs secreting IL-12 is important for sustaining the effects of αGalCer. In general, the immunomodulatory functions of αGalCer activation of iNKT cells seem to be mediated through communication with other immune cells, especially with DCs that regulate the ensuing immune response (Fujii et al., 2003; Hayakawa et al., 2001; Kitamura et al., 1999; Tomura et al., 1999). The adjuvant-like effect of αGalCer-mediated induction of Th1 immunity seems to be contradictory to the well-documented role of αGalCer-mediated prevention of autoimmunity. The αGalCer induced IL-4 production, followed by Th2-dominated immunity, seems to be protective against autoimmunity in NOD mice (Hong

et al., 2001; Sharif et al., 2001). Another mechanism of the αGalCer-induced protective effect of iNKT cells involves tolerization through modulation of DC functions (Chen et al., 2005b; Kojo et al., 2005; Naumov et al., 2001). Taken together, the main role of αGalCer-activated iNKT cells in controlling autoimmunity may be to affect the cytokine-profile or to induce protective immunomodulation through tolerogenic DCs. Although αGalCer activation results in highly divergent immune responses depending on the immunological context, this substance may be utilized to exploit the immunomodulatory functions of iNKT cells. Currently, αGalCer-loaded onto *in vitro* derived DCs are undergoing Phase II clinical trials as an adjuvant against metastatic malignancies. The effect of αGalCer on human cancer is not yet known, but αGalCer therapy represents a promising way to harness the potent immunomodulatory functions of iNKT cells (reviewed by (Seino et al., 2006)).

NKT cells are activated during infectious diseases

NKT cells have been shown to promote immunity towards a wide-range of pathogenic microbes, including gram-positive and gram-negative bacteria, virus, fungi, parasites and helminths (reviewed by (Tupin et al., 2007)). In many types of infectious diseases, NKT cells mediate protective immune responses by rapid production of cytokines. But due to the potent immunomodulatory function of NKT cells, they may also cause tissue damage, for instance by enhancing liver injuries during *Salmonella* infection (Shimizu et al., 2002). The massive production of the Th1 cytokine IFN-γ or the Th2 cytokine IL-4 upon NKT cell activation are assumed to modulate the ensuing cell-mediated immunity or antibody-production, respectively. Another role of NKT cells during lung infections caused by *Pseudomonas aeruginosa* or *Streptococcus pneumoniae* was to promote recruitment of neutrophils (Nieuwenhuis et al., 2002; Kawakami et al., 2003). The role of dNKT cells in infectious disease has been studied in several infection models. The first report showing a functionally important role of dNKT cells analyzed encephalomyocarditis (ECMV-D) virus symptoms in CD1d-/- and Jα18-/- mice. The dNKT cells as well as the iNKT cell population protected against ECMV-D related tissue damage (Exley et al., 2001a). In contrast, during infection with the parasite *Trypanosoma cruzi*, dNKT cells caused severe tissue damage and increased fatality (Duthie et al., 2005). Diverse NKT cells transferred to RAG-/- mice expressing the hepatitis B virus transgenically in the liver caused tissue damage in a CD1d-dependent manner, whereas iNKT cells did not induce acute hepatitis in this model (Baron et al., 2002). During helminth *Schistosoma mansoni* infections, dNKT cells contributed to a protective Th2 response and iNKT cells were crucial for Th1 mediated anti-helminth response (Figure 7E) (Mallevaey et al., 2007). The dNKT cells mediate versatile functions in different infection models and can induce protection as well as excessive tissue damage. However, due to the large number of experimental factors such as the mouse strain, usage of the CD1d-/- model or the Jα18-/- models, the properties and the dose and route of administration of the pathogen and the parameters analyzed, there are many contradicting reports on the role of NKT cells in different infection models (reviewed by (Skold and Behar, 2003)).

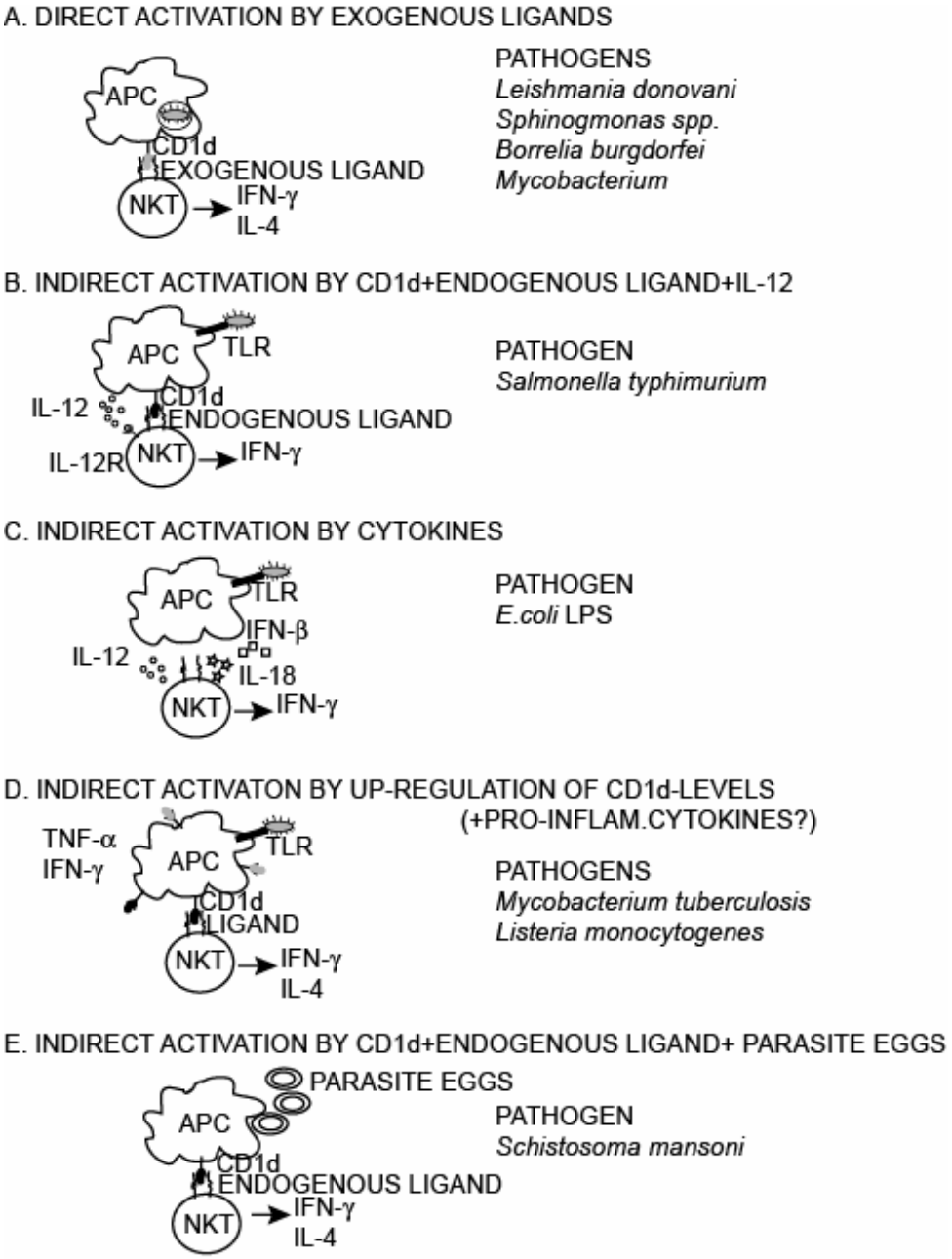

Figure 7. The activation mechanisms of NKT cells during infectious diseases. The different models of activation can mainly be divided into direct activation by exogenous antigens (A) and indirect activation that depends of presentation of endogenous glycolipids on CD1d and additional cytokine stimulation, pro-inflammatory cytokines, modulation of CD1d-levels and antigens derived from parasite eggs (B-E). Depending on the nature of the invading pathogen these activation mechanisms may contribute to inducing NKT cell functions such as secretion of cytokines during infectious diseases. Adapted from (Tupin et al., 2007).

In recent years there is an increased focus on how different infectious micro-organisms activate NKT cells (Figure 7). There appears to be two major mechanisms: direct activation or indirect activation (reviewed (Tupin et al., 2007)). Direct activation by presentation of microbial

glycolipids by the CD1d molecule has been convincingly shown for the gram-negative LPS-negative bacterial species *Sphingomonas* and *Borrelia burgdorferi* and also for the protozoan parasite *Leishmania donovani* (Figure 7A) (Amprey et al., 2004; Kinjo et al., 2005; Kinjo et al., 2006; Mattner et al., 2005). The scenarios of indirect activation are more complex and depend on production of the cytokines IL-12, IL-18 or IFN-β by APCs and presentation of self-antigen on the CD1d-molecule (Figure 7B). However, the pro-inflammatory cytokines IL-12 and IL-18 can activate NKT cells in the absence of CD1d, suggesting that cytokines alone can be sufficient stimuli for activation of NKT cells (Figure 7C). In addition, there is increasing recognition that regulation of the levels of CD1d on the surface of APCs is involved in NKT cell activation (Figure 7D) (Skold et al., 2005) and that CD1d-downmodulation is an efficient mechanism of immune evasion exploited by for instance herpes simplex virus (Yuan et al., 2006).

NKT cells in anti-tumour immunity

Tumour cells represent a difficult challenge for the immune system, due to the high degree of similarities between the transformed cells and normal non-cancerous cells. The NKT cells can provide the first signals that initiate the anti-tumour immune response against certain tumours both during the natural progression of anti-tumour immunity as well as after administration of exogenous stimuli. Activation of NKT cells by administration of IL-12 resulted in reduction of melanoma tumour growth and the number of metastatic nodules (Cui et al., 1997). The protective effects of αGalCer against cancer have been extensively documented (reviewed by (Seino et al., 2006)) and long-term αGalCer treatment reduced spontaneous tumour outgrowth induced by the a chemical carcinogen or in mouse models that are inherently cancer-prone (Hayakawa et al., 2003). The IFN-γ secreted from NKT cells rapidly activated NK cells, which sustains the anti-tumour immune responses (Smyth et al., 2002). IFN-γ has also been suggested to directly mediate anti-angiogenesis and thereby prevent the blood vessel formation within the tumour mass (Hayakawa et al., 2002). Clearly, NKT cells are important initiators of tumour immunity and their presence also regulates the immunogenicity of the tumour. Absence of NKT cells permitted the survival of more immunogenic tumour cells, suggesting that tumour cells evolved to evade NKT cell recognition during the natural progression of cancer (Smyth et al., 2000). There are major differences in the capacity to form anti-tumour immune responses depending on tissue of origin and phenotype of NKT cells. The $CD4^-$ (DN) NKT cells isolated from liver were most efficiently preventing tumour outgrowth. Thymic NKT cells were least efficient mediators of anti-tumour immunity, although their capacity to reduce the number of melanoma lung metastasis was enhanced in IL-4-/- mice (Crowe et al., 2005).

In contrast to the anti-tumour effect of iNKT cells, several studies have shown that dNKT cells prevent anti-tumour immunity. The CD1d-/- mice had a higher survival rate, fewer metastasis nodules and more efficient tumour rejection of fibrosarcoma, colon carcinoma and mammary carcinoma than wild-type mice or Jα18-/- mice. Hence, the dNKT cell population actively inhibit anti-tumour immunity (Terabe et al., 2005). The mechanism behind the dNKT cell ability to

prevent anti-tumour immunity is likely to involve the Th2 cytokine IL-13 that activates myeloid cells to produce TGF-β, which prevents $CD8^+$ cytotoxic T cells (CTLs) from killing tumour cells (Terabe et al., 2000; Terabe et al., 2003). Also, in a model of UV-light induced tumours, $Dx5^+$ NKT cells were shown to prevent anti-tumour immunity (Moodycliffe et al., 2000). Interestingly, dNKT cells seem to be implied in dampening anti-tumour immunity whereas iNKT cells generally promote anti-tumour immunity via IFN-γ production that activate NK cells and $CD8^+$ CTLs, thus leading to killing of the malignant tumour cells.

NKT cells can dampen autoimmunity

The immunomodulatory role of NKT cells in autoimmunity has been studied in mouse models of multiple sclerosis called experimental allergic encephalomyelitis (EAE) and in SLE, but the NKT cell-mediated dampening of autoimmune diabetes has received most attention. Several studies have found that NKT cells were protective against EAE and that CD1d-/- mice developed more severe EAE symptoms. The CD1d-restricted NKT cells enhanced the influx of TGF-β1 producing cells into the brain, thereby participating in the protection against neuronal damage (Teige et al., 2004). However, NKT cells have both been implied in protection and in detrimental autoantibody-production in different SLE-models (Yang et al., 2003; Zeng et al., 2000).

The ability of NKT cells to modulate immune responses plays a beneficial role by dampening the autoimmune destruction of pancreatic β-cells in the nonobese diabetic (NOD) mouse model of human Type 1 diabetes (reviewed by (Cardell, 2006)). The initial observation concerning NKT cells in NOD mice showed that the DN NKT-like population size was strongly reduced in the thymus of NOD mice. Transfer of these thymic NKT-like cells into young NOD mice delayed the onset of diabetes (Hammond et al., 1998). Furthermore, both $CD4^+$ and DN NKT cells in NOD mice were deficient in terms of IL-4 production and to a lesser extent IFN-γ production upon TCR-crosslinking (Gombert et al., 1996). The impaired NKT cell population in NOD mice may be one of the factors leading to autoimmune disease. Direct evidence of the protective effect of Vα14i NKT cells against autoimmunity came from studying Vα14i NKT cell transgenic mice on the NOD background. The Vα14 transgenic line with the highest frequency of NKT cells was significantly protected against diabetes, despite the presence of auto-reactive T cells. The regulation of autoimmunity was an active process and not just a consequence of dilution of the auto-aggressive T cells (Lehuen et al., 1998). Similar results were observed in the transgenic 24αβNOD dNKT cell mouse model (Duarte et al., 2004). Furthermore, the absence of NKT cells in CD1d-/- NOD mice resulted in an accelerated onset and higher degree of incidence of diabetes compared to wild-type NOD mice (Shi et al., 2001; Wang et al., 2001). The protection against diabetes provided by iNKT cells was further demonstrated by αGalCer treatment (Hong et al., 2001; Sharif et al., 2001). The mechanisms of the autoimmune preventive effects of NKT cells remain unclear. Presently there are at least three modes of immune-regulation described: cell-cell contact-dependent anergization of auto-aggressive T cells, release of cytokines especially IL-4 and finally, recruitment and maturation of tolerizing DCs to the pancreatic lymph nodes. Most

likely, all these mechanisms of NKT cell regulation are relevant in the immunologically complex NOD mouse model and are not mutually exclusive. The NKT cells may continually regulate the auto-aggressive cells from exerting the final destruction of the pancreatic β–cells. It seems that NKT cells are incapable of inhibiting the first stages of infiltration of the pancreas. The transgenic Vα14 iNKT cells prevented auto-aggressive transgenic $CD4^+$ T cells from becoming full-blown effector cells, but the Vα14 iNKT did not affect the initial proliferation and infiltration of the pancreas by the auto-aggressive transgenic $CD4^+$ T cells (Beaudoin et al., 2002). In terms of cytokine profile IL-4 may be protective by skewing the immune response towards humoral immunity, rather than Th1 cell-mediate immunity although there is also evidence showing that IFN-γ can be protective against diabetes (Laloux et al., 2001; Cain et al., 2006). The role of APCs in the development of autoimmunity, suggests that the DC dysfunction is contributing to autoimmunity and that inducing NKT cell-mediated maturation/activation of DCs is relevant for prevention of diabetes (Chen et al., 2005b; Naumov et al., 2001). Taken together, the protective role of NKT cells in the prevention of auto-aggression reveals their capacity to modulate immune responses, although the actual mechanisms apart from IL-4 or IFN-γ production are yet unidentified.

In autoimmunity, dNKT cells participate in the self-destructing pathology in lupus and ulcerative colitis but dNKT cells also function as important immunoregulatory cells that can dampen ongoing tissue destruction, for example in type 1 diabetes and in EAE. In the CD1d-restricted TCR transgenic Vα4.4Vβ9 mouse model (Cheng et al., 1996), the $CD4^+$, $CD8^+$ and DN transgenic splenic cells contributed to SLE-symptoms, whereas DN cells from the bone marrow protected against autoimmunity, probably via secretion of IL-4 (Zeng et al., 1998). The dNKT cells are also thought to be destructive in human patients suffering from ulcerative colitis (UC). The UC-patients had an increased CD1d-reactive dNKT cell population, compared to healthy controls and Crohn's disease patients. The dNKT cells isolated from UC colon produced IL-13, which potentiated *in vitro* cytotoxic killing of epithelial cells and thus may contribute to the tissue damage associated with UC (Fuss et al., 2004). On the other hand, dNKT cells that are sulfatide-reactive protected against EAE. The sulfatide-reactive dNKT cell subset strongly reduced the EAE symptoms after activation by sulfatide. The immunoregulatory effects of sulfatide-reactive dNKT cells dampened the overall cytokine production and proliferative capacity of the autoimmune T cells (Jahng et al., 2004). The sulfatide-reactive dNKT cell protection was described to be unaffected by anti-IL-4 antibodies, suggesting that the effector mechanism of sulfatide-reactive dNKT cells is not Th2-dependent. It seems likely that dNKT cells can mediate protection against autoimmunity via other mechanisms than Th2 cytokine production. In the 24αβNOD model, the Th1-tilted 24αβ dNKT cells are protective against autoimmune diabetes. The transgenic increase of 24αβ dNKT cells in the NOD mouse model of autoimmune diabetes resulted in protection against diabetes, despite the presence of endogenous, potentially auto-reactive T cells. Transfer of 24αβNOD spleen cells into NOD.SCID mice that lack all lymphocytes, revealed that the 24αβ dNKT cells could regulate the co-transferred T cells

from diabetic NOD mice and prevented and delayed autoimmune diabetes (Duarte et al., 2004). The cytokine profile of 24αβNOD dNKT cells was strongly skewed towards IFN-γ. Although IFN-γ is implied in the destructive Th1 immune responses in NOD mice, IFN-γ secretion by NKT cells was shown to be crucial for the regulation of autoimmune T cells by modulating APCs (Cain et al., 2006). However, the role of IFN-γ in protection by dNKT cells against autoimmunity is incompletely understood.

NKT cells in tolerance induction

Autoimmune manifestations may be devastating for an individual and since some auto-reactive T cell clones are not eliminated during thymic selection, there is a constantly ongoing process of peripheral tolerization. Peripheral tolerance is crucial for maintaining the integrity of immune-privileged sites such as the eye, the brain and the pregnant uterus. In addition to sequestering of the antigens uniquely expressed in the brain by the blood-brain barrier and of the foetus with the specialized environment of the placenta, these niches also actively inhibit immune responses (reviewed by (Niederkorn, 2006)). The most extensively studied model of tolerance induction is called anterior-chamber associated immune deviation (ACAID). After injection of antigen into the anterior-chamber of the eye, macrophages from the eye homed to the MZ in the spleen. In the MZ a complex interplay between NKT cells, $CD1d^{hi}$ MZ B cells and eye-derived macrophages resulted in the generation of regulatory $CD8^+$ T cells. The regulatory $CD8^+$ T cells dampened delayed-type hypersensitivity reactions against the same antigen that was originally injected into the eye. In this system, NKT cells recruited the eye-derived macrophages and $CD8^+$ T cells by production of the chemokine RANTES into the MZ region and the NKT cells also secreted the immunomodulatory cytokine IL-10 that mediated the differentiation of $CD8^+$ regulatory T cells (Faunce and Stein-Streilein, 2002; Sonoda et al., 2001). Mice lacking NKT cells were unable to generate ACAID and CD1d expression was required on the eye-derived macrophages as well as on the MZ B cells (Sonoda et al., 1999; Sonoda and Stein-Streilein, 2002). NKT cells play a pivotal role in tolerance induction and provide the mechanistic link between APCs and regulatory $CD8^+$ T cells that mediate systemic tolerization to antigens originating from the immune-privileged eye.

NKT cells enhance B cell antibody-responses

Recent studies have shown that NKT cells enhance humoral immune responses. Activation of iNKT cells by αGalCer could compensate for the absence of conventional $CD4^+$ Thelper cells in MHC class II-/- mice in terms of antibody-production against influenza-virus antigen. In addition to providing T cell help during the initiation of antibody-responses, αGalCer activated iNKT cells contributed to long-term B cell memory. The mechanism of iNKT cell help involves CD40L, IFN-γ for isotype-switching but the role of IL-4 can be replaced by other Th2 cytokines (Galli et al., 2007). NKT cells played a role in the induction of IgG-antibody production against *S. pneumoniae* polysaccharides, which is mainly mediated by MZ B cells and B1 B cells. The CD1d-restricted NKT cells together with $CD8^+$ T cells promoted IgG-isotype switching after

challenge with *S. pneumoniae* polysaccharides (Kobrynski et al., 2005). Thus, activated NKT cells could help B cells during antibody-production against TI antigens but also TD antigens in the absence of $CD4^+$ Th cells. NKT cells are also involved in antibody-production that initiates contact sensitivity reactions. By rapidly secreting IL-4, Vα14 iNKT cells residing in the liver enhanced the IgM production by the B1 B cells, thus promoting the early phase of the contact sensitivity reaction (Campos et al., 2003). These results indicate a functional connection between liver NKT cells and B1 B cells localized to the peritoneal cavity.

Interactions between MZ B cells and NKT cells in the immune system

MZ B cells express high levels of CD1d on their surface, suggesting that they may have a specialized function as APCs to CD1d-restricted NKT cells (Amano et al., 1998; Makowska et al., 1999; Roark et al., 1998). Interplay between MZ B cells and NKT cells has been suggested to occur during infections with the tick-borne pathogen *Borrelia hermsii*, during tolerance induction (see section "NKT cells in tolerance induction") and in lupus-models. The spirochete *Borrelia hermsii* is a blood-borne pathogen transmitted via ticks. *Borrelia* causes relapsing fever in mammals. Early antibody production against *Borrelia hermsii* is important for reducing the bacterial burden and NKT cells seemed to direct the MZ B cells towards a protective antibody-response (Belperron et al., 2005).

Mouse models of SLE can be divided into two categories: induced lupus caused by inflammation-promoting substances and spontaneous lupus models that are dependent on mouse strain genetics. In hydrocarbon oil induced lupus nephritis there is a correlation between a diminished NKT cell population and an expanded hyper-reactive IgM-secreting MZ B cell population. After administration of hydrocarbon oil, CD1d-/- mice suffered from more severe lupus-disease than wild-type mice, suggesting that NKT cells could regulate B cell autoimmunity (Yang et al., 2003). On the other hand, in (NZWxNZB) F1 mice, treatment with anti-CD1d antibodies that blocks the interaction between NKT cells and CD1d-expressing B cells significantly delayed disease onset although the mechanisms remain unknown (Zeng et al., 2000). In contrast, aged Jα18-/- and CD1d-/- mice developed lupus-like symptoms. There is a concomitant expansion of MZ B cells with age that may underlie the increased tendency of NKT cell-deficient mice to develop auto-antibodies and kidney damage (Sireci et al., 2007). In order to study if NKT cells can affect B cells, we have extensively characterized the splenic B cells in the transgenic 24αβ dNKT cell and transgenic Vα14Cα-/- iNKT cell mouse models. There was a trend towards an expansion of MZ B-like cells in the NKT cell transgenic mice compared to littermate controls. However, the CD23 expression was strongly up-regulated on all B cells in the NKT cell transgenic mice, which made it difficult to define the B cell subsets. The regulation of CD23 expression may not represent a physiological role of NKT cells, since there was no decrease in CD23 levels among B cells in CD1d-/- mice (our unpublished observations). The mechanisms of interaction between MZ B cells and NKT cells needs to be further elucidated in order to understand the role of this interplay in the immune system.

HUMAN NKT CELLS

In humans, many conventional T cells express NK cell receptors hence human NKT cells are identified based on CD1d-reactivity and CD1d-αGalCer multimers. In humans, 0.01-0.1% of the blood lymphocytes consists of αGalCer-reactive iNKT cells expressing the Vα24-Jα18 TCRα chain paired with Vβ11 TCRβ chain corresponding to the mouse Vα14-Jα18 iNKT cells. Human Vα24 iNKT cells are often analyzed based on their expression of the co-receptors CD4 and CD8 and are divided into $CD4^+$, $CD8^+$ and DN iNKT cells. $CD4^+$ iNKT cells in humans are capable of secreting both IFN-γ and IL-4, whereas DN iNKT cells mostly produce IFN-γ and express several NK cell receptors such as NKG2D (Gumperz et al., 2002; Lee et al., 2002). Since $CD8^+$ iNKT cells constitute a minor population its functional profile remained unknown until microarray studies comparing each of these three iNKT cells subsets after *in vitro* αGalCer expansion was performed. The microarray analysis revealed that $CD4^+$ iNKT cells are the only constitutively IL-4-producing iNKT cell subset. The genes over-expressed by the DN iNKT cell subset are associated with homing to the liver. The $CD8^+$ iNKT cell subset expressed high levels of NKG2D and STAT4, which is down-stream of the IL-12R, suggesting that these cells are extremely Th1-biased (Lin et al., 2006). Human Vα24 iNKT cells have been implied in tumour immunity and were shown to directly kill CD1d-expressing malignant cells or to secrete IL-2 and IFN-γ after αGalCer administration and thus activate NK cells (Metelitsa et al., 2001). Reduction of NKT cell numbers has been observed in several human cancer diseases and for instance the frequency of hepatic Vα24Vβ11 iNKT cells was found to be severely diminished in patients with hepatic metastases (Kenna et al., 2003). In summary, the similarities between human and mouse iNKT cells include the recognition of αGalCer presented on CD1d and potent cytokine-secretion upon activation. The relative frequency of Vα24 iNKT cells varies considerably between individuals and is low compared to the size of the Vα14 iNKT cell population in mice. Therefore, the contribution of Vα24 iNKT cells in immune responses or immunomodulation of autoimmunity may be harder to define in humans than in mouse models.

Human bone marrow and liver contain CD1d-reactive non-Vα24 NKT cells that by far outnumber Vα24-Jα18 iNKT cells. The human bone marrow-derived dNKT cell clones had a predominant Th2-cytokine profile, which may indicate that the human dNKT cell population modulate B cell functions. In contrast, in the liver the dNKT cell clones produced high levels of IFN-γ. These studies of human dNKT cells strongly suggest that non-Vα24 NKT cells participate in immune reactions occurring in the bone marrow or liver (Exley et al., 2002; Exley et al., 2001b). The total NKT cell population appears to be dominated by CD1d-reactive dNKT cells rather than by Vα24-Jα18 iNKT cells, suggesting that studies of dNKT cells in mouse is highly relevant for understanding the role of NKT cells in human diseases.

AIM OF THIS THESIS

In the immune system, different subsets of lymphocytes have distinct features and functions. Innate-like lymphocytes have unique properties and effector functions in comparison to conventional lymphocytes. The overall aim of this thesis is to expand our knowledge concerning innate-like lymphocyte characteristics and functions in different immunological settings. The specific aims were:

- **To determine the properties and genetic regulation of the enlarged marginal zone B cell population in the nonobese diabetic (NOD) mouse model of type 1 autoimmune diabetes.**

- **To determine the gene expression profile of invariant NKT cell and diverse NKT cell subsets, in order to identify common NKT cell signature genes and also genes that distinguish each of the NKT cell subsets.**

- **To determine the response of NKT cells during *Salmonella typhimurium* infection.**

BRIEF SUMMARY OF THE PAPERS

Paper I

The enlarged population of marginal zone/CD1d(high) B lymphocytes in nonobese diabetic mice maps to diabetes susceptibility region Idd11. (2005). Julia Rolf, Vinicius Motta, Nadia Duarte, Marie Lundholm, Emma Berntman, Marie-Louise Bergman, Lydia Sorokin, Susanna L. Cardell and Dan Holmberg. J Immunol. *174*, 4821-7.

The NOD mouse model of autoimmune type 1 diabetes is complex. The autoimmune disease is driven by self-antigen specific T cells that mediate the destruction of the insulin-producing β-cells in the pancreas. B cells are important as antigen-presenting cells that can initiate autoimmunity in the NOD mouse model (Serreze et al., 1998). The marginal zone (MZ) B cells can efficiently activate $CD4^+$ T cells, implying that MZ B cells may prime auto-reactive T cells in NOD mice (Attanavanich and Kearney, 2004). We identified an expansion of MZ B cells in NOD mice and hypothesized that the MZ B cell population participates in the development of autoimmunity. In this article, we characterized the population size, phenotype, localization and development of MZ B cells in NOD mice in comparison to non-autoimmune B6 mice. We showed that the MZ B cell population was expanded and developed earlier in NOD mice, but retained its features and localization. The genetic control of the MZ B cell population size was studied by a genome-wide screen of the F2-progeny of a (NODxB6)F1 intercross. The strongest genetic linkage was associated with the diabetes susceptibility loci Idd9/11 on chromosome 4. The MZ B cell population size in NOD is genetically regulated by chromosomal region correlated to autoimmunity, supporting the hypothesis that the expanded MZ B cell population may be a contributing factor in NOD autoimmunity.

Paper II

Molecular profiling reveals distinct functional attributes of CD1d-restricted natural killer (NK) T cell subsets. (2007). Julia Rolf, Emma Berntman, Martin Stenström, Emma Smith, Robert Månsson, Hanna Stenstad, Tetsuya Yamagata, William Agace, Mikael Sigvardsson and Susanna L. Cardell. Submitted manuscript.

Microarray technology is a powerful tool for providing a complete description of the gene expression profile of cell populations. There are two CD1d-reactive NKT cell subsets: invariant NKT cells and diverse NKT cells (Cardell et al., 1995). We performed gene expression profiling of these two NKT cell subsets in comparison to conventional $CD4^+$ T cells by microarray analysis. We hypothesized that the gene expression profile of the NKT cell subsets provides the basis for their properties and functions and should be distinct in the NKT cell lineage compared to conventional $CD4^+$ T cells. The common gene expression signature of NKT cells revealed that they share certain features with other innate-like T lymphocytes, such as the expression of NK cell receptors (Denning et al., 2007). The expression patterns of chemokine receptors that dictate migration of cells suggest that NKT cells are prone to home to inflamed tissues. Selective expression of integrins that mediate adhesion indicate that NKT cells may bind to extracellular

matrix components. Apart from the common NKT gene expression signature, each of the NKT cell subsets over-expressed a unique pattern of genes. The invariant NKT cell gene profile included both IFN-γ and IL-4 and in addition genes that promote cytotoxicity such as Slamf7 and FasL. Diverse NKT cells over-expressed genes associated with NK cell activation, suggesting that their functions may be coupled to enhancing immune responses against tumours and virally infected cells. We identified the expression pattern of the transcription factors Eomesodermin, Hhex, Id2 and T-bet and showed that these genes were induced during thymic development of NKT cells. These transcription factors may participate to the transcriptional regulation of the NKT cell features. The overall gene expression profile of dNKT cells, iNKT cells and the common NKT cell signature has expanded our understanding of the NKT cell lineage and will provide a basis for further studies to define their unique roles in immunity.

Paper III

The role of CD1d-restricted NK T lymphocytes in the immune response to oral infection with *Salmonella typhimurium*. (2005). Emma Berntman, Julia Rolf, Cecilia Johansson, Per Andersson and Susanna L. Cardell. Eur J Immunol. *35*, 2100-9.

The role of NKT cells is to rapidly secrete cytokines that will influence the activation of other immune cell populations such as NK cells and dendritic cells. We hypothesized that NKT cells become activated by the *Salmonella typhimurium* infection and that NKT cells can shape the early immune response against *Salmonella typhimurium* through cytokine secretion. We characterized the properties of NKT cells after oral infection with *Salmonella typhimurium*. The NKT cells became very strongly activated early during the infection and their cytokine-profile was dominated by IFN-γ secretion, which is known to enhance the immune response during *Salmonella* infection. However, NKT cells did not affect the outcome of the infection since the number of bacteria and recruitment of phagocytic cells in mice that lacked NKT cells were similar to control mice. In addition, we observed that CD1d levels on DCs were increased after exposure to *Salmonella in vitro*, but *in vivo* there was no general up-regulation of CD1d. In conclusion, NKT cells are strongly activated during *Salmonella* infection and respond rapidly by secreting the pro-inflammatory cytokine IFN-γ, thereby contributing to the defence against this lethal pathogen.

BACKGROUND - PAPER I.

Nonobese diabetic (NOD) mice represent a mouse model of human type 1 diabetes also known as insulin dependent diabetes mellitus (IDDM). The NOD mouse strain spontaneously develops autoimmune diabetes, characterized by destruction of the insulin producing β cells in the Langerhans islets in the pancreas (Makino et al., 1980). There are two phases during the progression of autoimmunity: insulitits (inflammation of the pancreas) and overt diabetes. All NOD mice develop insulitis. Insulitis is a term for the inflammation in the pancreas and the different stages of insulitits are scored based on the degree of mononuclear cell infiltration around the islets and the islet cell destruction. Overt diabetes arises after the destruction of 90% of all β cells and at this stage the insulin-production is too low to control the blood sugar levels. The diabetic stage in NOD mice is fatal. There is gender bias in the development of diabetes in NOD mice; 60-80% of the females have become diabetic, but only 40% of the males at 30 weeks of age. Peripheral regulation of autoimmunity halts the β-cell destruction at the stage of insulitits in the mice that do not become diabetic (reviewed by (Andre et al., 1996)). The autoimmune disorder developing in the NOD mice is complex. At 2-3 weeks of age there is a physiological wave of apoptosis in the pancreatic islets and β cell antigens derived from apoptotic cells are ferried by DCs to the pancreatic lymph nodes (PaLNs) where priming of auto-reactive T cells occur (Turley et al., 2003). Diabetes in NOD mice is a T cell driven disease, as shown by transfer of disease with $CD4^+$ T cells or $CD8^+$ T cells and absence of disease in various T cell deficient mouse models. $CD8^+$ T cells kill target cells and seem to be especially important in the early phase of the destruction of pancreatic β cells. The $CD4^+$ T cells are crucial both in the early and late phase of autoimmunity through production of cytokines that dictate the autoimmune inflammation. Th1/Th2 cytokine balance in NOD mice is considered important for the autoimmune disease to develop. Th1 domination is thought to contribute to the pathogenesis whereas a Th2 response may divert autoimmunity towards non-destructive responses (reviewed by (Anderson and Bluestone, 2005; Delovitch and Singh, 1997)). Although, NOD autoimmunity depends on T cell mediated destruction, immune cells isolated from the pancreatic lesions also include macrophages, DCs, B cells and NK cells. The autoimmune disease in NOD mice is the result of interplay of several leukocyte populations both in the pancreas and the PaLNs. The functional defects of NKT cells, macrophages, DCs and NK cells and the hyper-reactivity of B cells may also contribute to autoimmune diabetes.

B cells are crucial APCs that mediate autoimmunity in NOD mice

The crucial role of B cells in autoimmunity was determined in NOD mice devoid of all B cells by deletion of the Ig μ heavy chain locus. These NOD.Igμ^{null} (NOD.μMT-/-) mice were protected from diabetes and had less severe degree of insulitis (Akashi et al., 1997; Serreze et al., 1996). Injection of antibodies directed against Ig-molecules that abolish the B cell population in NOD mice also reduced diabetes incidence (Noorchashm et al., 1997). The B cells are crucial for

autoimmunity to progress into overt diabetes but how do B cells participate in the development of autoimmune diabetes?

Transfer of Ig-molecules from overt diabetic mice into NOD.Igμ^{null} mice did not give rise to diabetes. Therefore, secretion of auto-antibodies is not the primary mechanism of auto-aggression caused by B cells. Instead, it is considered that the role of B cells in autoimmune diabetes is mainly as APCs to T cells and transfer of NOD B cells could restore diabetes development in NOD.Igμ^{null} mice (Serreze et al., 1998). The B cells were suggested to capture self-antigen via their BCR rather than via Fc-receptor mediated up-take and then activate self-antigen specific CD4$^+$ T cells (Falcone et al., 1998). The presentation of auto-antigens to CD4$^+$ T cells is mediated by MHC class II molecules and the NOD mice express the MHC class II molecule I-A^{g7} that is strongly coupled to autoimmune disease (reviewed by (Delovitch and Singh, 1997)). B cell-specific genetic deletion of I-A^{g7} MHC expression results in nearly complete protection from diabetes. Therefore, B cells can be assumed to be the crucial APC-population that drives the CD4$^+$ T cell pathogenesis by presentation of self-antigen derived peptides on the I-A^{g7} MHC class II molecules (Noorchashm et al., 1999a). However, the NOD B cells are inefficient APCs. For activation-induced cell death (AICD) and IL-4 production to be initiated during an immune response, it is claimed that the T cells must undergo at least three cell-cycle divisions. In NOD mice, this number of cell-cycle divisions is not achieved with B cells as APCs. Consequently, auto-reactive Th1-tilted CD4$^+$ T cells are suggested to persist in a state of constant activation in NOD mice (Noorchashm et al., 2000). Another factor that contributes to the inefficient activation of T cells in NOD mice is the low expression of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) on APCs. The inefficient co-stimulatory signals in NOD mice probably prevent optimal CD4$^+$ T cell activation, thus allowing auto-reactive T cells escape AICD. In addition, the low level of CD86 has been suggested to result in deficient expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) that functions as an inhibitor of T cell activation (Dahlen et al., 2000). In summary, B cells are required for initiation of autoimmune diabetes in NOD mice through presenting self-antigens to auto-reactive T cells. Defects in the B cell-mediated activation of T cells seem to contribute to autoimmune disease in NOD mice.

NOD B cell characteristics

The importance of B cells in diabetes development highlights the need to understand the properties of B cells in NOD mice. The self-reactive Ig-molecules on the surface of B cells can capture self-antigens and then process and present self-peptides on MHC class II to CD4$^+$ T cells. Characterization of the BCR specificities in NOD mice has revealed that there is an increased propensity towards expression of germ-line encoded, non-mutated V regions of the IgH chain. These germ-line encoded IgH V regions are the same as anti-dsDNA antibodies isolated from lupus-prone mouse models, suggesting that self-reactive IgH chains may be a common denominator between NOD mice and spontaneous lupus mouse models (Leijon et al.,

1993; Thomas et al., 2002). The BCR specificity is crucial for the role of B cells in autoimmunity. NOD mice expressing an insulin-specific BCR-transgene had a higher incidence of diabetes than if an irrelevant non-pathogenic BCR was expressed on all B cells (Hulbert et al., 2001; Rojas et al., 2001; Silveira et al., 2002). Insulin-reactive transgenic B1 B cells in the peritoneal cavity were found to be the main producers of auto-antibodies (Rojas et al., 2001). The relative contributions of the three mature B cells subsets: B1 B cells, FO B cells and MZ B cells to diabetes development in wild-type NOD mice are unclear. Both B1 B cells and MZ B cells are innate-like lymphocytes that contain germ-line encoded, multi-reactive BCR-specificities (reviewed by (Martin and Kearney, 2000a)). Since the MZ B cell population contains multi-reactive BCR specificities and becomes activated in the absence of T cell help, the MZ B cells may play a role in initiating autoimmunity (reviewed by (Martin and Kearney 2002)). We hypothesized that the MZ B cell population participates in autoimmunity in NOD mice. Therefore, we performed an extensive study of the properties of the MZ B cells in NOD mice. Since the autoimmune diabetes in NOD mice is genetically linked we analyzed the genetic regulation of MZ B cells in order to understand if the MZ B cell population size was correlated to gene regions determining autoimmunity.

Experimental approach – genetic mapping

In order to study the genetics underlying autoimmunity in the NOD mouse model, genetic regions that are involved in the disease are being mapped. The genetic regions that have been shown to be linked to autoimmunity are termed diabetes susceptibility loci (Idd). Presently over 20 Idd loci have been identified, including Idd1 that contains the diabetes-associated MHC class II I-A^{g7} haplotype. The approach to genetic mapping studies generally involves crossing NOD mice with a non-autoimmune mouse strain. The F1 generation is then inter-crossed, thus giving rise to the F2-generation that will have a mosaic of genetic material from the two parental mouse strains. The genome of the F2 offspring is then genetically mapped with microsatellite markers that identify allelic differences between the parental mouse strains. The genetic regions defined by microsatellite markers are then correlated to quantitative trait such as the size of an immune cell population. After identifying a correlation between a quantitative trait with a genetic region, this genetic region can be studied in detail by creating congenic mouse strains. The congenic mouse strain has the exact same genetic material as one of the parental strain, except for the genetic locus specific to a particular congenic strain derived from the other mouse strain. In many cases, the congenic locus contains genetic material from the non-autoimmune parental mouse strain. This makes it possible to analyze the effect of a particular locus by comparing the features of the congenic mouse strain to wild-type NOD mice.

In order to study the genetic control of the expanded MZ B cell and $CD1d^{hi}$ B cell population in NOD mice, we performed a genome-wide screen. First we crossed NODxC57Bl/6 and then inter-crossed the F1-generation to obtain F2-generation mice with a mixture of genetic material from both parental strains. The F2 off-spring displayed a greater phenotypic variance in the MZ

B cell population size than the parental strains, demonstrating that genetic regions determining the MZ B cell population size segregated in this cross. Therefore, we deemed the MZ B cell population as a trait under genetic control that would be possible to study by genome-wide screening. To cover the entire genome 119 genetic markers with an average distance of 15 cM (centiMorgan: the distance separating two genetic loci that recombine with a frequency of 1%, also termed map unit) was used for the genetic mapping of the genome of the F2-generation mice.

RESULTS AND DISCUSSION - PAPER I.

MZ B cells in NOD mice

We observed an increase in the frequency and absolute numbers of MZ B cells in NOD mice compared to B6 mice (Paper I, Figure 1, 2 and Table I). The MZ B cell and CD1d^{hi} B cell populations were highly overlapping, although it seemed that some CD1d^{hi} B cells had the CD21^{hi}CD23^{hi} phenotype of transitional 2 B cells (Paper I, Figure 1B). Our results are in accordance with the description of an increased splenic B cell population expressing the complement receptor CD21, suggesting that MZ B cells were expanded in NOD mice. Treatment with antibodies against the CD21-marker resulted in disappearance of the MZ B-like cells, although it is unclear whether they were depleted or merely down-regulated the CD21-marker. Also, the follicular dendritic cells (FDCs) that present antigen to B cells express very high levels of CD21 and therefore the CD21-treatment may deplete these cells as well, which would impair FO B cell activation. The anti-CD21 antibody treatment increased resistance towards cyclophosphamide-induced diabetes, suggesting that MZ B cell depletion could reduce diabetes-incidence (Noorchashm et al., 1999b).

We studied the localization of MZ B cells in NOD mice in order to determine if there is a general expansion of MZ B-like cells. Although MZ B cells are generally sessile in the MZ region, expansion of MZ B/CD1d^{hi} B cells occurred in the intestine during chronic inflammation and in salivary glands in BAFF-transgenic mice (Groom et al., 2002; Mizoguchi et al., 2002). We reasoned that the ongoing autoimmune disease in NOD mice could potentially enhance the formation of MZ B cells in other organs than the spleen. However, in NOD mice there was no general expansion of MZ B cells or CD1d^{hi} B cells in the thymus, bone marrow, mesenteric lymph nodes (MLN), PaLN or peritoneal cavity (Paper I, Figure 5). The anatomical sites of initiation of autoimmune diabetes are thought to be the PaLNs or possibly the MLNs and surgical excision of the spleen did not affect the initiation of diabetes (Gagnerault et al., 2002; Jaakkola et al., 2003). During later stages of autoimmunity, the spleen becomes the largest reservoir of auto-reactive T cells. Transfer of equal numbers of lymphocytes from spleen, PaLN and MLN revealed that splenocytes caused diabetes at an earlier time-point compared to transfer of LN lymphocytes from adult donor mice (Jaakkola et al., 2003). Therefore, MZ B cells may sustain autoimmunity by promoting auto-reactive T cell activation in the spleen, since MZ B cells are more efficient APCs than FO B cells to naive CD4^{+} T cells (Attanavanich and Kearney, 2004). We found that the expansion of MZ B cells in NOD preceded insulitis (Paper I, Figure 7), indicating that the activated phenotype of NOD MZ B cells was not a secondary consequence of ongoing auto-immunity. Since we could not detect any MZ B cells in the PaLN at an early age, it remains uncertain whether the MZ B cells participate in the initial priming of auto-reactive CD4^{+} T cells that is thought to occur in the PaLN. Possibly, the MZ B cells maintain the auto-aggression by continuously activating CD4^{+} T cells in the spleen, rather than mediate the initial priming in the PaLNs.

The MZ B cell population have the potential to act as APCs to NKT cells via the high levels of CD1d constitutively expressed on their surface. Recent studies have shown that NOD DCs were dysfunctional in terms of stimulating iNKT cells after αGalCer injection. In contrast, in B6 mice the DCs were crucial APCs to iNKT cells and B cells rather inhibited the DC-mediated activation of iNKT cells. Thus, the B cells are the most important APC subset for activation of NKT cells in NOD mice (Bezbradica et al., 2005). It remains to be determined if the role of B cells, including MZ B cells, in NKT cell activation contribute to the functional defects in terms of impaired cytokine-secretion observed in NOD NKT cells (reviewed by (Cardell, 2006)).

After completing the study on MZ B cell features and genetic control, we set out to analyze the capacity of NOD B cells to respond to antigens from bacterial capsules. The antibody-response against these antigens is mediated by the innate-like lymphocyte subsets MZ B cells and B1 B cells. Challenge with *Streptococcus pneumoniae* elicits rapid IgM production against phosphorylcholine (PC) by these B cell populations (Martin et al., 2001). In preliminary studies in collaboration with Prof. J Kearney at University of Alabama at Birmingham, we have found that the NOD mice produced two-three fold higher levels of PC-specific IgM antibodies after challenge with heat-killed *S. pneumoniae* bacteria than B6 mice (Figure 8). The enhanced PC-specific antibody response in NOD mice suggests that the MZ B cell population is functional in terms of mounting an immune response. However, since B1 B cells also produce anti-PC antibodies further analysis is required to establish the relative contribution of MZ B cell to the high anti-PC antibody titers in NOD mice.

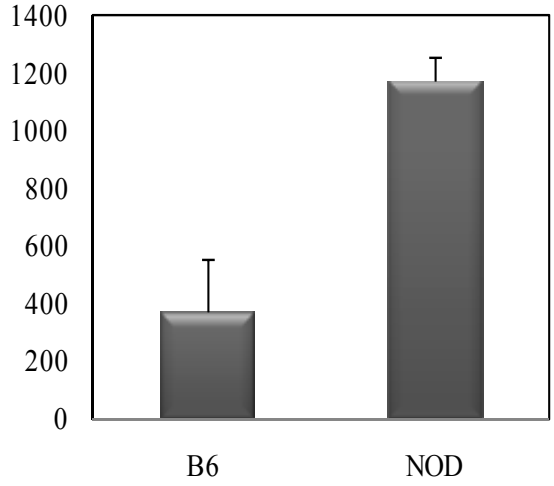

Figure 8. The PC-antibody IgM-levels (μg/ml) in B6 mice compared to NOD mice. B6 mice (n=5) and NOD mice (n=5) were intravenously injected with heat-killed *S. pneumoniae* bacteria. Serum was isolated on day 7 after challenge and the PC-specific antibody-titers were determined by ELISA.

Defects in NOD peripheral B cell tolerization

Since B cells are crucial for initiation of autoimmunity in NOD mice, the B cell population is likely to be auto-reactive to self-antigen and to have defects in tolerization. The NOD Transitional T1 B cell population was found to be reduced compared to B6 mice, suggesting that this stage of B cell selection may be affected by the break-down of tolerance in NOD mice (Silveira et al., 2004; Paper I, Table I). The central tolerance in the bone marrow to membrane-bound antigens was similar in NOD and B6 mice. However, in B6 mice the number of auto-reactive B cells recognizing soluble antigens was reduced. In contrast, in NOD mice the reduction of auto-reactive B cells was incomplete and the anergic state of the persisting B cells was highly reversible after stimulation. This suggests that peripheral deletion of T1 self-reactive B cells is defect in NOD mice and that auto-reactive B cells are maintained among mature B cells in NOD mice (Silveira et al., 2004). Reduced output of immature B cells from the bone marrow led to the reduction in the T1 B cell populations size. The overall stringency of peripheral B cell maturation was lower in NOD mice compared to B6 mice and self-reactive B cells were permitted to mature and become FO B cells or MZ B cells in the NOD mice (Quinn et al., 2006). Similar observations were made in transgenic mice expressing insulin-reactive BCR. The self-reactive B cells were anergic but allowed to persist among the mature B cells, especially in the MZ B cell population. Although the insulin-specific B cells did not secrete auto-antibodies, they up-regulated the co-stimulatory molecule CD86 after stimulation (Acevedo-Suarez et al., 2005). Defects in the peripheral B cell selection in the NOD mouse allow maturation of self-reactive B cells that can act as APCs to self-antigen specific $CD4^+$ T cells. Interestingly, the Idd9/11 loci contribute to the aberrant B cell properties in NOD mice. Genetic regions underlying B cell autoimmunity was analyzed in NOD mice congenic for Idd9/11 and Idd5.1/5.2. The congenic mouse models had restored capacity to anergize immature B cells after exposure to soluble self-antigen, unlike wild-type NOD mice that had deficiencies in the anergization of B cells. The diabetes incidence is lower after transfer of B cells from Idd9/11 and Idd5.1/5.2 congenic mouse stocks compared to wild-type NOD mice into NOD.Ig^{null} mice (Silveira et al., 2006). These results showed that B cell tolerization defects in NOD mice had a genetic basis, linked to the Idd9/11 and Idd5.1/5.2 regions and that the tolerization defects contributed to diabetes. The Idd9/11 loci are associated with the dysfunctional B cell tolerance in NOD mice and also with an increased MZ B cell population (Paper I, Figure 6).

The Idd9/11 loci are associated with B cells in autoimmune diseases

Genetic mapping of the frequency of MZ B cells and $CD1d^{hi}$ B cells in the F2-generation from NODxC57Bl/6 F1 inter-cross showed genetic linkage to several chromosomal regions (Paper I, Figure 6). The genetic region most strongly linked to the MZ B cell population was found between the markers D4Mit72 and D4Mit251 spanning 7cM on chromosome 4 and covered the Idd9/11 loci. There was also suggestive linkage to regions on chromosome 1 (LOD-score 3.2), 4 (LOD-score 4.0), 9 (LOD-score 3.0) and 19 (LOD-score 3.1), although none of these regions contain identified Idd loci. The Idd11 locus is found between the markers D4Mit119 to

D4Mit204 and covers 13cM (Brodnicki et al., 2000; Morahan et al., 1994). The Idd11 locus is contained within the Idd9.1 locus (Lyons et al., 2000). Interestingly, the distal region of chromosome 4, including the Idd9/11 loci contains genetic regions that promote lupus, autoimmune diabetes, autoimmune gastritis and sialadenitis (inflammation of salivary glands similar to the autoimmune disease Sjögren's syndrome) in genetically susceptible mouse strains (reviewed by (Johansson et al., 2003)). Dysfunctional B cell tolerance mechanisms may be a common denominator between NOD and several SLE models. NOD mice are known to develop lupus-like symptoms after challenge with *Mycobacterium bovis* and this lupus-like disease maps to lupus-associated loci rather than to Idd loci (Jordan et al., 2000). In conclusion, chromosome 4 contains several loci associated with B cell autoimmunity, although further analysis is required to identify the nature of candidate genes that could promote break-down of B cell tolerance.

We determined the MZ B cell population size to be most strongly linked to the Idd9/11 loci (Paper I, Figure 6). The Idd9/11 loci were estimated to contribute to 12 % of the genetic control of the MZ B cell population size in the F2 off-spring mice. The increase of the MZ B cell population is also most likely influenced by the four regions with suggestive linkage. The MZ B cell number is known to be affected by many genes that are involved in BCR signalling and localization (reviewed by (Pillai et al., 2005)). Therefore, it is reasonable that several genetic regions together promote the increase in MZ B cell population in NOD mice. Studies of the role of Idd11 in controlling MZ B cell population size was performed to explore the effect of this locus alone on MZ B cell population size by using congenic mouse strains. NOD mice congenic for B6 specifically covering the Idd11A locus or a larger region of the chromosome 4 were analyzed. The Idd11 and chromosome 4 congenic mouse strains were claimed to have an increased MZ B cell population similar to wild-type NOD mice. However, the variation between individual mice in all of the mouse strains was considerable and there were also indications of a reduction of MZ B cell in the NOD.B6Idd11A compared to the other NOD mouse strains analyzed. Therefore, a difference in MZ B cell frequency corresponding to the 12% of the regulation conferred by the Idd11 locus is difficult to determine from the few congenic mice analyzed in this study (Brodnicki et al., 2006). Still, our data as well as from Brodnicki et al indicated that the MZ B cell population size is determined by several genetic loci and not only by Idd9/11. It remains to identify the genes within the genetic regions associated with control of MZ B cell population size and how they may exert their regulation of the MZ B cell population size.

Concluding remarks

The B cell population consists of different maturation stages and also of phenotypically distinct populations, including the innate-like lymphocyte B1 B cells and MZ B cells. In this study we have extensively characterized the MZ B cell subset in the mouse model of autoimmune diabetes known as NOD mice. The MZ B cell population was two-three fold expanded in NOD mice in comparison to non-autoimmune B6 mice, but the phenotype and the localization of MZ B cells were similar in NOD mice and B6 mice. The MZ B cell population was formed at an early age in

NOD mice that precede the initiation of autoimmune diabetes. Therefore, the MZ B cell expansion was not secondary to autoimmune inflammation. The antibody-production against antigens present in bacterial capsules that are typically recognized by MZ B cells was higher in NOD than in B6. In order to find the underlying genetic regions regulating the MZ B cell population size, genetic mapping was performed using the F2-generation derived from an inter-crossed (NODxB6) F1-generation. The genetic loci most strongly associated with MZ B cell frequency were Idd9/11 on chromosome 4 that is known to contribute to diabetes. There was also suggestive linkage to genetic regions on chromosome 1, centromeric 4, 9 and 19, indicating that the MZ B cell population size was regulated by multiple gene regions. The genetic region encompassing Idd9/11 includes loci is associated with a variety of different autoimmune disorders such as lupus, which is characterized by auto-reactive antibody-production.

Future perspectives

In order to further study the role of MZ B cells in diabetes development in NOD mice, their capacity to induce diabetes should be analyzed. By performing *in vivo* transfer experiments of MZ B cells compared to FO B cells into NOD.SCID mice, the relative contribution of these B cell subsets as APC may be determined.

BACKGROUND - PAPER II.

In mice, less than half of the NK1.1$^+$TCRβ$^+$ cell population in the spleen consists of iNKT cells expressing the Vα14-Jα18 TCRα chain paired with a limited repertoire of TCR Vβ-chains. The remaining non-Vα14 NK1.1$^+$TCRβ$^+$ cell population consists of CD1d-restricted dNKT cells but also of MHC-restricted cells that have up-regulated NK cell markers. The CD1d-reactive dNKT cells contain many different TCR clones that have variations in antigen-specificities (Behar et al., 1999; Cardell et al., 1995; Jahng et al., 2004; Park et al., 2001). The dNKT cells are clearly playing unique roles in the immune system as shown in a model of hepatitis B where transfer of dNKT cells caused serious hepatic injury whereas iNKT cells did not attack the hepatocytes (Baron et al., 2002). In contrast to causing excessive inflammation, dNKT cell subsets are protective against autoimmune diseases such as type 1 diabetes in NOD mice and EAE (Duarte et al., 2004; Jahng et al., 2004). This versatility in immunomodulatory functions, including inflammation during infections and protection against autoimmune destruction, is a common feature of iNKT cells and dNKT cells. Therefore, it is highly relevant to identify the properties and functional capacities of dNKT cells in order to understand their role in diseases. In order to expand the understanding of the two NKT cells subsets, we have performed global gene expression profiling using microarray analysis of transgenic iNKT cells and dNKT cells. By combining both of these NKT cell subsets in a comparison with the gene profile of CD4$^+$ T cells, we identified the expression pattern of genes that belong to the NKT cell common signature. In addition, the genes that were specifically expressed in each of the NKT cell subsets were determined by microarray analysis.

NKT cell transgenic mouse models

In order to study the gene expression profile of the iNKT cells and dNKT cells TCR transgenic mouse models representing each of these subsets was utilized. The Vα14 transgenic mouse model uses a TCRα chain cloned from the CD1d-reactive DN32D3 hybridoma expressed under the endogenous Vα11 promoter. The Vα14 transgenic mice were bred onto a TCRα-/- background and were back-crossed for over 16 generations onto the B6 genetic background (Bendelac et al., 1996; Lehuen et al., 1998). The 24αβ transgenic line carries the Vα3.2Jα20 chain that pairs with Vβ9Jβ1.4 isolated from the CD1d-autoreactive VIII24 hybridoma. The two TCR transgenic chains are expressed under the natural enhancer and promoter regions for TCRα and TCRβ chains. The transgenic 24αβ dNKT cells have been shown to be CD1d-restricted by backcrossing them onto CD1d-/- mice, which resulted in a disruption of the transgenic 24αβ dNKT cell properties such as NK1.1 and CD122 expression (Cardell et al., 1995; Skold et al., 2000). Since the NK1.1$^+$TCRβ$^+$ cell population in wild-type mouse is contaminated by T cells that have up-regulated NK-markers but do not belong to the NKT cell lineage, studies of the dNKT cells is fraught with difficulties. The advantage of using the transgenic mouse models representing iNKT cells or dNKT cells, respectively, is that highly homogenous, well-

characterized cell populations that are known to be CD1d-restricted are used for the microarray. The transgenic 24αβ dNKT cells have many similarities to non-Va14 NK1.1^{+}TCRβ^{+} cells from wild-type mice, suggesting that 24αβ dNKT may represent some of the dNKT cells in wild-type mice (Stenstrom et al., 2004).

Experimental approach – microarray analysis

Microarray technology is a powerful method for studying the total mRNA transcript expression of cell-types or tissue-biopsies, in order to obtain a broad overview of the gene expression profile. The commercially available gene array chips provided by Affymetrix Inc were used in this study (www.affymetrix.com). The basis for Affymetrix's high definition microarray is the usage of probe-sets containing different nucleotide sequences. The general difficulty with microarray analysis is false positive data resulting from unspecific hybridization of the transcripts from the sample to probes on the microarray gene chip. Affymetrix's gene chip technology is based on the discrimination between perfect match (PM) and mismatch (MM) nucleotide sequences within the same probe-set. The perfect match probe has complementary nucleotide sequence to a certain gene-of-interest, whereas the mismatch probes is not perfectly complementary and represent unspecific background binding. The microarray data is modelled by PM/MM and transcripts from the sample that are considered to be present have higher PM values than MM values. We have chosen to use the dChip software package (http://biosun1.harvard.edu/complab/dchip/) to perform statistical analysis of the global gene expression. Genes were considered to represent biologically relevant gene expression differences when fulfilling the following criteria:

To be two-fold or higher differentially expressed between one population in comparison to the other two populations.
To fall within the lower bound 90 % confidence interval of dChip's calculation of fold change that weighs in the variation between the duplicate samples.
To have a difference in probe set signal value higher than 100 (the overall median of signal values is 141), thus excluding genes that are expressed at background levels.

The samples used in this microarray analysis were all deemed to be of high quality and showed reproducibility between the duplicate samples. Analysis of microarray gene chip-data poses statistical difficulties due to the sheer amount of data and the detection of false positive genes as well as missing relevant information due to false negative data is an inherent problem. However, by using stringent demands on the genes considered to be differentially expressed, the problem of detecting false positive genes was minimized in our analysis. The major risk of detecting false positive genes occurs for low abundance mRNA transcripts. By excluding all genes with signal values below 100, our analysis removed genes that are expressed at levels similar to background. However, the disadvantage is that genes transcribed at low levels were excluded completely from the analysis. Therefore, it is important to emphasize that the genes considered to be differentially

expressed in this microarray analysis were derived from the criteria stated above. In order to study the gene expression profile of NKT cells, we compared the NKT cell populations to $CD4^+NK1.1^-$ T cells. The NKT cells have certain fundamental similarities to $CD4^+$ T cells, such as the expression of TCR. However, the $CD4^+$ T cells are conventional T lymphocytes, whereas NKT cells are innate-like lymphocytes belonging to a different cell-lineage. Broad microarray analysis has revealed that approximately 10% of the genes transcribed in the immune system are preferentially expressed only by a certain population of immune cells (Hyatt et al., 2006). It can be assumed that by comparing $CD4^+$ T cells and NKT cells that are relatively similar, the genes found to be differentially expressed are quite specific to each of these two cell-lineages. Some of the genes found to be differentially expressed have been verified using quantitative real-time PCR with gene-specific primers. The correlation between microarray expression pattern and mRNA confirmations was high. Microarray data of expression pattern of some chemokine receptors and integrins was also verified on the protein level by using monoclonal antibodies and detecting the cell-surface expression levels using flow cytometry. The functionality of the chemokine receptors was evaluated by chemotaxis assays.

RESULTS AND DISCUSSION - PAPER II.

The gene expression profile of NKT cell subsets compared to $CD4^+$ T cells revealed differentially expressed genes common to both NKT cell populations. By performing hierarchical clustering analysis, which is a method of analysis of the degree of similarity between cell populations, the NKT cell subsets were shown to be more closely related to each other than to $CD4^+$ T cells (Paper II, Figure 1). The high degree of correlation in gene expression by NKT subsets supports the hypothesis that NKT cells have a common mRNA transcript profile. The number of differentially expressed genes represented in the Venn diagram indicated that the NKT cell common genes (180) in comparison to the $CD4^+$ T cell specific genes (132) together constituted the majority of genes varyingly expressed by these three cell-types (Paper II, Figure 1). In order to expand the knowledge on NKT cell subset-specific genes, the gene expression of each of the NKT cell subsets were studied. The 24αβ dNKT cells transcribed 57 genes more highly than Vα14 iNKT or $CD4^+$ T, whereas Vα14 iNKT cells over-expressed 72 genes in relation to diverse 24αβ dNKT cells and $CD4^+$ T cells. We could confirm the expression pattern of a set of genes detected in the microarray analysis by the real-time PCR (Paper II, Figure 1). In the following sections, the genes that are likely to play significant roles in mediating NKT cell-specific features are discussed in detail.

Innate-like properties

The innate-like lymphocytes CD8αα $TCR\alpha\beta^+$, γδ T and NKT cells all express different NK cell-related molecules such as NK1.1, Ly49G2, 2B4, DAP12 and the transcription factor Id2 (Denning et al., 2007; Paper II, Figure 2). The NK-related genes participate in the regulation of the activation and possibly also in the induction or maintenance of the specialized functions of innate-like T lymphocytes. We found that several genes that distinguish the gene profile of CD8αα T cells from conventional CD8αβ T cells, apart from NK-related genes, were also expressed by the NKT cell subsets in comparison to $CD4^+$ T cells (Denning et al., 2007; Yamagata et al., 2004; Paper II, Figure 2). Many of the positively regulating receptors present on NKT cells, including NK receptors, c-kit, prostaglandin E receptor, CD122, annexin and others are part of the innate-like profile, suggest that innate-like lymphocytes are more prone to respond to various stimuli than conventional lymphocytes. In contrast, conventional $CD4^+$ T cells over-expressed the chemokine receptor CCR7 and the enzyme TdT that inserts N-nucleotides into the TCR, which are associated with conventional lymphocyte properties (Yamagata et al., 2006).

Modulation of NKT cell activation status

NKT cells express a broad variety of receptors that influence their effector functions and that distinguish them from conventional T lymphocytes (Paper II, Figure 2, 3, 4). One example of additional receptors influencing NKT cells activities are the NK cell receptors. Mature NK cells express receptors that modulate their activation status and enable NK cells to identify viral infections or malignantly transformed cancer-cells. The NK receptors may either be inhibitory or

activating and together they maintain a fine-tuned balance regulating the NK cell status. The inhibitory NK receptors bind MHC class I molecules, thereby preventing killing of normal cells. Virus-infected or tumour cells that have down-regulated MHC class I on their surface as a means of avoiding targeting by $CD8^+$ CTLs no longer can inhibit NK cell killing. On the other hand, the activating NK receptors recognize specific ligands that often are up-regulated on virally infected or transformed cells. The inhibitory receptors seem to play a dominant role over the activating receptors under normal conditions, and thus yet another signal, for example by the cytokines IL-12 or IL-18, is required to override the inhibitory receptor signalling (Ortaldo and Young, 2003). Ly49 receptors are a large family of 13 inhibitory (exemplified by Ly49A, C, G, I) and 10 activating NK receptors (exemplified by Ly49D, H) (reviewed by (Dimasi and Biassoni, 2005)). The inhibitory Ly49-receptors are characterized by immune receptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail that recruit SHP1-phosphatase and thus prevent intracellular signalling pathways that may activate cells. The activating Ly49 receptors and NKG2D are dependent on the immune receptor tyrosine-based inhibitory motifs (ITAM) containing adaptor proteins DAP12 or DAP10 to mediate an intracellular signalling cascade that results in exocytosis of cytotoxic granules or cytokine secretion (Ortaldo and Young, 2005). The Ly49 expression pattern on NKT cells seems to be modulated by MHC haplotype and the Ly49 receptors are differentially expressed in thymus and spleen (Robson MacDonald et al., 1998; Skold and Cardell, 2000). The iNKT cells express Ly49C/I but the non-Vα14 $TCR\beta^+NK1.1^+$ cells are positive for Ly49G2, suggesting that the Ly49-receptor repertoire distinguishes the iNKT cell and dNKT cell populations (Skold et al., 2003). Our microarray analysis showed that NKT cells expressed 8 inhibitory receptors and 9 activating receptors more highly than $CD4^+$ T cells (Paper II, Figure 3). The expression of activating NK receptors suggested that NKT cells have a capacity to recognize virally infected, stressed or transformed cells that express ligands for these receptors. Possibly, the role of these activating receptors is especially important in the NKT cell type represented by 24αβ dNKT cells. The adaptor molecule DAP12 is over-expressed in the 24αβ dNKT cells, suggesting that activating signals via DAP12 are transmitted upon recognition of the ligands for activating NK receptors such as NKG2D. However, the role of DAP12 may also be to transmit signalling that prevents activation when the avidity of the ligands is low (reviewed by (Turnbull and Colonna, 2007)). In terms of inhibitory receptors, the expression of each of these receptors is heterogeneous when comparing 24αβ dNKT and Vα14 iNKT. The differences in inhibitory NK receptor profile between the two NKT cell subsets seemed to imply that the dNKT cells rely on Ly49-MHC class I inhibitory signals, whereas iNKT cells are more dependent on NKR-mediated negative regulation. Ly49 receptor signalling clearly influences the formation of NKT cells, as shown in Ly49A transgenic mouse model, where the NKT cell population size and Ly49A levels are influenced by the presence of the Ly49A ligand MHC $H\text{-}2D^d$ (Robson MacDonald et al., 1998). In another model where the activating Ly49D is over-expressed together with DAP12 and $H\text{-}2D^d$, the NKT cell population is severely diminished, probably through too strong ITAM-signalling. The NKT cell population can be partially rescued by transgenic expression of the inhibitory Ly49A receptor (Voyle et al.,

2003). It has also been shown that expression of Ly49A on dNKT cells reduce their proliferation after *in vitro* stimulation with CD1d-transfected B cell hybridoma (Skold and Cardell, 2000). Taken together, NKT cell development and effector functions are in part regulated by the Ly49 receptor-family, suggesting that the Ly49 receptors contribute to a balance between signals that promote activation (ITAM) and signals that are correlated to unresponsiveness (ITIM). The expression of DAP12-associated Ly49 receptors may be an important feature of dNKT cells.

The homeostasis of NKT cells is dependent on the cytokine IL-15 while a combination of the cytokines IL-12 and IL-18 enhances NKT cell effector functions. Microarray analysis showed that NKT cells over-expressed the IL-2Rβ chain (CD122) which is shared by both the IL-2R and the IL-15R (Paper II, Figure 2 and 4). The IL-15R is crucial for NKT cell survival and mice lacking IL-15 have a dramatic reduction of mature NKT cells (Matsuda et al., 2002). The cytokine IL-18 plays a general role in enhancing cytokine production in iNKT cells upon activation (Leite-De-Moraes et al., 1999). The cytokine-receptor profile in Vα14 iNKT cells included over-expression of IL-12Rβ1 compared to $CD4^+$ T and 24αβ dNKT, which indicated that Vα14 iNKT cells may be more sensitive to IL-12. The expression of receptors for IL-12 and IL-18, may explain the role of these cytokines in anti-tumour immunity (Baxevanis et al., 2003). Vα14 iNKT cells also transcribed more IL-2Rα(CD25) mRNA than either $CD4^+$ T cells or 24αβ dNKT cells. Our microarray data confirmed the importance of the cytokines IL-15, IL-12, IL-18 and IL-2 for NKT cell survival and activation.

Similarly to NK cells, NKT cells can bind to the constant part of secreted immunoglobulins via Fc-receptors. Fc-receptors on NK cells provide activating signals and can enhance NK cell cytotoxicity. Our microarray data showed that FcεRIγ was abundantly expressed in both NKT cell subsets (Paper II, Figure 2 and 4). The FcεRIγ seems to be utilized for signalling in innate-like lymphocytes such as 2B4 TCRα transgenic cells with γδ-like properties and also in CD8αα lymphocytes (Petersson and Ivars, 2001; Yamagata et al., 2004). The FcγRIII/CD16 binding to IgG was present at high levels in 24αβ dNKT cells. The FcγRIII ITAM signalling is central for the pathogenic cytokine production by NKT cells in a model of autoimmune joint inflammation, suggesting that NKT cells can be activated via Fc-receptors (Kim et al., 2006). Taken together, the ITAM-containing Fc-receptors FcεRIγ and FcγRIII are likely to positively stimulate NKT cell effector functions.

Two members of the SLAM family of receptors were highly expressed by NKT cells namely 2B4 (CD244) and CRACC (Slamf7) that was most abundant in Vα14 iNKT cells (Paper II, Figure 2 and 4). The 2B4 molecule exists as a short and a long splice form and depending on the size of the molecule it has been shown to be activating or inhibitory (reviewed by (Veillette, 2006). CRACC promotes activation of NK-cell mediated cytotoxicity (Bouchon et al., 2001). It seems that NKT cells utilize SLAM family members to promote their activation and the

prominent CRACC expression by Vα14 iNKT suggests a role for this molecule in induction of cytotoxicity.

In conclusion, the microarray analysis of Vα14 iNKT and 24αβ dNKT revealed that they express a broad panel of receptors that regulate their survival, activation status and effector functions. The high levels of activating NK receptors, CRACC and other anti-tumour immunity associated receptors such as CD226, implies that NKT cells are more directly involved in cytotoxicity against malignant or infected target cells than $CD4^+$ T cells.

Effector functions

NKT cells possess an array of different effector molecules, including secreted cytokines, cytotoxic effector molecules, tumour necrosis factor super family (TNFSF) members, secreted enzymes and others (Paper II, Figure 2, 3 and 4). In terms of cytokine profile, Vα14 iNKT cells transcribed very high levels of IL-4 mRNA, corroborating the well-established fact that Vα14 iNKT are rapidly and potently modulating immune responses via this Th2 cytokine. As expected, both NKT cell subsets over-expressed IFN-γ mRNA during homeostasis (Stetson et al., 2003). The chemokine CCL5 (RANTES), which is associated with homing of immune cells to inflammatory sites, was more highly transcribed by NKT cells than $CD4^+$ T cells. Interestingly, the NKT cell-expressed genes that are involved in cytotoxicity represent the different steps required for tumour cell killing, including recognition of target cell (activating NK receptors such as NKG2D), enhancement of cytotoxic capacity (CRACC, 2B4, CD226, Pglypr1) and execution of the actual killing of a target cell (FasL, granzyme A). Since NKT cell-mediated activation of NK cells is one important effector function in anti-tumour immunity, the high expression of LIGHT (Tnfsf14) may provide a novel mechanism of NKT cell stimulation of NK cell effector functions. LIGHT has been shown to bind to the receptor herpes virus entry mediator expressed on NK cells, thereby activating IFN-γ production by NK cells. IFN-γ primes $CD8^+$ CTLs to induce cytotoxic killing of tumour cells (Fan et al., 2006).

Localization and adhesion

Chemokine receptors bind specific ligands that regulate leukocyte homing. Certain chemokines are homeostatically expressed and involved in the re-circulation of immune cells through secondary lymphoid organs, whereas others are induced by microbial infections and inflammation. We found that the CCR7 chemokine receptor that is crucial for homing to T cell areas in LNs is elevated on $CD4^+$ T conventional cells (Paper II, Figure 5). Interestingly, 24αβ dNKT cells expressed higher levels of CCR7 than Vα14 iNKT cells, which is in accordance with a previous study showing that $Ly49G2^+$ and $Vα14^-NK1.1^+$ T cells have higher levels of CCR7 and increased migration towards the ligand CCL21 compared to Vα14i NKT cells (Johnston et al., 2003). Therefore, dNKT cells seem to be more prone than iNKT cells to home into T cell areas in LNs. Among the chemokine receptors over-expressed by NKT cells, several have been shown to mediate important homing functions *in vivo* (Paper II, Figure 2 and 5). During lung

infection, the chemokine receptor CCR2 binding its ligands CCL2 induced homing of NKT cells into the inflamed tissue and thus contributed to clearance of the microbes (Kawakami et al., 2001). The chemokine receptor CXCR6 is strongly expressed by NKT cells and binding of CXCR6 to its ligand CXCL16 on liver endothelium and Kupffer cells was important for NKT cell patrolling and survival within the liver (Geissmann et al., 2005). In a model of heart transplant, the CXCR6-CXCL16 interaction was crucial for NKT cell-mediated allograft tolerance (Jiang et al., 2005). Clearly, CXCR6 expression by NKT cell is an important prerequisite for homing to the liver or transplanted organs and for performing the NKT cell effector functions at these sites. The binding of the chemokine receptor CXCR3 to its ligands CXCL9 (MIG) or CXCL10 (IP-10) is known to be of importance for homing of activated memory/effector cells to inflamed tissues. *In vitro* chemotaxis assay demonstrated the migratory capacity to CXCL10 by both 24αβNKT and Vα14 iNKT cells, thereby showing that the CXCR3 expression is functional and likely to play a role in the recruitment of NKT cells into inflamed peripheral tissues (Paper II, Figure 5). Although NKT cells are not a prominent population among gut-homing lymphocytes during homeostasis, the high level of CCR9 on a portion of Vα14 iNKT cells suggests that iNKT cells may be poised to migrate to the gut (Stenstad et al., 2007; Svensson et al., 2002). *In vitro* chemotaxis assays showed that the Vα14 iNKT cells migrated towards the CCR9-ligand CCL25/TECK (Paper II, Figure 5). In addition to chemokine receptors required for homing to inflamed tissues, the secreted enzyme matrix metallo-protease 9 (MMP9) was transcribed in Vα14 iNKT cells. MMP-9 is important for cleaving of basement membrane components, thus permitting migration across blood vessels and possibly facilitating homing into inflamed tissues. Taken together, the chemokine receptor profile of NKT cells suggest that their capacity to home to peripheral tissues and sites of inflammation is enhanced compared to $CD4^+$ T cells.

Integrins are molecules that mediate adhesion between cells or between cells and extracellular matrix components. They form heterodimers composed of one α-chain and one β-chain. Integrins can assume three different conformational shapes, which control their binding capacity to their respective ligands. Knowing that integrins are central for adhesion of immune cells, we analyzed the integrin profile of NKT cells compared to $CD4^+$ T cells by microarray and flow cytometry (Paper II, Figure 6). In addition to its importance for homing, integrins can modulate T cell activation. The integrin pair LFA-1 ($\alpha_L\beta_2$) binding to the cell-adhesion molecule ICAM negatively regulated the production of IL-4 after αGalCer stimulation (Matsumoto et al., 2004). However, the major role of integrins is to mediate adhesion and interestingly, NKT cells showed enhanced expression of extracellular-matrix binding integrins such as $\alpha_1\beta_1$ (collagen), $\alpha_2\beta_1$ (collagen), $\alpha_5\beta_1$ (fibronectin, laminin), $\alpha_v\beta_3$ (fibronectin, laminin). NKT cells overexpressed α4 which forms pairs with different β-chains: $\alpha_4\beta_1$ binding VCAM-1 or $\alpha_4\beta_7$ binding MAdCAM. The latter integrin pair was highly expressed on 24αβ dNKT cells. The adhesion molecules VCAM and MAdCAM are expressed on stromal cells and endothelial cells present in the

marginal zone and the red pulp. It seems likely that by using the integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$, NKT cells can adhere to endothelium. Although the localization of NKT cells within the spleen has not been extensively studied, Ly49G2$^+$ 24αβ dNKT cells seemed to localize both to the T cell area and also to the red pulp (see Figure 1; and Sandahl and Cardell, unpublished observations). Possibly, the 24αβ dNKT cells utilize their high expression levels of integrins binding to extracellular-matrix and VCAM-1 to maintain their localization in the red pulp, in contrast to CD4$^+$ T cells that are only present in the T cell areas in the white pulp. In conclusion, the chemokine receptor pattern and integrin profile of NKT cells suggest that NKT cells can home to peripheral tissues and possibly also adhere directly to extracellular matrix and stromal cells within the tissues.

Transcriptional regulation in NKT cells

Transcriptional regulation provides the basis for inducing and maintaining the NKT cell gene expression profile. Several of the NKT cell over-expressed transcription factors are crucial for embryonic development of different anatomic structures such as retinal ganglion (Dlx2) or the heart (Hod). All of the transcription factors studied in detail: Id2, Hhex, Eomesodermin (Eomes) and T-bet mediate development of immune cell subsets or induction of immunological effector functions (Paper II, Figure 7). The mRNA levels of all the four transcription factors were up-regulated in the CD44$^+$ (stage 2) in transgenic thymocytes. Their expression was increased further in CD44$^+$NK1.1$^+$ (stage 3) thymocytes that represent mature thymic NKT cells (see Figure 6). The expression of Id2, Hhex and T-bet was similar between transgenic Vα14 iNKT cells and CD1d-αGalCer tetramer$^+$ cells from the thymus of B6 mice. The expression pattern of these four transcription factors implies that they may participate in shaping the NKT cell lineage gene profile already during thymic development.

Id2. The transcription factor Id2 (inhibitor of DNA-binding) is transcribed by NK cells, DCs and the innate-like lymphocyte CD8αα subset. Id2 negatively regulates E-proteins by preventing their DNA-binding (Yamagata et al., 2004). The effects of genetic deletion of Id2 in mice were pleiotropic and include defects in secondary lymphoid organ formation and impaired NK cell development (Yokota et al., 1999). Recently, it has been demonstrated that Id2 played a crucial role in the survival of effector/memory CD8$^+$ T cells after infection (Cannarile et al., 2006). It seems likely that Id2 could be important for the normal development of the innate-like lymphocyte subsets NKT cells and CD8αα T cells, since we find that Id2 is induced already during thymic development (Paper II, Figure 7).

Hhex. During embryonic development, the transcription factor Hhex (hematopoietically expressed homeobox, also called Prh and Prhx) regulates the formation of endothelium, the heart, liver, and thyroid gland. Due to its crucial role in organogenesis Hhex-/- mice have an embryonic lethal phenotype. Hhex has been implicated in early development of B cells, T cell development and maintenance and in T cell lymphomas (Bogue et al., 2003; George et al., 2003;

Mack et al., 2002). We found that Hhex was highly expressed by NKT cells in the thymus and spleen in transgenic and wild type mice, and also by NK cells. In fact, Hhex was strongly induced already at the stage 2 ($CD44^+NK1.1$-) of NKT cell development, suggesting that Hhex represents one important factor distinguishing the NKT cell lineage from conventional T cells. The diversity of Hhex functions during immune cell differentiation and tumour progression suggest that Hhex may also be involved in the genetic regulation of NKT cell maturation.

T-bet and Eomes. Recently, the transcription factor T-bet (Tbx 21) was shown to be crucial for the final maturation step of Vα14 iNKT cells, when typical NKT cell features such as expression of granzyme B, Perforin, IFN-γ, CXCR3 and several NK receptors are up-regulated (Matsuda et al., 2007; Matsuda et al., 2006; Townsend et al., 2004). Since T-bet was expressed in a similar pattern in transgenic Vα14 iNKT cells and 24αβ dNKT cells, it seems likely that T-bet is crucial also for the progression of dNKT cell development (Paper II, Figure 7). There is overlap in the role of the transcription factors T-bet and Eomes in terms of promoting CD122 expression, production of IFN-γ and cytolytic molecules in memory $CD8^+$ T cells and NK cells (Intlekofer et al., 2005; Pearce et al., 2003). Both of these genes belong to the T box family of transcription factors that contain an approximately 200 amino acids long DNA-binding motif. The redundancy of T-bet and Eomes is also suggested by T-bet-/- NK cells retaining the capacity to secrete IFN-γ, most likely due to the expression of Eomes in NK cells (Townsend et al., 2004). We found that Eomes was expressed in the transgenic NKT cell subsets and most highly by thymic $CD44^+NK1.1^+$ (stage 3) transgenic cells. However, Eomes was undetectable in wild-type B6 Vα14 iNKT cells from thymus or spleen (Paper II, Figure 7). Since the expression of Eomes seems to vary depending on phenotype, activation status or organ it is possible that Eomes may be expressed in wild-type iNKT cells after they receive the appropriate signals. The level of Eomes is up-regulated after activation in γδ T cells and Eomes together with T-bet controls the IFN-γ production by these innate-like lymphocytes (Chen et al., 2007).

Concluding remarks

Microarray analysis of the gene expression profile of 24αβ dNKT, Vα14 iNKT and $CD4^+$ T cells has revealed a gene signature specific for the two NKT cell subsets in relation to $CD4^+$ T cells. The genes highly transcribed in NKT cells can be grouped according to their putative functional roles. The NKT cells expressed a broad spectrum of genes, including receptors that modulation activation and localization, signalling pathway components, immunological effector molecules, transcription factors and enzymes. The gene expression profile also identified many relatively unknown genes whose roles in NKT cell biology remain to be established. The global gene expression profile of NKT cells revealed that they have common denominators with the other innate-like T lymphocytes subsets CD8αα T cells and γδ T cells, including the enhanced expression of several activating and inhibitory NK cell receptors. The fact that NKT cells can be modulated by a broad panel of these NK-related receptors adds another dimension to their capacity to respond to the same stimuli as NK cells. In order to transcribe a unique gene

signature, certain transcription factors that can induce or maintain this profile are required. The four transcription factors: Id2, Hhex, Eomes and T-bet are all central for development or induction of effector functions. The confirmation that these genes were present already during thymic maturation suggests that they are likely to be involved in the specific gene expression profile of the NKT cell populations. The gene expression profile of each of the NKT cell subsets suggested novel features that provide clues to the mechanisms underlying the different roles of these two subsets. The 24αβ dNKT cells expressed high levels of activating NK receptors, which suggest that 24αβ dNKT cells may potentiate NK cell effector functions by IFN-γ secretion and expression of LIGHT. Vα14 iNKT cells maintained a cytokine-profile including both IL-4 and IFN-γ simultaneously and in addition seemed poised for performing cytotoxic functions, which confirmed the diversity of Vα14 iNKT cell functions. In conclusion, the global microarray analysis has expanded the understanding of NKT cells and provided novel insights into NKT cell features.

Future perspective

The microarray analysis has provided a comprehensive description of genes that are associated with NKT cells and also specific to each of the NKT cell subsets. It would be of great interest to study the role of the transcription factors Id2, Hhex, Eomes and T-bet in determining the NKT cell features. Thymic development can be simulated *in vitro* by using stromal cells called OP-9δ cells that promote T cell development. The contribution of these four transcription factors to NKT cell lineage can be determined by inhibiting or enhancing their expression in the *in vitro* model of thymic development. Another important set of molecules to analyze further are the effector molecules that mediate NKT cell functions. The effector molecule LIGHT expressed by NKT cells may be central for activation of NK cells, as occurs during anti-tumour immunity. Studies of LIGHT-deficient NKT cells in tumour models may indicate if LIGHT together with IFN-γ can enhance NK cell anti-cancer immune responses. By further exploring the role of NKT cell-specific genes identified by the microarray analysis, profound new insights into NKT cell development and functions can be obtained.

BACKGROUND - PAPER III.

Salmonella species are gram-negative, facultative intracellular pathogens that infect mammals, including humans and mice, via ingestion of contaminated food or water. In mice, virulent *Salmonella enterica* serovar *typhimurium* cause a systemic disease similar to typhoid fever in humans. The invading *Salmonella typhimurium* bacteria transverse the M cells overlying the Peyer's patches (PP) or induce epithelial cells to modify their actin cytoskeleton, leading to engulfment of the whole bacteria. *Salmonella* bacteria are capable of surviving in vacuoles within phagocytic cells, by inhibiting generation of reactive oxygen species and nitric oxid (reviewed by (Hornef et al., 2002)). During the early stages of infection, the recruitment and activation of phagocytic cells in PP and mesenteric lymph nodes (MLN) are important for the outcome of the *Salmonella* infection (reviewed by (Wick, 2004)). However, for resolution of the *Salmonella* infection, the adaptive immune response needs to be activated and mice lacking $CD4^+$ T cells were shown to be unable to eradicate an attenuated *Salmonella* strain (Hess et al., 1996). The role of different leukocyte subsets has recently been delineated and recruited monocytes are crucial for production of NO and TNF-α, whereas $CD11c^+$ DCs are central for activation of $CD4^+$ T cells in PP (Rydstrom and Wick, 2007). *Salmonella* infection induces production of pro-inflammatory cytokines such as TNF-α and IFN-γ. These cytokines are crucial for the immune cell activation, resulting in the killing of *Salmonella* bacteria. The cytokine IL-12 also plays an important role through maintaining high levels of IFN-γ production (John et al., 2002). During the early stages of infection, the production of pro-inflammatory cytokines is mediated by innate immune population such as macrophages and neutrophils. However, $NK1.1^+$ cells, constituting both of NK cells and NKT cells, also produced high levels of pro-inflammatory cytokines and were strongly activated during the early phase of *Salmonella* infection in the spleen (Kirby et al., 2002). Therefore, we reasoned that oral *Salmonella* infection represented a model for exploring the progress of natural NKT cell activation and effector functions during infection with an intracellular pathogen.

NKT cell responses to Salmonella infections

The role of $NK1.1^+$ cells in production of pro-inflammatory cytokines suggested that NKT cells could enhance Th1 responses and macrophage-mediated killing of *Salmonella* bacteria. However, two previous studies had shown that the contribution of NKT cells in *Salmonella* subspecies *choleraesuis* intraperitoneal infection was mainly to produce IL-4 that dampened the IL-12 production by peritoneal macrophages. Injection of blocking IL-4 antibodies decreased the pathogen burden, implying that IL-4 production by NKT cells inhibited the Th1 immune response against *Salmonella choleraesuis* (Enomoto et al., 1997; Naiki et al., 1999). Also, liver Vα14 iNKT cell activation by *Salmonella choleraesuis* caused liver damage (Ishigami et al., 1999). The mechanism of liver injury was suggested to depend on the expression of FasL on NKT cells (Shimizu et al., 2002). Thus, it had been shown that NKT cells became activated by *Salmonella choleraesuis*, but the ensuing immune response may either be skewed towards non-

protective IL-4 production or cause liver damage through FasL expression. In contrast to the studies using *Salmonella choleraesuis*, during oral infection with *Salmonella typhimurium*, NKT cells and NK cells had been shown to the produce Th1 pro-inflammatory cytokines TNF-α and IFN-γ (Kirby et al., 2002). The natural infection route of *Salmonella* involves dissemination from the PP in the small intestine to MLN, spleen and liver. Relatively high numbers of NKT cells are found in the spleen and liver. Activation of NKT cells occurs when the *Salmonella* bacteria spread systemically within the first days of the infection. This study also described a reduction of $NK1.1^{+}TCR\alpha\beta^{+}$ cells during the course of the infection. The decline in NKT cell numbers may relate to the down-modulation of the NK1.1-marker that occurs after strong stimulation of NKT cells (Chen et al., 1997a). Taking into account the rapid activation of NKT cells and the problems associated with using the NK1.1 marker to identify activated NKT cells during infections, the role of NKT cells during *Salmonella* infection needed to be further elucidated. In order to determine the NKT cell response to *Salmonella* infection, we studied the NKT cell cytokine-production and indications of activation-status during the natural progression of *Salmonella* infection. By studying the impact of the *Salmonella* infection in CD1d-/- mice compared to CD1d+/- or wild-type mice, the contribution of NKT cells to defence against *Salmonella* was clarified. These two approaches a) identified the effect of the *Salmonella* infection on the activation and effector functions of NKT cells and b) investigated the role of NKT cells on the outcome of the lethal *Salmonella* infection.

Experimental approach – Salmonella infection model

The progression of *Salmonella typhimurium* infection and the immune cells involved in the immune response were studied in orally infected mice in an experimental model established by Prof. M J Wick. The virulent bacteria *Salmonella enteric* serovar *typhimurium* strain χ4666 causes a systemic disease that includes spreading to the MLN, spleen and liver. The mice succumb to the lethal infection after approximately 7 days. The susceptibility of different mouse strain to *Salmonella* infection is highly correlated to the Nramp1 locus on chromosome 1. The C57Bl/6 (B6) mouse strain is sensitive to *Salmonella* infections. The CD1d-deficient mice were generated in the 129 mouse strain, but were subsequently backcrossed onto the B6 mouse strain. These mice are therefore likely to have the B6 Nramp1 susceptibility allele. Although all mouse strains used in these studies are susceptible to *Salmonella* infection, in order for all the mice to be infected the bacteria dose must exceed 10^9 live *Salmonella* bacteria. It was crucial that all mice became infected in a synchronous fashion that would give similar immune response in all of the mice after the challenge. In effect, this *Salmonella* model allows analysis of the activation phase of the immune system during *Salmonella* infection, but long-term protective immunity is impossible to determine due to the rapid lethal outcome in this infection model. By using this model we could analyze the NKT cells during the early phase of the *Salmonella* infection. In order to detect the effector functions of NKT cells, namely cytokine-production, the *ex vivo* cytokine-profile was determined using flow cytometry. In addition, we analyzed the cytokine-production after *in vitro* re-stimulation to study the skewing of the cytokine-profile that we first

detected in *ex vivo* isolated immune cells. The other aspect of the project was focused on the role of NKT cells in the immune system. In order to determine the contribution of the NKT cells during *Salmonella* infection we studied the immune cell dynamics and the number of bacteria in normal mice and mice lacking NKT cells. We assumed that if NKT cells are crucial for the defence against *Salmonella*, it would be possible to detect changes in the immune cell composition in the organs or the number of bacteria isolated from each organ when comparing CD1d-/- to CD1d+/- mice that have normal NKT cell numbers.

The mechanism of NKT cell activation during *Salmonella* infection was unknown when this project was initiated. Therefore, we were interested in studying the CD1d-levels on APCs both *in vivo* and *in vitro* in order to determine whether increased CD1d-expression could mediate NKT cell activation during *Salmonella* infection.

RESULTS AND DISCUSSION - PAPER III.

Activation of NKT cells during Salmonella infection

We have shown that the NKT cell population was dramatically affected during *Salmonella* infection. Already at day 3 post-infection (p.i.) the NKT cells had an increased cell-size, which suggests that they were in an activated state. The NKT cells had also up-regulated the early activation marker CD69. The NK1.1 marker, which often is used to define NKT cells was significantly down-regulated at day 3 p.i. in the liver and was almost completely absent on day 5 p.i. in both the liver and spleen. Thus, due to the down-modulation of NK1.1, the definition of CD1d-restricted NKT cells as $NK1.1^{+}TCR\beta^{+}$ was no longer valid and only iNKT cells could be detected by using CD1d-αGalCer tetramers. Previously, the "disappearance" of $NK1.1^{+}TCR\beta^{+}$ cells after stimulation *in vivo* had been interpreted as activation-induced cell death. Our studies showed that the numbers of CD1d-αGalCer $tetramer^{+}$ cells remained similar during *Salmonella* infection. Most iNKT cells did not undergo activation induced cell death after stimulation, instead the NKT cells transiently down-regulated the NK1.1-marker (Paper III, Figure 1; Wilson et al., 2003). The reason for down-modulation of NK1.1 by *Salmonella* is not fully understood but may prevent NKT cells from obtaining stimulation via the NK1.1-molecule when they are already in a fully activated state (Wilson et al., 2003). During *Salmonella* infection the TCR levels as detected by the CD1d-αGalCer tetramer were not reduced, suggesting that TCR expression is maintained during NKT cell activation by a naturally occurring pathogen (Paper III, Figure 1). This result is in contrast to stimulation with the artificial ligand αGalCer that induced down-modulation both of NK1.1 and TCRβ on iNKT cells (Crowe et al., 2003; Wilson et al., 2003). However, the αGalCer-stimulation of iNKT cells is extremely strong and therefore may be different from physiological activation signals that are encountered during an infection.

Studies on the activation mechanism of NKT cells during *Salmonella* infection turned out to be important for understanding the interplay between APCs and NKT cells during the early phase of an infection. Antibody-blocking of IL-12 and CD1d reduced the capacity of liver iNKT cells to secrete IFN-γ during *Salmonella* infection, showing that the NKT cell activation was dependent on the cytokine IL-12 and recognition of CD1d+endogenous antigen provided by the APCs (Brigl et al., 2003). The activation of NKT cells during *Salmonella* infection by CD1d+endogenous ligands and IL-12, circumvents the requirement for cognate recognition of bacterial antigens by the NKT cells. Part of the reason for the rapid responses of NKT cells may be that they can be become activated by endogenous glycolipids and hence the NKT cells do not need to directly recognize the bacterial-derived molecules (Figure 7B and reviewed by (Tupin et al., 2007)). The iNKT cell activation is instead relying on APCs recognizing bacterial products, probably via Toll-like receptors, and then starting to secrete IL-12. However, infections with microbes containing glycolipids that can be presented directly on CD1d such as *Sphingomonas* glycosylceramides, represents a distinct mechanism of NKT cell activation different from the indirect activation occurring during *Salmonella*-infections (Mattner et al., 2005).

CD1d levels of APCs after exposure to Salmonella

The antigen-presentation of endogenous glycolipids on CD1d by APCs is crucial for the activation of iNKT cells during *Salmonella* infection (Brigl et al., 2003; Mattner et al., 2005). Therefore, an increase of the CD1d expression on APCs would result in a relative higher TCR-CD1d avidity and thus more efficient activation of NKT cells. We wished to establish whether CD1d was up-regulated as a consequence of *Salmonella* infection. In this paper it is demonstrated that the levels of CD1d were increased two-three fold on bone-marrow derived dendritic cells (BMDCs) exposed to live *Salmonella* bacteria *in vitro*. However the CD1d-levels on $CD11c^+$ DCs *in vivo* remained similar during the progress of *Salmonella* infection (Paper III, Figure 2). The BMDCs *in vitro* were most likely directly exposed to and/or infected by the bacteria, whereas in *vivo* only a minor population of the DCs are in contact with the *Salmonella* bacteria (Sundquist and Wick, 2005). Since the number of DCs that have directly encountered *Salmonella* bacteria is very low, it is possible that an up-regulation of CD1d occurs on the few APCs that are in direct contact with the bacteria. The CD1d-levels alone are unlikely to regulate the activation status of NKT cells during *Salmonella* infection, although a role of increased CD1d-levels on the minority of DCs exposed directly to the bacteria cannot be excluded. Several articles published after this study was completed have shown that CD1d-levels are up-regulated during infection with *Mycobacterium* or *Listeria monocytogenes* and/or in the presence of pro-inflammatory cytokines (Raghuraman et al., 2006; Skold et al., 2005). Our data show that general CD1d up-regulation on DCs is not a feature common to all infection models *in vivo*. Therefore, further studies are warranted to elucidate the control of CD1d-expression in the presence of different pathogenic species and pro-inflammatory cytokines.

The cytokine-profile of iNKT cells during Salmonella infection is skewed towards IFN-γ

Both NKT cells and NK cells in the spleen are known to rapidly start producing IFN-γ during oral infection with *Salmonella*. But due to the down-modulation of NK1.1 during the course of the infection, the contribution of NKT cells alone was unknown (Kirby et al., 2002). By detecting the iNKT cells with the CD1d-αGalCer tetramer, it was possible to identify the iNKT cells specifically. We found that 10% of the IFN-γ-producing cells were iNKT cells on day 3 p.i. Together the iNKT and NK cells corresponded to more than half of the total IFN-γ producing cells detected *ex vivo* on day 3 of the *Salmonella* infection (Paper III, Figure 3). Our results showed that NKT cells in the spleen and liver promoted IFN-γ dominated Th1-immunity during *Salmonella typhimurium* infection via the natural route. The strongly IFN-γ skewed profile of iNKT cells in our study is in contrast to the IL-4 production by NKT cells observed after intraperitoneal challenge with *Salmonella choleraesuis* (Enomoto et al., 1997; Naiki et al., 1999). The differences in cytokine-profile may be related to the bacterial strain or the site of pathogen entry and factors such as the nature of the APCs activating the NKT cells.

During *Salmonella* infection, the cytokine-production profile after re-stimulation *in vitro* of iNKT cells was dominated by IFN-γ production and the IL-4 secretion was completely

abolished. This Th1-skewing after exposure to *Salmonella* infection is in contrast to the Th0, meaning combined IFN-γ and IL-4 cytokine profile, that characterizes iNKT cells during homeostasis (Paper III, Figure 6). *Salmonella* infection is known to induce high levels of the Th1-tilting cytokine IL-12 secreted from APCs. The IFN-γ dominated cytokine profile of iNKT cells after infection is therefore likely to reflect that iNKT cells are activated in the presence of IL-12 and self-antigen presented on CD1d. This mode of activation seems to skew the iNKT cell cytokine profile towards Th1 (Brigl et al., 2003). In contrast, to infection with *Salmonella*, the iNKT cell cytokine release immediately after αGalCer stimulation is claimed to be impervious to skewing towards Th1 or Th2, unlike activated conventional $CD4^+$ T cells that are either Th1 or Th2 tilted (Matsuda et al., 2003). However, our data showed that activation of iNKT cells during *Salmonella* infection skewed the cytokine-profile of iNKT cells strongly towards IFN-γ. Therefore, iNKT cells are responsive to the activating signals and the local milieu where the priming occurs and most likely, the APCs play a central role in directing iNKT cells towards Th1 or Th2 immunity.

Lethal Salmonella infection is not controlled by NKT cells

NKT cells had previously been shown to be activated during challenge with *Salmonella typhimurium*, although the contribution of NKT cells to the defence against the infection had not been studied (Kirby et al., 2002). Since the NKT cells produced IFN-γ early during the infection, they may help controlling the infection and decrease the bacterial load by promoting immune cell homing to the infected organs. Therefore, these two parameters were analyzed in CD1d-/- compared to CD1d+/- mice. The bacterial load reflects the bacterial replication as well as the capacity of the immune cells, mostly macrophages and neutrophils, to kill ingested bacteria. However, there was no difference in the bacterial load measured as colony forming units/organs in CD1d-/- compared to CD1d+/- mice. (Paper III, Figure 4). The immune cell recruitment to the infected organs in CD1-/- and CD1d+/- mice, suggested that the most apparent effect of *Salmonella* infection was increased numbers of phagocytic cells. The macrophages and neutrophils, are important for controlling the *Salmonella* infection by being able to kill ingested bacteria (reviewed by (Wick, 2004)). In the spleen and liver, the macrophages and neutrophils expand more than two-fold. The increase of macrophages was especially prominent in the liver on day 5 of infection. However, the recruitment of macrophage and neutrophil populations occurred independently of NKT cells during virulent *Salmonella* infection, since the number of macrophages and neutrophils were similar in CD1d-/- and CD1+/- mice (Paper III, Figure 5). Thus, the progression of the lethal *Salmonella* infection was not prevented by NKT cells.

Concluding remarks

In this model of *Salmonella typhimurium* infection via the oral route, it is reasonable to assume that the NKT cell response closely mimics a naturally occurring infection. All NKT cells became very strongly activated by the *Salmonella* infection, as was obvious from their up-regulation of the early activation marker CD69 and down-modulation of the NK1.1 marker. The disappearance

of NK1.1 was previously interpreted as NKT cells undergoing apoptosis after stimulation. However, our data show that the iNKT cells were still present but that they down-modulate their expression of NK1.1 and therefore only the iNKT cells are detectable by staining with the CD1d-αGalCer tetramers.

Oral infection with *Salmonella* activated NKT cells *in vivo* and strongly skewed their cytokine profile towards IFN-γ production. The capacity of NKT cells from uninfected mice to produce both IFN-γ and IL-4 simultaneously was completely abolished by the ongoing *Salmonella* infection, since no IL-4 could be detected even upon re-stimulation *in vitro*. The IFN-γ produced by NKT cells is particularly prominent during the first days of infection. Clearly, NKT cells contribute to the pro-inflammatory Th1 response against *Salmonella*. Although, NKT cells enhance the immune response against *Salmonella,* the NKT cells did not seem to influence the severity of the infection. The *Salmonella* infection developed similarly in mice lacking NKT compared to mice with normal NKT cell numbers. Although, NKT cells did not affect the outcome of this lethal *Salmonella* infection, they became strongly activated and produced large amounts of IFN-γ in response to *Salmonella* infection via the oral route.

Future perspectives

In order to expand the knowledge of the NKT cell response during *Salmonella* infection it would be useful to infect the mice with a less virulent *Salmonella* strain. It would then be possible to determine whether NKT cells may be able to influence the immune response if the immune system can combat the bacteria efficiently. It would also be interesting to study the effector potential of NKT cells further and determine if they produce other cytokines or effector molecules, in addition to IFN-γ. When the infection is resolved, the degree of skewing of the cytokine profile of NKT cells could be explored in order to understand whether this Th1-skewing of the cytokine profile only occurs during the acute infection. In conclusion, by using a less virulent *Salmonella* strain, the role of NKT cells and their effector functions could be further studied.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunförsvarets främsta funktion är att skydda vår kropp mot hot från inkräktande organismer. Nyckeln till bekämpning av farliga infektioner är att immunceller effektivt kan särskilja främmande ämnen från kroppens egna beståndsdelar. Immunförsvaret består av flera delar. Mest urprungligt är det medfödda immunförsvaret som utgörs av celler och proteiner som kan identifiera de vanligaste främmande ämnena. Det medfödda immunförsvaret är väldigt snabbt och aktiveras inom minuter eller timmar för att skydda vår kropp. Dessutom har det medfödda immunförsvaret ytterligare en viktig funktion - att förmedla aktiverande signaler till den icke-medfödda grenen av immunförsvaret. Den del av immunförsvaret som inte är medfödd utan ett resultat av de infektioner eller vaccineringar som vår kropp tidigare utsatts för kallas det adaptiva eller specifika immunförsvaret. Det adaptiva immunförsvaret utgörs av vita blodkroppar också kända som lymfocyter. Det finns två olika sorter av lymfocyter: B lymfocyter som tillverkar antikroppar som kan markera eller blockera farliga organismer och T lymfocyter som reglerar immunsvar via flertalet olika signaler. Det adaptiva immunförsvaret har en obegränsade förmåga att identifiera främmande ämnen, vilket försvårar för till exempel virus att undkomma upptäckt. Däremot innebär den oerhörda spännvidden av olika ämnen som känns igen av det adaptiva immunförsvaret att vi även riskerar att bli angripna av våra egna lymfocyter. Exempel på sjukdomar då lymfocyterna går till attack mot kroppens egna celler är Typ 1 diabetes (ungdomsdiabetes) och ledgångsreumatism. Därför är det en ständig balans mellan faktorer som aktiverar immunförsvaret och de faktorer som bidrar till att dämpa immunförsvarets attacker.

Det finns även en tredje gren av immunförsvaret, som utgörs av medfödda lymfocyter och är ett mellanting mellan det medfödda och det adaptiva immunförsvaren. Tidsmässigt blir medfödda lymfocyter aktiverade mycket snabbare än vanliga lymfocyter och kan utföra sina uppgifter inom ett dygn. Aktivering av medfödda lymfocyter underlättas av att de ofta kommer i direkt kontakt med invaderande organismer och att de har en låg tröskel för att inleda ett immunsvar mot inkräktare. I denna avhandling undersöker vi två olika typer av medfödda lymfocyter, kallade marginal-zons (MZ) B lymfocyter och naturliga mördar T (NKT) lymfocyter.

Tidigare studier har visat att MZ B lymfocyter är väldigt aktiva och kan lätt starta ett immunsvar. Därför ville vi studera MZ B lymfocyter i möss som drabbas av typ 1 diabetes, för att förstå om MZ B lymfocyter kan spela in i utvecklingen av typ 1 diabetes. Vi fann ett större antal MZ B lymfocyter i mjälten hos möss från den mus-stammen som får diabetes jämfört med möss som tillhör en frisk mus-stam. Denna skillnad i antal uppstod redan vid tidig ålder, vilket antyder att MZ B lymfocyterna kan vara viktiga för utvecklingen av diabetes. Vi fann att ökningen av antalet MZ B lymfocyter regleras av gen-områden som är kopplade till typ 1 diabetes. Våra resultat stödjer således antagandet att MZ B lymfocyters antal har ett samband med typ 1 diabetes.

I en annan studie har vi undersökt genprofilen hos två olika undergrupper av NKT lymfocyter. Egenskaper hos de två undergrupper av NKT lymfocyter skiljer sig åt. Men det är inte tidigare känt exakt vilka gener som bidrar till deras olika egenskaper. Vi har kartlagt skillnader i bland annat aktiverande receptorer, migreringsmönster och funktioner i immunförsvaret mellan de två undergrupperna av NKT lymfocyter. En viktig grupp gener som särskiljer genprofilen mellan olika cell-typer kallas transkriptionsfaktorer. Transkriptions-faktorer styr vilka proteiner som finns i en cell. Vi upptäckte att vissa centrala transkriptionsfaktorer uttrycktes redan under NKT lymfocyternas utveckling. Vi anser att dessa transkriptionsfaktorer är viktiga för att styra genprofilen hos NKT celler. Sammantaget har vår analys av genprofilen hos NKT lymfocyterna utökat förståelsen av deras egenskaper.

Slutligen har vi undersökt NKT lymfocyters roll vid infektion med den skadliga bakterien *Salmonella typhimurium*, som ger tyfus-liknande sjukdom i möss. NKT lymfocyterna blev starkt aktiverade under *Salmonella* infektionen. Efter aktivering började de snabbt producera en viss molekyl som är viktig för immunförsvarets bekämpning av *Salmonella*. Detta visar att NKT lymfocyter bekämpar *Salmonella*-infektionen, speciellt i ett tidigt skede. Däremot kunde NKT lymfocyterna inte skydda mössen mot *Salmonella* infektionen. Men avsaknad av skydd beror troligen på att *Salmonella* infektionen har dödlig utgång på grund av den höga dosen farliga bakterier. Därför är mycket svårt att mäta ett skydd mot en så allvarlig infektion. Men oavsett utfallet av immunförsvarets kamp mot *Salmonella* så har vi visat att NKT lymfocyter bidrar till tidigt starta bekämpandet av *Salmonella*.

Medfödda lymfocyter blir snabbt och starkt aktiverade, vilket gör dem lämpade för att skydda kroppen mot infektioner. Studier i denna avhandling har utforskat medfödda lymfocyters egenskaper. Vi har undersökt MZ B lymfocyters koppling till typ 1 diabetes och funnit att de troligen bidrar till sjukdomen. Vi har också beskrivit genprofilen hos två undergrupper av NKT lymfocyter. Dessa två ovannämnda studier har ökat våra kunskaper om medfödda lymfocyters egenskaper. För att också förstå medfödda lymfocyters funktioner har vi följt NKT lymfocyters svar under en livshotande *Salmonella*-infektion. Vi har funnit att NKT lymfocyter bidrar till immunförsvarets kamp, speciellt under den tidiga fasen av en infektion.

Under de senaste åren har forskning riktats mot medfödda lymfocyters roll i immunförsvaret. Men det finns ännu många obesvarade frågor. Medfödda lymfocyter har strategiskt viktiga egenskaper som är hämtade både från det medfödda och det adaptiva immunförsvaret. De kan därför ofta bidra kraftfullt till att forma ett immunsvar, till exempel under en infektion eller vid typ 1 diabetes. Våra kunskaper om medfödda lymfocyter kan hjälpa oss att förstå olika sjukdoms-förlopp som idag saknar förklaring.

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