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Diabetes Pathogenesis
- Role of Natural Killer Cells, T Cells and a Novel Neuropeptide

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Stockholm 2010

Cover picture shows a confocal micrograph of a section of the pancreas from an 8.5 week-old NOD mouse, stained with an antibody against NKp46 (green) and antiserum against insulin (red). NKp46 positive NK cells are seen inside and right next to the insulin producing beta cells.

Published by Karolinska Institutet. Printed by Larserics Digital Print

ISBN 978-91-7409-980-5

ABSTRACT

Diabetes is a major health problem affecting nearly 5% of the population world wide. It is a heterogeneous group of diseases with the autoimmune type 1 diabetes (T1D) and the metabolic type 2 diabetes (T2D) being the major types. Here we have investigated immune mechanisms of the pathogenesis of T1D and properties of a novel anorexogenic peptide in the pancreatic islets and in T2D.

One drawback with an efficient immune system is the risk of developing autoimmune diseases, where immune reactions are mounted against self. T1D is an example of an autoimmune disease where the insulin producing beta cells in the pancreas are destroyed by the body's own immune system. As for most autoimmune diseases, the pathophysiology of T1D is complex, involving several susceptibility genes and environmental triggering factors. Autoreactive T cells are key players in the pathogenesis of T1D, but many other immune cells are likely also involved. Studies on T1D are complicated by the difficulty to access the target-organ of the autoimmune attack: the pancreas. The non-obese diabetic (NOD) mouse spontaneously develops an autoimmune disease that well resembles the human T1D. Much of our knowledge on T1D is therefore derived from studies in the NOD mouse.

We have investigated the involvement of natural killer (NK) cells in T1D in the NOD mouse, with a particular focus on the pancreas infiltrating NK cells (**paper I**). The NK cells were found to infiltrate the pancreas of NOD mice early during disease progression. They have distinct phenotype and function consistent with activation but also display signs of exhaustion, suggesting that NK cells may play a role in the pancreas during T1D pathogenesis. To test this hypothesis we depleted NK cells from the NOD mouse with an antibody to the IL-2/15Rβ and found that the mice were protected from T1D (**paper II**). This supports, but does not fully prove, the notion that NK cells are pathogenic in T1D, since a minor subset of T cells also was affected by the treatment. We further investigated the effect on T1D by the signalling molecule DAP12, which associates to activating receptors on NK cells, myeloid cells and some T and B cells (**paper III**). DAP12 deficiency led to accelerated T1D in the simplified BDC2.5/B6^{g7} animal model for T1D, possibly as a result of impaired suppression by regulatory T cells (Treg) of the T cell priming in the pancreatic lymph node (PLN).

The other major type of diabetes is T2D. It is a metabolic disease where impaired beta cell secretion of insulin is the main pathogenic event together with a decreased sensitivity to insulin in liver and non-hepatic cells. Many mediators are shared between the central nervous system (CNS) and the endocrine pancreas. Nucleobindin-2 is a protein with wide distribution in the CNS, which was recently shown to have anorexogenic properties. In **paper IV** we for the first time demonstrate nucleobindin-2 expression in human pancreatic islets, opening up for a possible involvement of this protein in glucose homeostasis.

In conclusion, we have identified new immunologic mechanisms of importance in the pathogenesis of T1D, particularly a novel subset of pancreatic NK cells and DAP12 associated Treg control of autoreactive T cell priming in the PLN. We have also demonstrated nucleobindin-2 in human pancreatic islets.

LIST OF PUBLICATIONS

I. **Brauner H**, Elemans M, Lemos S, Broberger C, Holmberg D, Flodström-Tullberg M, Kärre K, Höglund P. **Distinct phenotype and function of NK cells in the pancreas of nonobese diabetic mice.** *J Immunol. 2010 Mar 1;184(5):2272-80.*

II. **Brauner H**, Johansson S, Hall HT, Flodström-Tullberg M, Kärre K, Höglund P. **Depletion of IL-2/15Rβ positive cells close to disease onset protects from diabetes in non obese diabetic mice: role of natural killer cells and a subset of T cells.** *Manuscript*

III. Hall HT*, Sjölin H*, **Brauner H**, Tomasello E, Dalod M, Vivier E, Höglund P. **Increased diabetes development and decreased function of CD4+CD25+ Treg in the absence of a functional DAP12 adaptor protein.** *Eur J Immunol. 2008 Nov;38(11):3191-9.*
**equal contribution*

IV. Foo KS*, **Brauner H***, Östenson CG, Broberger C. **Nucleobindin-2/nesfatin in the endocrine pancreas: distribution and relationship to glycaemic state.** *J Endocrinol. 2010 Mar;204(3):255-63.*
**equal contribution*

CONTENTS

LIST OF ABBREVIATIONS

ADCC	antibody dependent cell-mediated cytotoxicity
AgRP	agouti-related protein
APC	antigen presenting cell
BrdU	bromodeoxyuridine
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CNS	central nervous system
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
GAD	glutamate decarboxylase
GK	Goto-Kakizaki
GLP-1	glucagon-like peptide-1
GWAS	genome wide association study
HGP	hepatic glucose production
Hsp60	heat shock protein 60
IA-2	insulinoma-associated protein 2
IGRP	islet-specific glucose-6-phosphatase catalytic subunit–related protein
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
KIR	killer immunoglobulin receptor
KLRG1	killer cell lectin-like receptor G1
MHC	major histocompatibility complex
NK	natural killer
NOD	non-obese diabetic
NPY	neuropeptide Y
PLN	pancreatic lymph node
POMC	pro-opiomelanocortin
MCMV	mouse cytomegalovirus
T1D	type 1 diabetes
T2D	type 2 diabetes
TCR	T cell receptor
TLR	toll like receptor
TRAIL	tumour necrosis factor related apoptosis inducing ligand
Treg	regulatory T cell
TREM	triggering receptors expressed on myeloid cells

1 INTRODUCTION

Diabetes is a heterogeneous group of diseases commonly characterised by hyperglycaemia. It is a major health problem globally, affecting nearly 5% of the population and therefore a highly prioritized field of research. The major types of diabetes are the autoimmune type 1 diabetes (T1D) and the metabolic type 2 diabetes (T2D).

The immune system protects from infections and tumours and is instrumental in maintaining health. Immunity can also cause disease by attacking cells from the own body (autoimmune diseases) or via exogenous antigens not normally associated with disease (allergy). In healthy individuals unwanted immune reactions are prevented by central and peripheral tolerance mechanisms.

T1D is an autoimmune disease resulting from immune destruction of the insulin producing beta cells in the pancreas. Since the beta cells do not regenerate, the patients require life long treatment with insulin. In the T1D pathogenesis T lymphocytes specific to beta cell antigens are the main immunological mediators, but many other immune cells, such as natural killer (NK) cells, are probably also involved [1]. A better understanding of the complex pathogenesis of T1D is an important scientific challenge, which may lead to the development of new treatments.

The pancreas and its draining lymph nodes are important sites for the development of T1D [2, 3]. Emerging evidence suggests that tissue specific factors largely influence the local immune response, stressing the importance of investigating immune processes at these sites [4]. Studies on the pathogenesis of T1D in humans are complicated by the inaccessibility of the autoimmune target-organ and the long silent phase of immune destruction usually preceding the diagnosis. Much of our knowledge of the pathogenesis on T1D therefore stems from studies in animal models of the disease, such as the non-obese diabetic (NOD) mouse, which spontaneously develops an autoimmune disease resembling T1D [1]. Using the NOD mouse and related models we have investigated several aspects of the immune pathogenesis of T1D. A detailed analysis of the pancreas infiltrating NK cells (**paper I**) has been performed and in **paper II** a new immune preventive treatment of T1D is described. In **paper III** molecular events affecting the activation of autoreactive T cells in the pancreatic lymph nodes have been studied.

T2D shares many of the clinical features of T1D, with the primary defect being in the pancreas, although with different pathogenesis. T2D is a metabolic disease, where decreased insulin secretion from beta cells combined with impaired insulin sensitivity leads to hyperglycaemia [5]. However, recent evidence suggests an immune component also in the pathogenesis of T2D [6]. A major risk factor for the development of T2D is obesity, since it decreases insulin sensitivity. The hypothalamus, a region in the central nervous system (CNS), is involved in regulation of feeding [7]. It is well known that many proteins and peptides are shared between CNS and endocrine organs such as the pancreas. The protein nucleobindin-2 has recently been shown to have anorexogenic properties in the CNS [8]. In the present studies the distribution in the pancreas of this protein has been investigated as well as its relation to glycaemic state (**paper IV**).

2 DIABETES

2.1 GLUCOSE HOMEOSTASIS

Since the availability of glucose is crucial for all cellular activities, the glucose homeostasis is tightly regulated by several metabolic hormones, such as insulin. The endocrine pancreas is composed of several highly specialised cell types producing some of these hormones. Beta cells represent approximately 70% of the endocrine islet cells and produce insulin. Glucose is an important stimulator of insulin release. Insulin secretion could also, under certain conditions, be stimulated by other nutrients such as amino acids and fatty acids. The pancreatic alpha cells produce glucagon that is stimulated by hypoglycaemia and counteracts the effect of insulin. The pancreatic delta cells produce somatostatin that inhibits the secretion of both insulin and glucagon. Other hormones of importance for glucose regulation are catecholamines (adrenaline and noradrenaline), cortisol and growth hormone, which are all triggered by hypoglycaemia. Catecholamines increase blood glucose by stimulating hepatic glucose production while cortisol and growth hormone rescue the blood glucose levels mainly by decreasing the insulin sensitivity in muscles and adipose tissue. Signals from the nervous system also regulate insulin release, where inhibition is conferred by sympathetic pathways and stimulation by parasympathetic pathways. In addition to the classical neurotransmitters, noradrenaline and acetylcholine, several neuropeptides have been shown to regulate insulin secretion, e.g. galanine, cholecystokinin (CCK) and pro-opiomelanocortin (POMC) [9-11]. In **paper IV** we explored the novel anorexogenic neuropeptide nucleobindin-2 in the pancreas. This protein will therefore be introduced in greater detail in section 6.1.

2.2 DIABETES

In diabetes there is an absolute or relative lack of insulin, due to impaired or abolished insulin secretion and often also decreased sensitivity to insulin. This can lead to insufficient glucose uptake of insulin sensitive cells, augmented HGP and consequently hyperglycaemia [5]. The elevated plasma levels of glucose in combination with other metabolic disturbances such as dyslipidemia can cause late complications, e.g. retinopathy, neuropathy, nephropathy and cardiovascular diseases. Both major types of diabetes, T1D and T2D, have become more common in Sweden over the last decades [12]. A dramatic increase in the incidence of T2D is also observed particularly in low and middle income countries [13].

2.3 TYPE 1 DIABETES

Sweden has one of the world's highest incidences of T1D with around 30 new cases per 100 000 inhabitants/year. The typical age of onset of T1D is in childhood or early puberty, but the disease can develop at any age. The symptoms of untreated T1D patients include hyperglycaemia, polyuria, polydipsia, fatigue, weight loss and in severe conditions also ketoacidosis and eventually coma. The pathophysiology of T1D depends on a combination of predisposing genes and polymorphisms and largely unknown environmental factors. Contributing susceptibility genes include the human MHC class II haplotypes DR3-DQ2 and DR4-DQ8 and polymorphisms in genes encoding insulin, IL-2 and IL-2Rα and negative regulators or T cell function such as PTPN22, CTLA-4 and PTPN2 [14, 15]. Since the concordance rate for T1D in genetically identical twins is less than 50%, triggering factors such as viral infections are believed to contribute to the pathogenesis [16]. The pathogenic mechanisms include

autoimmune destruction of the insulin producing beta cells, causing overt T1D when 80-90% of the beta cells are destroyed, as sufficient insulin levels can no longer be maintained. Since beta cells do not regenerate, patients with T1D require life-long insulin treatment.

In **paper I, II and III** we have investigated the T1D pathogenesis using NOD mice, B6 mice congenic for the NOD mouse MHC class II H-2^{g7} ($B6^{g7}$) and $CD4^+$ T cell receptor (TCR) transgenic mice bearing a diabetogenic TCR on the $B6^{g7}$ or NOD background (BDC2.5/$B6^{g7}$ and BDC2.5/NOD). These animal models are described below.

2.3.1 Animal models of T1D used in the present studies

2.3.1.1 The non-obese diabetic mouse

The non-obese diabetic (NOD) mouse was originally found during selective breeding of cataract-prone mice from the outbred JcI:ICR mouse line. A subgroup of those spontaneously developed diabetes and a separate inbreeding of these established the NOD strain [17].

In NOD mice, T cells are the most prominent contributors to T1D [1]. Pancreas antigens are presented in the pancreatic lymph nodes (PLN) at around 2-3 weeks of age, leading to the priming of autoreactive T cells and initiation of insulitis at 3-4 weeks of age (a process commonly referred to as "checkpoint 1"). Over the next months the insulitis progressively increases but remains respectful to the beta cells, i.e. non-destructive. At about 15 weeks of age the insulitis turns aggressive and rapidly destroys the beta cells ("checkpoint 2") and clinical diabetes occurs [18]. In addition to T cells, several other immune defects have been described in the NOD mouse, including impaired functions of DCs, B cells, NK cells and NKT cells [1]

The disease in NOD mice resembles human T1D in many ways, making this one of the most used animal models of T1D. Both display hyperglycaemia due to immune destruction of beta cells, can be treated with insulin and have circulating autoantibodies [1]. Likewise, they have increased co-morbidity with other autoimmune manifestations such as thyroiditis [19]. One important difference is that T1D affects slightly more males in humans but predominantly affects female NOD mice. From an evolutionary perspective it is also relevant to point out that NOD mice present with diabetes from approximately 15 weeks of age, i.e. well after the age when mice are able to reproduce. In humans, on the contrary, most T1D cases are diagnosed in childhood or puberty [1].

2.3.1.2 The BDC2.5 TCR transgenic mouse

The BDC2.5/$B6^{g7}$ mouse shares many of the general pathogenic features with the NOD mouse but is a simplified model of T1D [18]. These mice have a transgenic expression of BDC2.5 $CD4^+$ T cells which represent a diabetogenic T cell clone shown to be both self-reactive and involved in causing disease [20]. The great majority of T cells in this mouse thus express this pathogenic T cell receptor. BDC2.5 TCR transgenic mice generated on the B6 mouse background bearing the NOD mouse MHC class II (H-2^{g7}) (BDC2.5/$B6^{g7}$) rapidly develop T1D [21]. At three weeks of age insulitis abruptly develops, followed by a quick influx of lymphocytes into the pancreas and thereafter diabetes usually develops within weeks [22]. One benefit of the BDC2.5/$B6^{g7}$ model is that the rapid and more synchronous development of T1D allows for studies on disease modulating treatments [21]. It has been speculated that the rapid precipitation of disease

in the BDC2.5/B6^{g7} mouse is partly due to less efficient regulatory mechanisms, such as Tregs, of the B6 background compared to the NOD mouse [23].

2.4 TYPE 2 DIABETES

About 85% of the diabetic patients in Sweden have T2D. In contrast to T1D, T2D normally develops after the age of 40 years, but can occur at any age. Patients with T2D often demonstrate mild symptoms, or are even diagnosed without any obvious symptoms at all.

Genetic susceptibility combined with lifestyle factors such as obesity, physical inactivity, stress and tobacco use contribute to the development of disease. The pathophysiology of T2D is complex and involves several organs. Many defects have been proposed to underlie the development of hyperglycaemia in T2D, including dysregulation of pancreatic hormone release (diminished insulin and exaggerated glucagon secretion), impaired insulin sensitivity (insulin resistance) in liver and extra-hepatic tissues, decreased secretion of glucagon-like peptide-1 (GLP-1) from the gut and neurotransmitter dysfunction [5] (**figure 1**). Recent studies also indicate the involvement of the immune system, including pancreatic islet inflammation [6, 24, 25]. A variety of beta cell properties are altered in patients and experimental models of T2D including defects in the stimulus-secretion coupling for glucose and in the machinery regulating insulin exocytosis [26]. Strikingly, in recent genome wide association studies (GWAS) most of the associated polymorphisms are related to insulin secretion rather than insulin sensitivity or obesity [27]. This highlights the need to focus efforts at understanding local processes within the beta cells.

The importance of physical exercise and weight loss should not be underestimated, since it leads to increased peripheral sensitivity to insulin. It is therefore recommended to all patients. For patients with mild T2D such lifestyle changes can be sufficient [28]. Since T2D is a progressive disease, most patients will eventually require treatment with oral anti diabetic drugs or insulin.

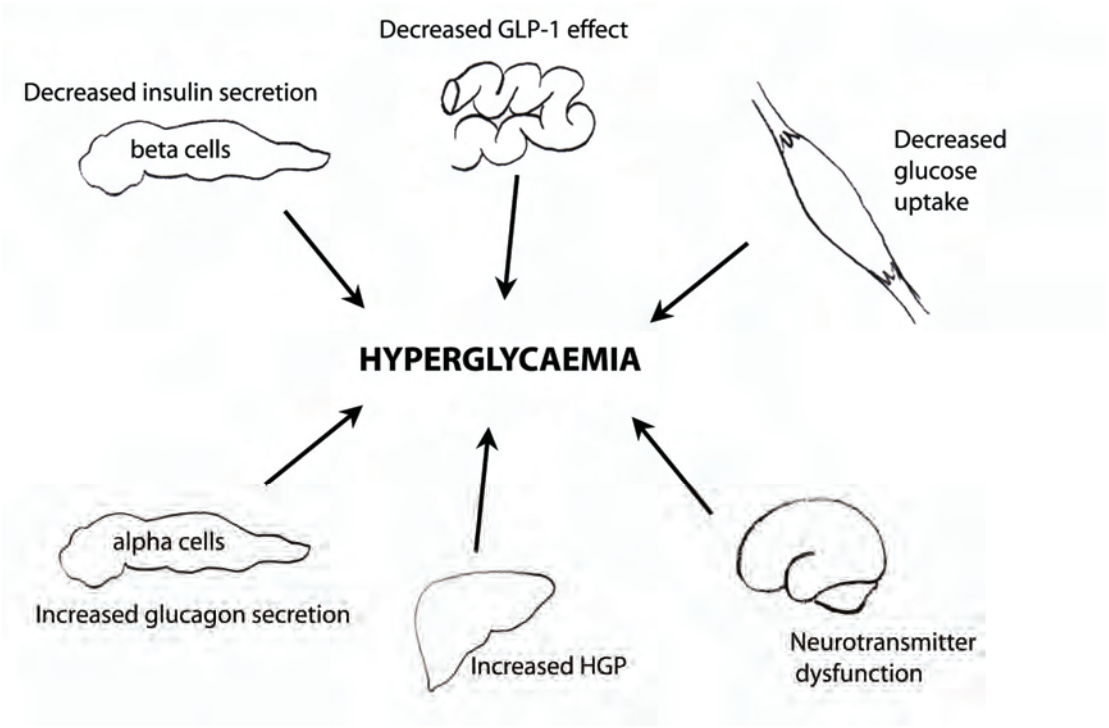

Figure 1. *Illustration of the defects which have been proposed to underlie the development of hyperglycaemia in T2D: dysregulation of pancreatic hormone release (diminished insulin and exaggerated glucagon secretion), impaired insulin sensitivity (insulin resistance) in liver and extra-hepatic tissues, decreased secretion of glucagon-like peptide-1 (GLP-1) from the gut and neurotransmitter dysfunction.*

2.4.1 Animal models of T2D used in the present studies

2.4.1.1 The Goto-Kakizaki rat

The Goto-Kakizaki (GK) rat is a non-obese model for T2D which develops hyperglycaemia already at 1-2 weeks of age [29]. The GK rat is derived through selective inbreeding of mildly hyperglycaemic Wistar rats and normal Wistar rats can therefore be used as control animals. The hyperglycaemia of the GK rats depends mainly on impaired insulin secretion [30]. Some colonies of GK rats also have alterations in the islet morphology, size and metabolism, which could influence insulin secretion. In the present studies GK rats from the Stockholm colony have been used (**paper IV**). The size of the pancreatic islets in this colony is similar in the GK and Wistar rats [30], facilitating the interpretations of experiments investigating beta cell function.

3 THE IMMUNE SYSTEM

The immune system can be divided into the more rapid, innate, response and the more specific, adaptive, response. The innate immune responses include granulocytes, dendritic cells (DC), monocytes, macrophages and natural killer (NK) cells. These cells recognise pathogens fast, form the first line of defence and activate and influence the adaptive immune responses. The adaptive immune responses are made up of T and B lymphocytes, whose receptors are generated through gene rearrangement yielding a great variation of receptor specificity. Upon activation, the adaptive cells with useful receptors undergo clonal expansion over some days, mounting a strong specific immune response. Since the adaptive immunity can develop memory, a more rapid response occurs in future exposures to the same antigen. One crucial feature of the immune system is the ability to distinguish self from non-self. Such immune tolerance is induced through control mechanisms both during immune cell development and through peripheral regulation.

3.1 CYTOKINES

Cytokines are soluble proteins produced by immune cells as well as other cells, and are important mediators of immune reactions. They affect the behaviour of themselves (through autocrine action) or other cells (through paracrine or endocrine action) and can exert multiple effects [31]. **Table 1** summarizes the cytokines of highest importance for the current studies, including their main cellular source and actions.

Cytokines signal through cytokine receptors, which can be grouped on the basis of their structure. For example, receptors for interleukin (IL)-2, IL-4, IL-7, IL-9 and IL-15 belong to the same group of cytokine receptors. They are heterodimeric and share the common γ subunit. IL-2 and IL-15 also share β subunit and the IL-2 receptor has a third, high affinity, α subunit [32]. The IL-2/15Rβ is expressed on NK cells and small subsets of T cells, including $CD8^+$ and $CD4^+$ T cells with memory and regulatory properties [33-35]. IL-2/15Rβ was targeted with a monoclonal antibody in **paper II**.

3.2 THE MAJOR HISTOCOMPATIBILITY COMPLEX MOLECULES

The *m*ajor *h*istocompatibility *c*omplexes (MHC) are central molecules in the immune system, since they present parts of pathogens and transformed cells to evoke immune responses. The MHC molecules are highly polymorphic and a particular combination of MHC alleles is called the MHC haplotype. Two types of classical MHC molecules exist, the MHC class I and II. MHC class I, consisting of an α chain and a smaller, non-polymorphic β2 microglobulin chain, is expressed by most cells in the body and display peptides derived from the cytosol. Three classical MHC class I genes exist: the human HLA-A, -B and –C and the murine H-2D, H-2K and H-2L. MHC class II molecules are mainly expressed by antigen presenting cells and display peptides from exogenous antigens that have been taken up by the cells. MHC class II genes in humans are HLA-DR, DQ and DP and in mice H-2A and H-2E.

Non-classical MHC molecules are structurally related to the MHC class I molecules but display little or no polymorphism. Examples of non-classical MHC are the human HLA-E, G and F, the murine Qa-1b and the CD1 molecules [36].

Cytokine	Producer	Actions	References
GM-CSF	Macrophage T cells NK cells	DC, macrophage and granulocyte expansion and differentiation	
IFNα/β	Leukocytes DC Infected cells Fibroblasts	Antiviral MHC I increase Stim IL-15 production + presentation NK cell cytotoxicity and proliferation	[37]
IFN-γ	T cells (Th1, CD8) NK cells	Macrophage activation MHC increase IgG2a switch Th2 suppression/Th1 induction NK cell activation	
IL-1β	Macrophages Epithelial cells	Fever T cell activation Macrophage activation Beta cell destruction	 [25]
IL-2	T cells (Th1, some CD8)	T cell proliferation and apoptosis NK cell proliferation, survival, apoptosis Treg activation B cell proliferation	 [32] [38]
IL-4	T cells Mast cells	Th2 induction B cell activation IgE switch	
IL-5	T cells (Th2) Macrophages NK cells	Eosinophil growth and activation B cell activation	 [39, 40]
IL-10	Monocytes NK cells T cells (Th2, Treg)	Macrophage suppression	
IL-12	DC Macrophages	NK cell activation Th1 induction	
IL-15	Macrophages Monocytes DC	T cell stimulation NK cell stimulation	[32, 41]
IL-18	Macrophages Kupffer cells	IFN-γ production by T and NK cells Th1 induction	
IL-22	NK cells	Pro-inflammatory Induces acute-phase proteins	[42-45]
TGF-β	Monocytes T cells (Treg) NK cells	Anti-inflammatory Inhibits cell growth IgA switch	
TNF-α	Macrophages NK cells T cells	Pro-inflammatory Endothelial activation	

Table 1. *Cytokines of importance for the current studies (adapted from Janeway's Immunobiology, seventh edition, 2008).*

3.3 IMMUNE CELLS OF IMPORTANCE FOR THESE STUDIES

In these studies one of our main interests has been to investigate immune processes leading to T1D, with a particular focus on the pancreas and PLN. The role of NK cells in the pancreas of NOD mice and in the disease pathogenesis has been explored as well as T cell priming in the PLN and the influence of regulatory T cells. The key immune cells in the present investigations are thus NK cells, T regulatory cells and T effector cells. Each of these cells and their possible role in T1D will therefore be introduced in more detail.

3.3.1 T cells

3.3.1.1 Conventional T cells

Conventional T cells develop in the bone marrow and mature in the thymus to $CD8^+$ T cells or $CD4^+$ T cells, recognising MHC class I or II respectively. The TCR of conventional T cells usually consists of an α and a β chain and the TCR repertoire is formed by somatic recombination, generating a wide variety of receptor specificity (estimates vary between 10^{15} to 10^{18}).

The cytotoxic $CD8^+$ T cells mediate perforin/granzyme and Fas/FasL dependent killing of target cells upon stimulation with MHC-peptide complexes. $CD8^+$ T cells also produce cytokines such as IFN-γ and TNF-α, thereby exerting anti-viral and pro-inflammatory effects [46]. Whereas most $CD8^+$ T cells do not subspecialise further, naïve $CD4^+$ T helper cells differentiate into for example Th1, Th2, Th17 or adaptive regulatory T cells. These processes are not completely understood, although it is clear that initial signals from the antigen presenting dendritic cells play an important role. Cytokines are important modulators of $CD4^+$ T cell differentiation, where IL-12 and IFN-γ for example induce Th1 cells, which control intracellular pathogens, produce IL-2 and IFN-γ and activate macrophages [47]. Instead, IL-4 induces the development of Th2 cells, which provide help in B cell activation and produce various B cell stimulating factors including IL-4 and IL-5. The cytokines TGF-β and IL-6 induce Th17 cells, which promote neutrophil recruitment to the site of inflammation [48]. Adaptive regulatory T cells develop from conventional T cells in the periphery and produce the immune suppressive cytokines IL-10 and TGF-β [49]**.**

3.3.1.2 Unconventional T cells

Some T cells do not develop into conventional T cells, but instead become regulatory T cells, NKT cells or γδ T cells. Like conventional T cells they develop in the bone marrow and mature in the thymus.

Several types of regulatory T cells exist, both $CD4^+$ and $CD8^+$ T cells. The naturally occurring regulatory T cells develop in the thymus and the adaptive regulatory T cells differentiate from conventional T cells in the periphery [49]. The best characterised subset is the natural regulatory $CD25^+CD4^+FoxP3^+$ T cell (here called Treg), an important immune regulator mediating peripheral tolerance [50]. Altogether the Tregs form 1-10% of the $CD4^+$ T cells in humans and mice [50]. They have diverse TCRs, but with higher degree of self-antigen specificity than conventional T cells [51]. Phenotypically Tregs are defined by the expression of high levels of CD25 and the transcription factor FoxP3, which is critically linked to the suppressive function [52, 53]. They also display co-stimulatory molecules including a constitutive expression of CTLA-4. The effector mechanisms of Tregs have mainly been explored *in vitro* and

important mechanisms of action likely include suppression via cytokines such as IL-10 and TGF-β [54] and perforin/granzyme mediated cytotoxicity [55]. Tregs can suppress the proliferation and cytokine production of T cells, DC, NK cells, NKT cells, B cells and macrophages [56]. For example, IL-2 production by T cells is suppressed by Tregs. This leads to decreased T cell proliferation but also acts as a negative feed-back regulator of the Tregs themselves, which are critically dependent on IL-2 for their activation [38]. In line with this, deficiency of IL-2, IL-2Rα or IL-2Rβ leads to uncontrolled autoimmunity [57-60], at least partly due to loss of Treg activity.

NKT cells represent another type of unconventional T cells with innate properties and regulatory functions [61]. The best characterised subset of NKT cells are the invariant NKT (*i*NKT) cells. They express a semi-invariant TCR, with an invariant α chain, Vα24-Jα18 in humans and Vα14-Jα18 in mice, coupled to a Vβ11 chain with limited variation [62]. As opposed to other T cells they recognize glycolipids presented by the non-polymorphic MHC class I molecule CD1d. Upon activation *i*NKT cells rapidly produce large amounts of cytokines such as IL-4, IL-13, IL-10, IL-17, TNF-α and IFN-γ, thereby influencing the immune response in a variety of ways [62].

A third type of unconventional T cells is the γδ T cells with one subgroup residing in the lymphoid tissues and one in the epithelium [63]. These T cells recognize small non-peptide antigens such as parts of pathogens or necrotic cells and have innate properties. The γδ T cells do not require MHC presentation of the antigen. Upon activation, γδ T cells respond quickly with cytotoxicity and secretion of cytokines such as IFN-γ and TNF-α.

3.3.2 NK cells

Natural killer (NK) cells are lymphocytes belonging to the innate immune response and were first described in 1975 [64-67]. NK cells are instrumental in the early immune defence against certain viruses and tumours, as well as being involved in immune processes influencing the outcome of bone marrow transplantation. NK cells are able to kill infected or transformed cells with perforin/granzyme mediated lysis or apoptosis via for example Fas/FasL [68]. They also have regulatory properties and can modulate the adaptive immunity [69]. Upon stimulation NK cells can rapidly produce cytokines including IFN-γ, TNF-α, GM-CSF, IL-5, IL-10 and IL-13 [37, 70, 71], thereby being able to exert both pro-inflammatory and immune regulatory roles. Although NK cells are part of the innate immune response recent studies indicate that NK cells, similar to the adaptive immune response, could form memory to cytomegalovirus and contact hypersensitivity antigens in animal models [72, 73]. These findings open up for a potentially much broader role of NK cells in the immune system than what has been previously estimated.

3.3.2.1 NK cell development

The NK cells mainly develop in the bone marrow through several defined maturation steps [74, 75]. IL-15 is the most critical cytokine promoting NK cell development and in mice IL-2/15Rβ (CD122) expression characterises the first committed NK cell progenitor [74, 76-78]. Other factors of importance for NK cell development include stromal derived cytokines such as Flt3 ligand and c-kit ligand and the transcription factors Ets-1, GATA-3, T-bet and Id2 [79]. The recently described E4BP4 is the only

known NK cell specific transcription factor. E4BP4 -/- mice completely lack NK cells and NK cell function but have no defects in other lymphocyte lineages [80].

Phenotypically the NK cell developmental stages are characterised by sequential expression of CD122, NK1.1, c-kit, Dx5 and CD11b [74] and it is only during the final stages of maturation that NK cells gain capacity to produce cytokines and mediate cytotoxicity [74, 76]. Recently, a distinct subset of thymus derived NK cells was described [81]. It is characterised by its dependency on IL-7 and GATA-3, has a distinct phenotype and is expressed in high frequency in the lymph nodes. NK cell development from early NK cell precursors has also been described in human lymph nodes [82], the liver and the spleen [83].

3.3.2.2 NK cell receptors

Unlike T and B cell receptors, the receptors on NK cells are encoded by genes that do not undergo rearrangement. Still, NK cell receptors display more variability than other receptors in innate immunity, for example the toll like receptors (TLR). Despite their name, NK cell receptors are also expressed on δγ T cells and some activated memory or effector αβ T cells. Each NK cell expresses several activating and inhibitory receptors and the integrated input from these receptors dictates whether the NK cell will become activated (**figure 2**). NK cell receptors can be classified on the basis of their structure, e.g. the Ly49 receptors, KIRs and CD94/NKG2 receptors, each group containing both inhibitory and activating receptors.

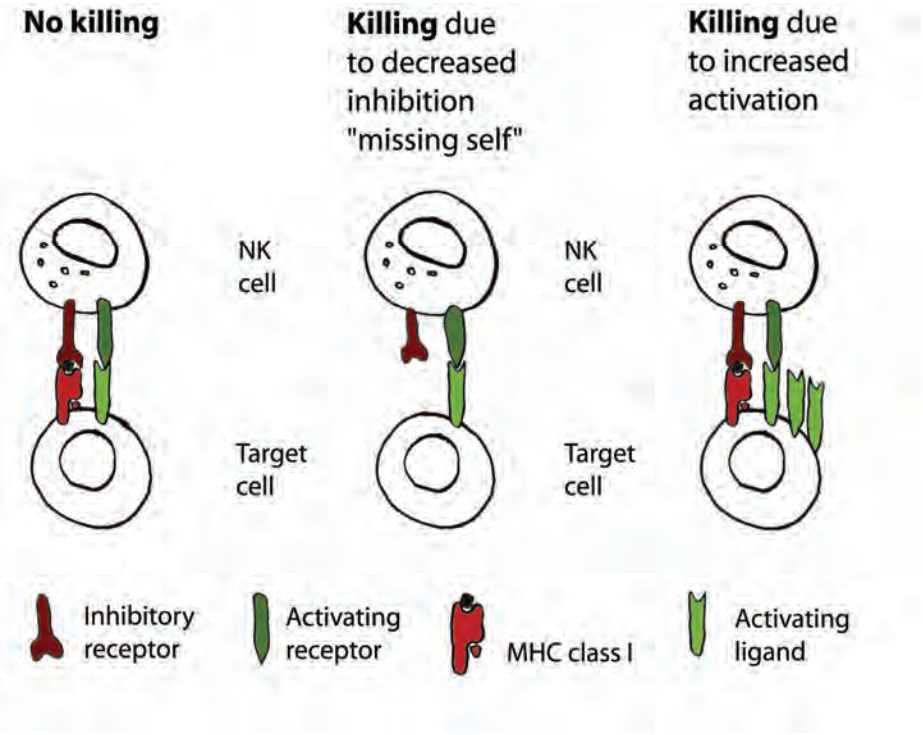

Figure 2. *The balance of inhibitory and activating signals dictates whether the NK cell will become activated and kill the target cell. Killing is promoted either by decreased inhibition, as stipulated in the "missing self" hypothesis, or by increased activation.*

3.3.2.2.1 Inhibitory NK cell receptors

Inhibitory receptors are pivotal in preventing unwanted NK cell activation. Many inhibitory NK cell receptors recognise self-MHC class I molecules, which are expressed by all cells in the body, with the rare exceptions of erythrocytes and resting neurons. Insufficient inhibitory input from self-MHC class I molecules leads to NK cell activation as stipulated in the "missing self" hypothesis [84, 85]. The inhibitory receptors interacting with MHC are called killer immunoglobulin receptors (KIR) in humans and Ly49 receptors in mice. To avoid NK cell activation against self the KIR/Ly49 receptor repertoire and NK cell reactivity is shaped through interactions with self-MHC class I molecules during NK cell development [86]. The CD94/NKG2A receptor is another type of receptor which binds the non-classical MHC Ib (HLA-E in humans and Qa-1b in mice). This receptor-ligand pair is peculiar in that MHC Ib preferentially presents peptides from other MHC class I molecules. As opposed to the KIR and Ly49 receptors CD94/NKG2A shows no polymorphism and the expression levels of this receptor can be modulated by cytokines [87]. Other inhibitory receptors have non-MHC ligands, such as glycoproteins or not yet identified ligands. The KLRG1, NKRP1 and siglec7 are examples in this category.

All inhibitory NK cell receptors signal through an *i*mmunoreceptor *t*yrosine-based *i*nhibition *m*otif (ITIM). ITIMs are highly conserved intracellular signalling motifs that associate with inhibitory receptors in a broad range of immune cells. Upon stimulation of the receptor the tyrosine residue of the ITIM is phosphorylated, phosphatases are recruited and a complex array of signalling pathways are initiated, ultimately leading to suppressed cytotoxicity and cytokine production [87]. Importantly, these events are very confined, both spatially and temporally. A previously inhibited NK cell can therefore later become activated, indicating that the same NK cells could be engaged in several interactions with potential target cells [87].

3.3.2.2.2 Activating NK cell receptors and signs of activation

Many activating receptors are expressed by a majority of NK cells, including the NKG2D receptor, NKp46, DNAM-1 and 2B4 [87]. Others, like the activating KIR and Ly49 receptors, are expressed in a variegated fashion. Only few ligands to activating receptors have so far been determined. Interestingly, at least one of the activating ligands is expressed by certain pathogens: the mouse cytomegalovirus (MCMV) encoded protein m157 recognised by Ly49H. The NKG2D is another activating NK cell receptor with identified ligands, that are structurally similar to MHC molecules: the human MICA/B and ULBP and murine rae-1, H60 and MULT-1 [87]. NKG2D is constitutively expressed on all NK cells, all human $CD8^+$ T cells and most γδ T cells. In mice, however, $CD8^+$ T cell expression of NKG2D requires cell activation and is present only on a fraction of circulating $CD8^+$ T cells [88]. The NKG2D ligands are induced by stress, by for example infections or tumours. Tumours often express NKG2D ligands but can also downregulate NKG2D receptor expression through release of soluble MIC proteins or TGF-β [89, 90].

Activation of NK cells via receptor stimulation almost always requires the engagement of more than one activating receptor simultaneously, at least *in vitro* [91]. Only the low affinity Fc-receptor CD16, which mediates antibody dependent cell-mediated cytotoxicity (ADCC), can independently activate NK cells to perform cytolysis and

cytokine release. When NK cells are activated to kill a target cell they rapidly reorganise their shape to form a synapse with the target cell and release granules with pre-formed cytokines and cytotoxic perforine and granzyme [87]. The inner surface of these cytotoxic granules is coated with the *l*ysosomal-*a*ssociated *m*embrane *p*rotein-1 (LAMP-1 or CD107a) which is displayed on the outer cell membrane upon degranulation [92, 93]. Following activation, the NK cells undergo several other phenotypic changes including downregulation of the lymph node homing marker CD62L and upregulation of the activation markers CD69 and CD25. Another NK cell receptor which is upregulated following stimulation and maturation is the inhibitory killer cell lectin-like receptor G1 (KLRG1) [94-96].

Many activating NK cell receptors signal via an *i*mmunoreceptor *t*yrosine-*b*ased *a*ctivation *m*otif (ITAM). Three ITAM containing adaptor proteins exist in NK cells: FcεRIγ, CD3ζ and DAP12 [87]. The role of DAP12 in the development to T1D was explored in **paper III**, and this particular adaptor molecule will therefore be described in more depth.

3.3.2.3 DAP12

The adaptor protein DAP12 was initially characterised in NK cells [97, 98] and couples to several activating receptors lacking signalling capacity, e.g. activating Ly49 receptors, KIRs and CD94/NKG2. Later DAP12 has been shown to be widely expressed in myeloid cells such as dendritic cells, monocytes, macrophages, neutrophils and mast cells [99, 100], mediating signalling of the *t*riggering *r*eceptors *e*xpressed on *m*yeloid cells (TREM receptors) [101]. DAP12 is also expressed in subsets of T and B cells [100].

DAP12 couples to its receptors by an extracellular disulfid bond and a transmembrane non-covalent binding [100]. The main part of DAP12 is however localised in the cytoplasm, where it confers signals through an ITAM. Phosphorylation of the ITAM recruits and activates Syk tyrosine kinases in myeloid cells and Syk and ZAP70 in NK cells. Paradoxically, DAP12 signalling inhibits immune responses in certain situations, adding further complexity to its role [102].

In humans, loss of function mutations in the gene encoding for DAP12, *tyrobp*, results in a recessively inherited disorder named *p*olycystic *l*ipomembranous *o*steodysplasia with *s*clerosing *l*eucoencephalopathy (PLOSL) or Nasu-Hakola disease [103]. In the absence of a functional DAP12 these patients develop bone cysts in their wrists and ankles and rapidly progressive pre-senile dementia [103, 104].

3.3.2.3.1 DAP12 deficient mice

Functional insight into the role of DAP12 has been gained from DAP12 deficient mice. Two different types of mice have been generated: one with a targeted gene disruption of the *Dap12* gene leading to a total absence of DAP12 expression [105]. Since the adaptor protein is required for stable receptor expression on the cell surface these mice have few DAP12 associated receptors. The other mouse model has a knock-in loss of function mutation with one of the tyrosine residues in the ITAM deleted [106], leaving the DAP12 expression normal but the signalling properties non functional [107]. The

DAP12 deficient mice have several alterations in the immune system including increased numbers of DCs in the skin, intestinal mucosa and Peyer's patches. As expected, NK cell cytotoxicity via DAP12 associated receptors such as Ly49D was also markedly reduced [105]. In **paper III** mice with knock-in loss of function mutation of DAP12 were explored [106, 107]. One important benefit with this model is that the extracellular part of DAP12 is intact and the association with, and surface expression of, the receptors therefore unaltered.

3.3.2.4 Cytokine activation of NK cells

In addition to the specific regulation of NK cells via their receptors for membrane bound ligands, several cytokines influence the activity, development, proliferation and effector functions of NK cells, including IL-2, IL-12, IL-15, IL-18 and IFNα/β [37, 108, 109]. During viral infection for example, IFNα/β produced by DC and infected cells [110] induce NK cell cytotoxicity and proliferation [37, 108, 109, 111]. IFNα/β also stimulates the IL-15 production by DCs, monocytes and macrophages. In addition to the important role of IL-15 during NK cell development and differentiation [32, 77, 112] this cytokine promotes NK cell proliferation, survival and cytotoxicity [32, 113]. IFNα/β induced trans-presentation of IL-15 by DCs was recently shown to prime NK cells for stimulation [114]. This finding challenges the traditional view on NK cells as unnecessary to prime due to their "natural" cytotoxicity [64-67]. IL-12 is produced in certain viral infections, including MCMV and influenza, and is together with IL-18 a potent inducer of IFN-γ production by NK cells [115-117].

3.3.2.5 Mature NK cell subsets

Mature NK cells can be divided into functionally distinct NK cell subsets [118-120], in humans defined by their expression level of the surface marker CD56 [118]. The NK cells with bright expression of CD56 are predominantly found at inflammatory sites and have immunoregulatory functions [121], including production of high quantities of TNF, IFN-γ, IL-10 and GM-CSF. This subset also has high expression of receptors facilitating homing and entry to lymph nodes and secondary lymphoid organs, such as L-selectin (CD62L) and the chemokine receptor CCR7. The $CD56^{dim}$ NK cells on the contrary constitute the majority of the NK cells in peripheral blood and are poor cytokine producers. Instead they have strong cytotoxic capacity [118].

Mouse NK cells, on the other hand, do not express CD56 but instead their expression of Mac-1 (CD11b) and CD27 can be used for defining subsets with distinct functional and phenotypic properties as well as tissue distribution [119, 120, 122]. The four subsets appear to be consecutive maturation stages with $Mac\text{-}1^{low}CD27^{low}$ cells being the most immature cells, followed by step-wise CD27 and Mac-1 upregulation and ultimately CD27 downregulation to form the most mature $Mac\text{-}1^{hi}CD27^{low}$ cell. $Mac\text{-}1^{hi}CD27^{hi}$ NK cells exhibit high cytotoxicy to classic NK cell targets and high cytokine production upon IL-12/18 stimulation or DC co-culture [119]. $Mac\text{-}1^{hi}CD27^{low}$ NK cells are tightly regulated by a high expression of inhibitory Ly49 receptors. However, genes involved in cytotoxicity are expressed in increasing levels as the cell matures within this program, indicating that this most mature subset has high cytotoxic capacity [120]. The inhibitory receptor KLRG1 is also associated to maturation of NK cells and is upregulated following homeostatic proliferation or activation due to infections [94-96]. $KLRG1^{+}$ NK cells have impaired proliferative and cytokine producing capacity and have been speculated to represent an end stage of NK cell maturation [94].

3.3.2.6 Tissue specific NK cells

Tissue specific NK cell subsets with distinct functional and phenotypic characteristics have been described [4, 42-45, 123]. We have in the present studies investigated the pancreatic NK cells (**paper I**). NK cells in the uterus and gut represent interesting comparisons to the pancreatic NK cells, infiltrating non-hematopoietic solid organs, and will therefore be described.

Pregnancy represents a special immunological challenge, with a strict need for immune tolerance between the mother and the developing foetus. Interestingly, the majority of all lymphocytes in the uterus are NK cells, both in mice and humans [123]. Murine uterine NK (uNK) cells are implicated to be important providers of IFN-γ, required for the physiological angiogenesis of spiral arteries ensuring sufficient oxygen transport to the foetus [123, 124]. In humans, as opposed to in mice, the uNK cells are present in the uterus before fertilization, which opens up for their possible involvement in facilitation the actual implantation. Both human and mice uNK cells are characterised by a dense granularity, but have impaired cytotoxic capacity [123]. Instead they are potent producers of GM-CSF, IFN-γ, TNF-α, TGF-β and angiogenic factors [123, 125]. Phenotypically, most human uNK cells are $CD56^{bright}$ and have a high expression of NK cell receptors with specificity to HLA-C, the only polymorphic MHC class I expressed on trophoblasts [126, 127]. Mouse uNK cells also have a unique receptor repertoire [125] and were recently shown to belong to two distinct subsets. The most abundant uNK subset expresses the NK cell marker NKp46 but lacks both Dx5 and NK1.1, which are commonly expressed by all mature NK cells [74, 125]. Despite this, murine uNK cells express Ly49 receptors, which are usually not expressed until the later maturation stages, possibly reflecting that uNK cells represents a distinct developmental pathway.

Another specialised subset of lymphocytes expressing NK cell receptors was recently described in the gut in both mice and humans [42-45]. It is still debated whether "gut NK cells" truly represents an NK cell subset since they lack some classic NK cell features. Mouse gut NK cells are for example not dependent on IL-15 for their development, express low levels of NK1.1 and have very low capacity of both cytotoxicity and cytokine production. They however resemble conventional NK cells in their expression of the NK cell receptors NKp46 and NKG2D. Mouse gut NK cell also display high levels of CD127, CD69 and c-kit but lack, or have significantly decreased levels of, CD122, Dx5 and Ly49 receptors. Most gut NK cells belong to the immature $Mac\text{-}1^{low}CD27^{low}$ population [42, 44, 45]. For their development, the gut NK cells are dependent on the commensal microflora and the transcription factor RORγt and they produce IL-22 upon activation. A human counterpart to the murine gut NK cells have been described in the mucosa-associated lymphoid tissue (MALT) including both the Peyer's patches in the gut and in the tonsils [43]. Similar to the murine gut NK cell subset they produce large amounts of IL-22. IL-22 in turn increases the murine gut epithelial expression of the antimicrobial peptides RegIIIβ and γ, which are involved in protection of the epithelium and are important in the immune response against certain infections [42, 44, 45]. Furthermore IL-22 induces human epithelial cells *in vitro* to secrete IL-10, proliferate and up-regulate pro-mitogenic and anti-apoptotic factors [43]. Taken together, gut NK cells are speculated to play a role in tissue repair and protection against local infections.

4 AUTOIMMUNITY

Autoimmune diseases result from lack of immune tolerance, followed by an attack on self. Some autoimmune diseases affect one single organ (or part of an organ), such as the pancreas in T1D or the thyroid in Grave's disease, and are thus called organ-specific autoimmune diseases. Systemic lupus erythomatosus (SLE) on the other hand is an example of systemic autoimmunity where multiple organs are affected.

The pathogenesis of autoimmune diseases is generally complex, involving several susceptibility genes, such as MHC haplotypes, and largely unknown environmental triggers [128]. The mechanism behind the powerful influence of the MHC is not fully clear. Possibly particular MHC molecules only weakly bind autoreactive peptides in the thymus, thereby allowing self-reactive T cells to escape into the circulation [129]. Despite the suggested weak peptide binding in the thymus these T cells could become activated in the periphery in the presence of higher concentrations of antigen or as a consequence of strong co-stimulation. In recent years the advances in GWAS have dramatically increased our knowledge on gene polymorphisms linked to autoimmunity [15, 130].

Autoreactive T cells mediate the tissue destruction of most organ-specific autoimmune diseases. B cell produced autoantibodies on the other hand play important roles in for example SLE and Grave's disease [131]. Most people are spared from autoimmunity thanks to several mechanisms assuring immune tolerance against self. An important event in avoiding autoimmunity is the induction of central tolerance in T and B cells during the hematopoietic development. In this process the potentially autoimmune lymphocytes undergo negative selection and are deleted. Some autoreactive T and B cells however escape these control mechanisms and enter the circulation [132]. This is probably not just an unwanted and unpreventable event, but ensures sufficient immunity towards foreign peptides with epitopes resembling a self epitope. Autoreactive cells are usually hindered from causing disease by peripheral tolerance mechanisms [133].

The initial triggers of autoimmune diseases are poorly understood. One possible scenario could be that normally hidden self-antigens are exposed, following abnormal cell-death during an infection for example. Previously quiescent self-reactive lymphocytes could then become activated in the presence of co-stimulatory molecules and cytokines induced by the ongoing infection. Such general cellular destruction could lead to the simultaneous exposure of multiple antigens and explain the broad range of autoantigenic peptides (epitopes) often present in autoimmune disease. The hypothesis of "epitope spreading" instead suggests that a single epitope initiates the autoimmune destruction [134]. The autoreactive tissue destruction causes exposure of new epitopes, and thereby further damage. Such sequential addition of epitopes amplifies the immune response, which eventually becomes chronic. An alternative way by which self-directed immune responses could be elicited is by "molecular mimicry", where an exogenous antigen closely resembles an antigen in the affected tissue. Immune reactivity towards the exogenous antigen could potentially cross-react with self and cause tissue damage. Reactive arthritis following enteric infections and rheumatic fever after streptococcal infections may develop due to molecular mimicry [135, 136].

5 IMMUNE PATHOGENESIS OF T1D

Increasing evidence points towards a critical immune modulating role of the tissues under attack in organ-specific autoimmune disease [4], i.e. the pancreas in T1D [137]. Therefore, in order to understand organ-specific autoimmunity it is necessary to take the targeted organ into account. In the following sections, I will try to set the stage for such a perspective, introducing the pancreas and also the PLN. Thereafter the key cells and events at these sites are introduced in a simplified scenario illustrating the current dogma of the pathogenesis of T1D (**Figure 3**).

5.1 THE PANCREAS

The pancreatic microenvironment affects the recruitment of lymphocytes to the tissue, by expression of specific chemokine ligands. In mouse the pancreatic beta cells for example exhibit CXCL9 and CXCL10 and thereby recruit $CXCR3^+$ lymphocytes, such as subsets of activated T cells, NK cells and plasmacytoid DCs [138, 139]. Ligands for other immune receptors are also present on beta cells, including rae-1, a ligand for the activating NK cell receptor NKG2D, enabling direct activation of effector functions at this site [140, 141]. Furthermore, beta cells can produce various cytokines [6]. Beta cell production of IFN-α for example causes MHC class I upregulation and increased $CD8^+$ T cell mediated cytotoxicity [137].

Several pancreatic proteins also act as autoantigens in T1D. For example insulin, glutamate decarboxylase (GAD), insulinoma-associated protein 2 (IA-2), islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP), heat shock protein 60 (Hsp60) and the most recently identified ZnT8, a pancreas specific zink transporter [142, 143]. These autoantigens are important predictors of disease, and 80% of the T1D patients have detectable autoantibodies at the diagnosis. One explanation for the broad range of autoantigens in T1D is that epitope spreading occurs. Evidence for this hypothesis is the requirement of immunity against proinsulin in order to mount IGRP directed T cell responses [144]. Further supporting a pivotal role for insulin is the protection from T1D in mice lacking pre-proinsulin 1 or the insulin epitope InsB:9-23 [145, 146] and the association of gene polymorphisms in the *insulin* gene with T1D, both in humans and NOD mice [147].

5.2 THE PANCREATIC LYMPH NODES

The pancreatic lymph nodes (PLN) are located close to the pancreas and are the sites where the antigen presenting cells (APC) display pancreatic antigens to naïve T cells. Depending on the level of maturation of the APC as well as other, not fully understood factors, this interaction either results in deletion or priming of the T cells [2, 148]. It is also possible that Tregs are simultaneously primed [149], regulating the activity of the effector T cells. Interestingly, a preferential drainage of the gut to the PLN enables presentation of enteric proteins to autoreacitve T cells in the PLN, and represent a possible way by which environmental antigens may influence T1D [150]. The processes occurring in the PLN are still not fully understood and it is of highest importance to further delineate what regulates the balance between T cell priming and induction of Tregs at this interface between the target organ and the potentially autoreactive immune system.

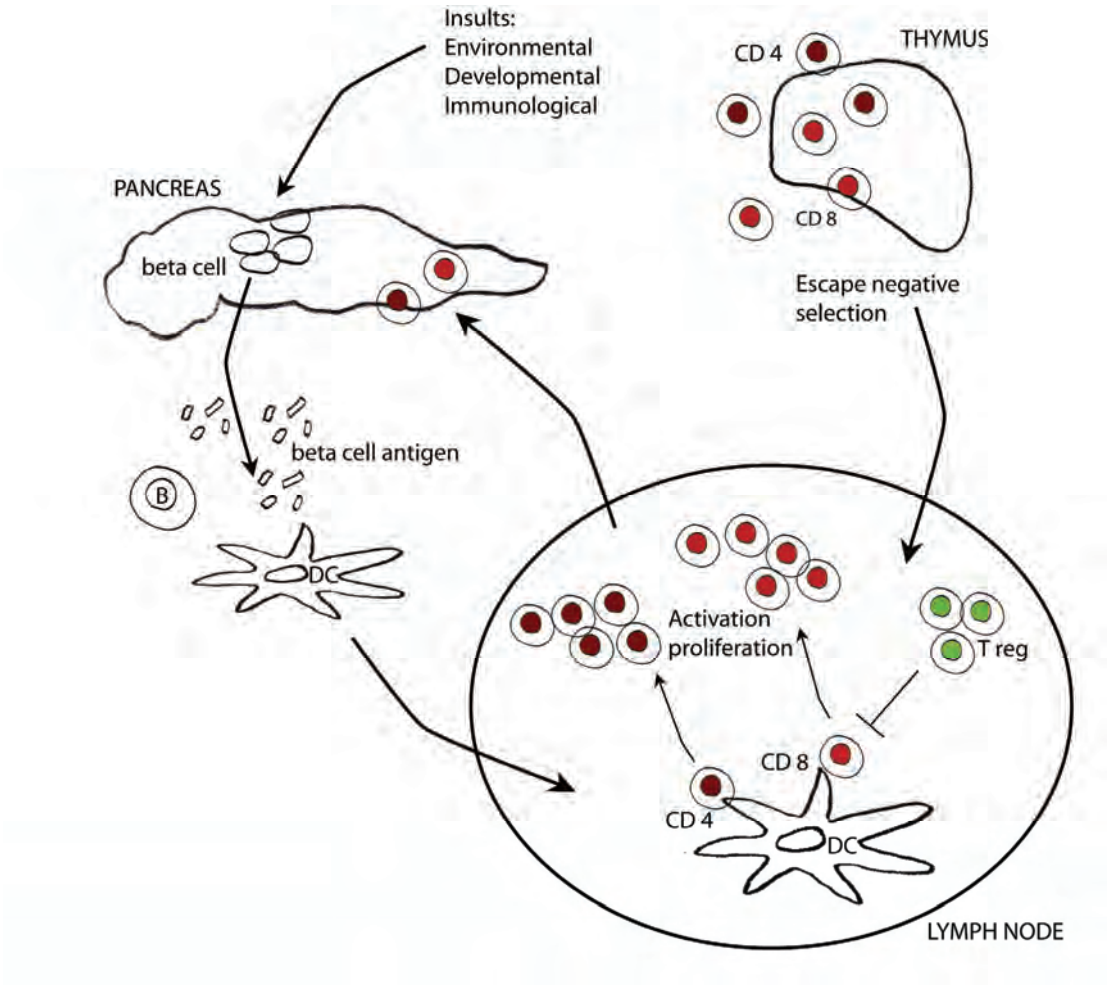

Figure 3. A scenario leading to T1D
T cells reactive to beta cell proteins escape negative selection during the development in the thymus. In a genetically susceptible person [15], an environmental trigger such as an infection leads to beta cell damage in the pancreas [151]. Previously hidden beta cell antigens are then exposed and phagocytosed by pancreas resident APC, e.g. immature dendritic cells (DC) [152]. The ingestion of antigen promotes DC maturation and migration to the PLN. In the PLN the DC primes autoreactive T cells [2] and T regs influence this process. The primed autoreactive cells then home to the pancreas and infiltrate the pancreatic islets. The pancreatic infiltrate also contains other immune cells such as B, NK, NKT cells, Tregs and macrophages, reflecting the complexity of the T1D pathogenesis and a possible involvement also of these cells [1]. The insulitis is initially non destructive, but later mediates beta cell destruction and ultimately failure to produce enough insulin, leading to overt T1D [18].

5.3 ROLE OF T CELLS AND NK CELLS IN T1D

5.3.1 T cells in T1D

T cells are well established key players in the pathogenesis of murine T1D [1, 153]. Adoptive transfer experiments where spleen cells from pre-diabetic or diabetic NOD mice were transferred to neonates, irradiated adult NOD mice or T cell deficient NOD mice suggest that both $CD4^+$ and $CD8^+$ T cells are important in inducing diabetes [154, 155]. $CD8^+$ T cells alone do not infiltrate the pancreas and $CD4^+$ T cells alone cause insulitis but not diabetes [156]. In addition, both MHC class I and II restricted NOD mouse derived diabetogenic T cells can independently transfer disease [20, 157]. The

evidence that T cells are instrumental also in patients with T1D is more limited. However, T cells have been found in pancreatic infiltrates [158, 159], autoreactive T cells can be obtained from the circulation at the time of diagnosis [160] and anti CD3 treatment affects T1D beneficially [161, 162]. There is also strong evidence that MHC molecules are involved in the T1D pathogenesis. Certain MHC class II variants, DR3-DQ2 and DR4-DQ8, represent the most prominent genetic association in T1D in humans and the NOD mouse has a very uncommon MHC class II haplotype, H-2^{g7} [1, 14]. The MHC class I types HLA-A and HLA-B are also associated with T1D (and foremost HLA-B*39) [163], which opens up for a more direct role of $CD8^+$ T cells in the diabetes pathogenesis also in humans.

Tregs on the contrary are important suppressor cells protecting from T1D [50]. Humans with a mutation in the *foxp3* gene, leading to a complete lack of FoxP3, suffer from the rare disorder *I*mmunodysregulation *p*olyendocrinopathy *e*nteropathy *X*-linked (IPEX), characterised by multi-organ autoimmunity including the development of T1D [164]. FoxP3-/- mice also succumb to multi-organ autoimmunity, which can be reversed by transfer of Tregs [165, 166]. The mechanisms for the Treg control of T1D are not fully understood. NOD mouse Tregs however progressively lose their function during the diabetes development, which could represent a pathogenic factor [167-170]. Tregs normally interact with antigen-loaded DCs in the PLN of NOD mice and thus efficiently prevents interaction and priming of autoreactive T effector cells [171]. Defect co-stimulation could be another possible mechanism, since deficiency of the co-stimulatory pathways CD28/B7 or CD40/CD40L leads to impaired T regulatory function and increased T1D development [169, 172]. The progression from respectful to aggressive insulitis correlates with reduced frequencies of Tregs both within the PLN and the pancreas [170]. In support for a local role of Tregs within the pancreas is the finding that FoxP3-/- BDC2.5/NOD mice rapidly progress from insulitis to diabetes, via a loss of suppression of pancreatic NK cells [173].

Also *i*NKT cells are protective in the pathogenesis of T1D [62] and CD1d-/- NOD mice rapidly succumb to T1D [174]. In line with a protective role of *i*NKT cells, transfer, stimulation or transgenic overexpression of *i*NKT cells in NOD mice decrease disease [62]. *i*NKT cell mediated suppression of T1D likely depend on several mechanisms, not necessarily involving interaction with CD1d [175]. One possibility is that *i*NKT cells are an important source of Th2 cytokines such as IL-4, and the production is impaired in NOD mice [176]. Another possible mechanism is that *i*NKT cells affect the APCs, by inducing the recruitment of tolerogenic DCs for example [177, 178]. Intriguingly, *i*NKT cells have been shown to also have pathogenic roles in T1D, by supporting autoreactive $CD8^+$ T cells [179].

5.3.2 NK cells in T1D

The role of NK cells in T1D is only shortly described here since it is a major issue in the present work and will be described in more depth in the results and discussion section (**paper I and II**).

In T1D the NK cells are impaired in function, both in peripheral blood from patients [180] and in NOD mice spleen [181, 182]. In addition, 3/6 newly diagnosed T1D patients had massive pancreatic NK cell infiltrate and concomitant pancreatic coxackie

virus B4 infection [151]. The role of NK cells in T1D is however controversial, with studies suggesting both a pathogenic and a protective role [183-185]. For example, a protective role for NK cells was demonstrated in NOD mice treated with complete Freund's adjuvant (CFA). A pathogenic role on the contrary was suggested by other studies [184, 185]. For example, NK cells accelerated T1D in BDC2.5/NOD mice where disease was induced by treatment either with the cytostatic drug cyclophosphamide or antibodies to the co-stimulatory receptor CTLA-4 [185]. Depletion of NK cells also protects from T1D in RIP-IFN-γ NOD mice, which have transgenic IFN-γ expression in the beta cells [184].

5.4 IMMUNE THERAPY OF T1D

The implementation of a successful immune therapy in T1D is complicated by the silent progression of the disease. When overt T1D presents, most of the destruction of beta cells has already occurred. The lack of reliable biomarkers to follow the pathogenesis and predict individuals at risk is also troublesome. One predictor is the presence of autoantibodies to pancreatic antigens, which are present in approximately 80% of the patients at diagnosis. Autantibodies however exist in the healthy population and new tools to detect the earliest events of T1D are probably required in order to successfully implement an immunologic treatment.

Several immune therapies for T1D have been tested in humans. The cytostatic cyclosporin was the first immune modulating therapy showing positive effects [186]. However, the serious renal adverse events and lack of long-lasting effects made this an unattractive treatment [187]. More recent trials with different types of modified anti-CD3 antibodies have given promising results with decreased need for insulin treatment and improved HbA1c [161, 162]. Approximately 10% of the patients however discontinued the treatment due to side effects, such as influenza like symptoms and mononucleosis [188]. The mechanism behind the beneficial effect of anti-CD3 treatment in patients is not resolved, but may resemble the situation in NOD mice where a similar treatment induces adaptive T regulatory cells in the PLN [189]. Another immune therapy tested in the clinic is anti-CD20 treatment. This antibody targets B cells and gives moderate but significant improvement of clinical parameters in newly diagnosed T1D patients [190]. Interesting clinical studies are performed with IL-1 antagonists and have curiously proven beneficial effects both in T1D and T2D patients [191, 192]. The mechanism behind protection with this treatment could be impaired T cells stimulation or decreased beta cells apoptosis, both functions linked to IL-1β.

An alternative way to module T1D is by tolerance induction via administration of autoantigens. Insulin, or parts of insulin, has been widely studied in this respect, both in patients with recent onset T1D and in individuals at risk [188]. Different administration routes and adjuvant have been investigated. Disappointingly, however, no convincing effects have been seen so far, despite a well established protective effect of this treatment in NOD mice. One possible explanation for the failures of this strategy could be that epitope spreading has already started at the time of the initiation of insulin treatment [188]. Clinical trials have also been performed with the autoantigen GAD, suggesting that this treatment may be beneficial if initiated close to disease onset [193].

6 PEPTIDES REGULATING APPETITE

Obesity is a major risk factor for T2D and can precipitate disease in genetically susceptible individuals. Therefore, the factors controlling the appetite are of high importance to understand. Several peptides that regulate appetite are produced in the CNS, where the hypothalamus is a critical region [7]. Both orexogenic and anorexogenic peptides are produced or exert their effects there. Orexogenic peptides from the hypothalamus include neuropeptide Y (NPY) and agouti-related protein (AgRP). The *c*ocaine- and *a*mphetamine-*r*egulated *t*ranscript (CART) and *p*ro-*o*pio*m*elano*c*ortin (POMC) are instead examples of neuropeptides with anorexogenic effects. Interestingly, in addition to its effect on feeding behaviour, POMC neurons also regulate glucose homeostasis [9]. Another curious finding, linking neuropeptide pathways and diabetes pathogenesis, is that gene polymorphisms in the melanocortin-4 receptor (MCR4), which POMC related peptides signal via, correlate to T2D [194]. One recent addition to the hypothalamic appetite regulators is the anorexogenic protein nucleobindin-2 [8]. However, all appetite regulating peptides are not produced in the CNS. For example, leptin is secreted from adipocytes and acts on the hypothalamus to decrease food intake [7]. Other examples include the orexogenic peptide ghrelin and the anorexogenic peptides CCK and glucagon-like peptide-1 (GLP-1), which are produced in the gut and affect nervous signalling in the hypothalamus or via the vagus nerve.

6.1 NUCLEOBINDIN-2

Nucleobindin-2 is a protein with a broad distribution in the central nervous system (CNS), mainly localized in the hypothalamus and the brain stem [8, 195, 196]. This highly conserved protein is suggested to be processed into at least three fragments: nesfatin-1 (aa 1-82), nesfatin-2 (aa 85-163) and nesfatin-3 (aa 166-396) [8].

Two CNS regions with high nucleobindin-2 expression are the arcuate nucleus and the paraventricular nucleus (PVN) of the hypothalamus [8, 196]. Both these regions are involved in the regulation of food intake [7], suggesting that nucleobindin-2 could be regulating appetite. Indeed, a peptide fragment of nucleobindin-2 (nesfatin -1) has anorexogenic properties [8]. Intracerebroventricular injection of nesfatin -1 reduces food intake. Additionally, the food intake was increased upon administration of anti-nesfatin-1 antibody or nucleobindin-2 antisense. No anorexogenic effect was seen after administration of nesfatin-2 or nesfatin-3, suggesting that nesfatin-1 specifically is a cleaved and secreted anorexogenic neuropeptide. The other fragments of nucleobindin-2 have putative DNA-binding domains, indicating a yet not fully understood intracellular role of these protein fragments [8, 197].

It is not fully established that nesfatin-1 acts a classical neuropeptide, since it can not be detected in the neuronal axons, from which neuropeptides are usually secreted [196]. It is also not certain whether the antibody to nesfatin-1 detects the cleaved protein fragment or the whole nucleobindin-2. In the present study, the term nucleobindin-2 will be used for both the full length nucleobindin-2 and possible peptide fragments thereof, detected by the anti-nesfatin-1 antibody.

7 AIMS

The overall aim of the present studies has been to generate new knowledge of the processes leading to and associated with diabetes, and thereby contribute to the scientific platform essential for the design of successful new treatments.

Our group and others have shown that NK cells in the circulation of T1D patients and NOD mice are impaired in function. At the initiation of the present studies tissue microenvironments were starting to attract interest as important regulators of NK cell phenotype and functions. We believed that it was crucial to understand the local immune processes within the target organ under attack in T1D and hypothesised that pancreatic NK cells in the NOD mouse would have distinct characteristics. **The aim of paper I was to investigate the presence, phenotype and function of pancreatic NK cells in the NOD mouse during the disease development.**

Several studies had also pointed towards a role for NK cells in the pathogenesis of T1D. However, conflicting data had been presented in different experimental settings using transgenic or induced models of T1D. Based on our findings in paper I, that the NK cells in the pancreas of NOD mice were present early in the islets and bore signs of activation, maturation and possibly exhaustion, we favoured the hypothesis that NK cells are pathogenic in T1D. **The aim of paper II was to investigate if NK cells are pathogenic in T1D, by depleting the NOD mouse NK cells *in vivo*, using an antibody to the beta chain of the IL-2/15 receptor.**

The intracellular adaptor protein DAP12 associates to activating receptors on NK cells, myeloid cells and some T and B cells. When the current studies were initiated DAP12 deficiency had recently been shown to cause resistance to MOG peptide induced experimental autoimmune encephalomyelitis (EAE) by affecting the T cell priming in the lymph nodes. Based on these findings we hypothesised that DAP12 deficiency also would lead to decreased T1D incidence. **The aim of paper III was to explore the role of DAP12 the in the development of T1D, with a particular focus on processes in the pancreatic lymph nodes.**

Many signalling peptides are shared between the central nervous system (CNS) and the endocrine pancreas. Nucleobindin-2 is a protein with a broad expression in the CNS, including expression in areas regulating food-intake. It was recently described to have anorexogenic properties. At the initiation of the present studies nucleobindin-2 had mainly been characterised in the CNS. We hypothesised that nucleobindin-2 would also be expressed in the endocrine pancreas and possibly regulate energy homeostasis also in the periphery. **In paper IV our aim was to investigate the presence of nucleobindin-2 in the pancreas, using a model for T2D, and its relation to the glycaemic state.**

8 METHODOLOGICAL CONSIDERATIONS

The material and methods of relevance for the studies are described in each paper. I will here discuss some of the most important methodological considerations in more depth.

8.1 RETRIEVING PANCREATIC NK CELLS

The retrieval of cells from the pancreas poses particular difficulties, as the organ is difficult to access and it produces digestive enzymes with the potential to destroy the cells during tissue handling. In order to analyze pancreatic NK and T cells (**paper I and II**), we developed a method to minimise contamination of lymphocytes from the blood or adjacent draining lymph nodes. This method includes flushing of PBS through the ascending aorta to empty the blood from the vessels and thereafter dissecting the lymph nodes under microscopy.

The process of retrieving cells from the pancreas includes digestion with collagenase. Careful controls ensured that collagenase treatment did not alter the NK cell expression of the phenotypic markers investigated. Nevertheless, in order to generate meaningful comparisons the NK cells from all tissues were treated with collagenase for the functional experiments, to avoid any unspecific influence of this treatment on the results.

8.2 POLYCHROMATIC FLOW CYTOMETRY

Flow cytometry is a powerful method that allows quantitative evaluation of cell subsets and their phenotype and function (used in **paper I, II, III**). The approximate size and granularity/complexity of the cells are assessed on the basis of how they diffract a laser and fluorochrome-conjugated antibodies allow assessment of the expression of surface bound and intracellular antigens such as cytokines.

The recent years' technical advances in the field now make it possible to perform very complex flow cytometry experiments where many parameters are assessed simultaneously. Along with more advanced staining options, however, comes a greater risk of technical caveats. Particular considerations have therefore been applied when flow cytometry experiments have been designed. For example, doublet cells and dead cells may give unspecific signals and have been excluded from the analysis in most experiments. This is particularly important in tissues such as the pancreas which contains much debris. Furthermore, any unspecific signals due to leakage between the detection channels or background staining of the negative population have been assessed with control samples containing only one antibody, or all but one antibodies [198]. Lastly, to ensure the integrity of the fluorochromes special care has been taken when sensitive tandem conjugates have been used, such as APC-Cy7 and PE-Cy7, including rapid analysis within a few hours after antibody labelling [199].

8.3 THE CD107A ASSAY TO ASSESS NK CELL DEGRANULATION

The traditional way to measure NK cell cytotoxicity *in vitro* involves labelling of target cells with radioactive chromium, incubation of NK cells with target cells and finally measurement of the released chromium as a read-out of destroyed cells. This method

requires relatively large numbers of NK cells and does not allow analysis of NK cell responses on a single cell level. A newer method to indirectly measure NK cell cytotoxicity is to detect surface levels of the *l*ysosomal-*a*ssociated *m*embrane *p*rotein-1 (LAMP-1 or CD107a) [92]. CD107a coats the inner layer of granzyme and perforin containing cytolytic granules, which are fused with the outer cellmembrane when the cell is activated to perform cytotoxicity [93]. Levels of CD107a correlate well with the chromium release assay in detecting cytotoxicity of MHC deficient target cells [92]. However, the intracellular distribution of CD107a and perforin is partially non-overlapping and there is not a complete correlation of NK cell CD107a expression and cytotoxicity in all systems [200]. An advantage with the CD107a assay is nevertheless that it allows concurrent analysis of cytokine production and phenotypic markers (**paper I**).

8.4 BRDU INCORPORATION TO MEASURE PROLIFERATION

Bromodeoxyuridine (BrdU) is a synthetic analogue to thymidine. If BrdU is present during the synthetic (S)-phase of mitosis it will be incorporated into the replicating DNA instead of thymidine. BrdU incorporation therefore defines cells that have passed through the S-phase during the labelling period [201]. BrdU is detected by fluorescent antibodies and analysed with flow cytometry. Harsh fixation and permeabilisation are required for the BrdU antibody to reach between the DNA strands and some cellular antigens may be destroyed by this treatment. To assure a successful and reliable staining of surface antigens in combination with the BrdU, careful testing of the antibody panel with and without the BrdU protocol is mandatory.

Since lymphocytes recirculate, we can not distinguish pancreatic NK cells which have proliferated within the pancreas from those who have proliferated elsewhere and later been recruited into the pancreas (**paper I**). A short time period of BrdU injections reduces the risk that recirculation of lymphocytes blurs the interpretations of the results, and is the reason for the relatively short puls of BrdU in our study. To more fully understand the dynamic processes of proliferation and death of NK cells within the pancreas a careful kinetic of BrdU incorporation in the pancreas, spleen, blood and PLN could be analysed in mathematical models [201].

8.5 CFSE LABELLING TO TRACE CELLS *IN VIVO* AND MEASURE PROLIFERATION

The 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) is a fluorescent dye which can be taken up by cells during incubation *in vitro*. The CFSE labelled cells can then be assessed in *in vitro* cultures or adoptively transferred *in vivo* and traced as in **paper II and III**. CFSE distributes equally between daughter cells during cell proliferation. Therefore the number of divisions the cells have undergone can be measured, where decreased levels of CFSE content correspond to more divisions. It is however not possible to trace the originally CFSE labelled cells individually, and more detailed investigations of cell proliferation require computational modelling [202].

8.6 HISTOLOGICAL IMMUNOFLUORESCENCE

Immunofluorescence and confocal imaging have been performed in **paper I and IV**, to visualize the localization of different cells within the pancreas. The fixation procedure greatly affects the preservation of the tissue and may also destroy antigens. In our studies we have performed transcardial perfusions with a buffer containing 4% paraformaldehyde, creating a rapid fixation and good retention of tissue morphology [203]. Antibody specificity is vital for reliable immunofluorescence results and has

been assessed in various ways. In paper I mice depleted of NK cells were used to determine antibody specificity and control stainings revealed minimal unspecific bindings by the secondary antibodies (data not shown). In paper IV pre-absorption with the antigen prior staining abolished the signal, indicating antibody specificity. Immunofluorescence signals were enhanced by the *t*yramide *s*ignal *a*mplification (TSA) protocol [204].

8.7 NK CELL DEPLETION *IN VIVO*

In vivo administration of antibodies can lead to the depletion of the cells they have bound. This effect is mediated by complement activation and antigen dependent cell-mediated cytotoxicity (ADCC). To deplete NK cells from mice one can use antibodies against NK1.1 (PK136), the glycolipid asialo-GM1 and IL-2/15Rβ (TMβ1) [33, 205-207]. Unfortunately none of these antibodies are completely NK cell specific, and subsets of T cells are also depleted. The anti-NK1.1 antibody only causes minimal reduction of cytotoxic T cell responses and is therefore the most commonly used depleting NK cell antibody. NK1.1 is however not expressed in many mouse strains, including the NOD mouse. The TMβ1 antibody affects cytotoxic T cell responses to a less extent than asialo-GM1 [33], and was therefore chosen for our studies (**paper II**).

9 RESULTS AND DISCUSSION

In the current study several aspects of the complex routes leading to T1D have been investigated, including both cellular (**paper I and II**) and molecular (**paper III**) events. The priming of pathogenic T cells in the draining lymph nodes has been studied (**paper II and III**) as well as the pancreatic NK cell compartment (**paper I**). As a follow up on paper I, **paper II** represents an attempt to prevent T1D by depletion of a small subset of lymphocytes. In **paper IV,** a novel anorexogenic protein initially described in the CNS has been studied in the pancreatic islets of healthy and T2D rats and in human islets.

Paper I-IV can be read in full at the end of this thesis. Here I will describe the main findings from the papers. I will also discuss these findings in a broader context, especially in relation to the most recent studies in the literature.

9.1 PANCREATIC NK CELLS

The impact of the tissue in shaping immune responses by NK cells is an area of research which currently attracts much attention [4]. Microenvironmental properties could affect the activation and maturation of recruited NK cells. Specialised subsets of organ-residing NK cells exist, and NK cell development from NK cell precursors has even been described to occur at sites outside the bone marrow [82]. Given the unique properties of NK cells in organs, we believe that it is crucial to focus more efforts at understanding organ-specific immunity, under physiological conditions as well as in disease. A better understanding of what influences NK cell subset specialisation, migration and function could possibly generate tools to selectively modulate the homing of certain NK cell subsets to specific organs.

Studies of NK cells in the NOD mouse render some particular precautions. It is for example interesting to note that the NOD mouse has an unusually large repertoire of activating Ly49 receptors, including some Ly49 receptors rarely seen in other strains [208]. One interesting possibility is that the NOD mouse NK cell receptor repertoire resembles the human KIR haplotypes termed “B”, characterised by many activating receptors [209]. The lack of NK1.1 receptor expression in certain strains, including the NOD mouse, limits the available tools for detection and manipulation of NK cells [33].

9.1.1 NK cells in healthy organs

NK cells have been described in healthy tissues, including the liver, gut, uterus and, by us, the pancreas [83] (**paper I**). The relatively novel concept of organ-resident NK cells is thought-provoking and invites speculation as to which role these NK cells may have at these sites. In organs where large numbers of pathogens are encountered, such as in the gut, NK cells could play a role as an early defence against invading bacteria. The NK cell-like lymphocytes that have been described recently in both human and mouse gut, are believed to develop under the influence of commensal bacteria and may be important in the response of certain pathogens [42-44, 210]. Uterine NK cells also represent an NK cell subset with very distinct demands, in order not to reject the foetus [123]. Yet another organ with NK cells under healthy conditions is the liver which is a site for haematopoietic development during foetal and early life. Although phenotypically immature and functionally impaired compared to conventional NK cells, the liver NK cells can mediate cytotoxicity via TRAIL (tumour necrosis factor related apoptosis inducing ligand) [211]. The role of the liver NK cells is not clear. One hypothesis, based on the finding that IFN-α and IFN-γ induce TRAIL expression is that these NK cells serve as inducible protection against pathogens or tumours [83].

We surprisingly found pancreatic NK cells in the non diabetes prone mouse strains (figure 6, **paper I**). Their role is not clear, but hypothetically they may be positioned in the pancreas to search for pathogens, although symptomatic infections in the pancreas are rare. Another possibility is that the pancreatic NK cells modulate local blood vessel growth, similar to uterine NK cells [123]. Although highly speculative, completely novel functions of pancreatic NK cells visavi the surrounding tissue could be envisioned, such as effects on hormone production or exocytosis.

9.1.2 Pancreatic NK cells during the progression to T1D

We decided to explore if NOD mouse pancreatic NK cells bear a specific phenotype, possibly reflecting that they play a role locally during the progression to T1D. The presence of NK cells within the pancreas was assessed in the pathogenesis to T1D, on tissue sections with immunofluorescence and in single cells suspensions with flow cytometry. For the analysis, a protocol was developed to retrieve lymphocytes with minimal contamination from blood vessels or the closely located PLN (see section 8.1). We found that NK cell infiltrated the pancreas of NOD mice at an early age, were detectable in the islets before T cells (figure 1, **paper I**) (**Figure 4**) and could home to the pancreas in the absence of a previous T cell-driven destruction of the tissue (figure 7, **paper I**), suggesting that pancreatic NK cells could have a role in the early stages of T1D development.

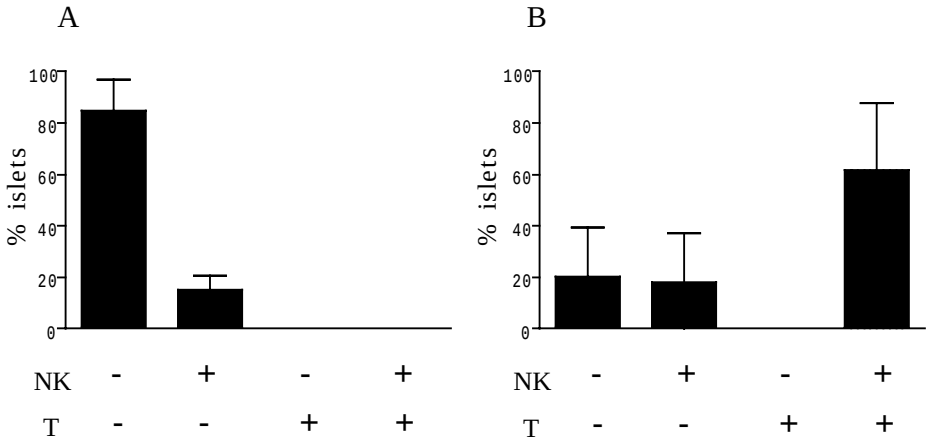

Figure 4. *The proportion of islets with infiltrating NK and/or T cells within the 4-5 week-old (A) and 9-10 week-old (B) NOD mouse pancreas.*

Interestingly, the NOD mouse pancreatic NK cells displayed a phenotype that differed from both spleen and PLN NK cells (figure 2, **paper I**). They showed clear signs of activation and the early activation marker CD69 was upregulated on the pancreatic NK cells already at 4 weeks of age, further supporting the notion that NK cells are involved in early events of the T1D pathogenesis (figure 4, **paper I**). Another surface marker upregulated on pancreatic NK cells is KLRG1. This is an inhibitory receptor which increases in expression on NK cells after activation and proliferation [94-96], implying that the pancreatic NK cells had been stimulated. Curiously, KLRG1 represents the only known phenotypic marker correlating with NK cell responsiveness after education [212, 213], suggesting that pancreatic NK cells may be have different functional properties due to altered education during their development or in the pancreas.

9.1.3 Functional properties of pancreatic NK cells

The activated phenotype and high expression of KLRG1 indicated that proliferation and maturation had occurred [94-96]. Indeed, there was a significantly higher BrdU

incorporation in NK cells from the pancreas than other organs (figure 3, **paper I**). NOD mice pancreatic NK cells also had a higher KLRG1 expressing fraction and increased proliferation as compared to non-diabetes prone strains. This indicates that proliferation is linked to conditions in the pathogenesis of T1D in the NOD mouse rather than being a general property of pancreatic NK cells.

We observed profound hyporesponsiveness in NOD mice pancreatic NK cells in response to stimulation *in vitro* (figure 5, **paper I**). In contrast, in the B6 mice the pancreatic NK cells responded equally well as spleen cells (**figure 5**). The hyporesponsiveness observed in NOD mice could be an inherent property of the pancreatic NK cell subset or alternatively result from exhaustion or be the consequence of active downregulation by other cells or factors.

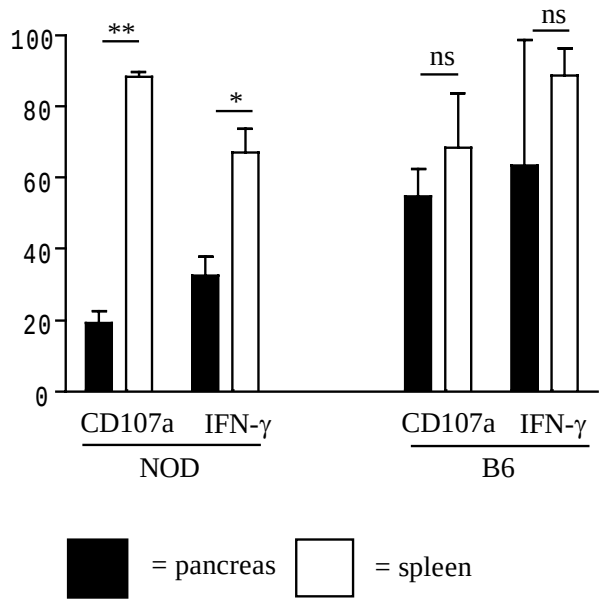

Figure 5. *A comparison between the intracellular IFN-γ and degranulation in NOD and B6 mouse in response to PMA and ionomycin stimulation.*
** = p<0.05, ** = p<0.01.*

Interestingly, a fraction of the pancreatic NK cells produced IFN-γ spontaneously, possibly reflecting an *in vivo* activation. Based on this finding and the in part similar phenotype reported for exhausted T cells [214], we propose a model where NK cells are activated and proliferate within the pancreas during the progression to T1D (**figure 6**). We believe that the NK cells participate in the pathogenesis, with for example IFN-γ production. Ultimately, however, they become exhausted, accounting for their hyporesonsiveness. In support for our theory is our observation that IL-15 and IL-18 restored the IFN-γ producing capacity in the pancreatic NK cells, arguing that the hyporesponsiveness is not an inherent property of these cells. Interesting in this respect, a defect in the *il15* gene was recently described in the NOD mouse [215] and supplementation of with IL-15/IL-15Ra complexes *in vivo* increased the frequency of NK cells and their cytotoxic ability and thereby restored their function.

Since we were not able to restore the GM-CSF producing capacity of pancreatic NK cells, we must consider other, not necessarily mutually exclusive, explanations for the

hyporesponsiveness. One such possibility is that at least a part of the inability to produce large amounts of cytokines represents an intrinsic property of the pancreatic NK cell subset. Reduced functional abilities could also be the consequence of active downregulation of specific effector functions, e.g. by regulatory cells acting locally in the pancreas. Recent data supporting this model comes from studies in BDC2.5/NOD mice with the *foxP3* gene under the diphtheria toxin (DT) receptor [173]. In this model, the $FoxP3^+$ Tregs are selectively ablated upon DT administration. The earliest and most striking effects of Treg ablation in the pancreas were on the NK cell compartment which expanded, became activated and provided large quantities of IFN-γ which was instrumental for $CD4^+$ T cell activation, destruction of beta cells and rapid development to diabetes [173].

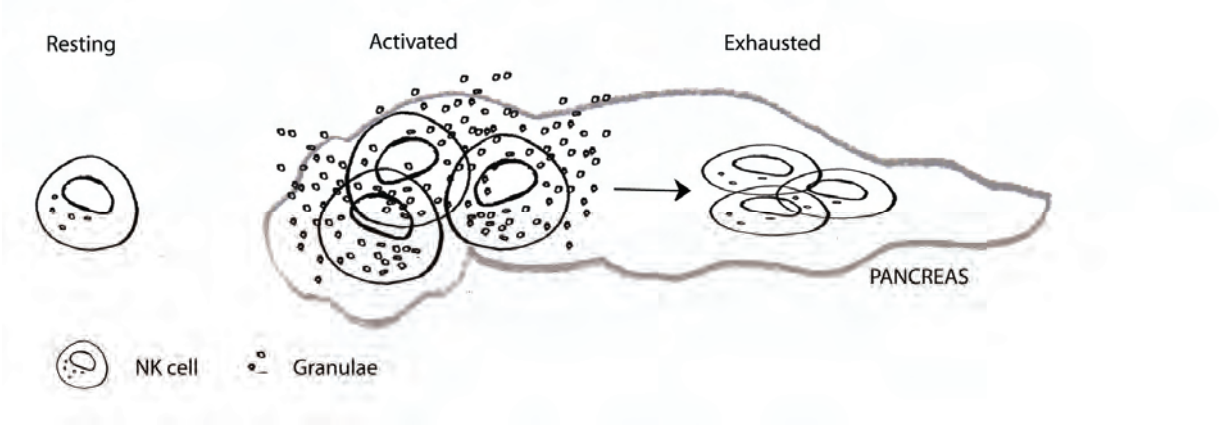

Figure 6. *Our proposed model for the function of pancreatic NK cells in NOD mice.* ***1.*** *Resting NK cells in the circulation.* ***2.*** *Newly arrived NK cells in the pancreas become activated, proliferate and secrete cytokines/cytotoxic granules. This leads to the upregulation of activation markers and KLRG1. If NKG2D receptor interaction occurs during this phase this receptor is also downregulated.* ***3.*** *Ultimately the NK cells become exhausted and unable to respond strongly to stimulation.*

9.1.4 Receptors for interaction with beta cells

A direct role for NK cells in the beta cell destruction could also be envisioned, for example mediated by the NK cell receptors PD-1 and NKG2D whose ligands are expressed on beta cells [140, 141] (figure 2, **paper I**). The NKp46 receptor was recently presented as another possible way by which NK cells could interact directly with beta cells and influence T1D development [216]. The ligand(s) for NKp46 is not known, but immunofluorescence with NKp46 and NCR1-Ig fusion proteins stained positive in the beta cells in the pancreas of both mice and humans, thereby presenting a new possible mechanism of NK cell mediated beta cells cytotoxicity.

9.1.5 Origin of pancreatic NK cells

Considering the possibility that pancreatic NK cells could contribute to T1D, a better understanding of the origin of these cells is required. Such studies could be of high clinical relevance as prevention of the development or migration of pathogenic NK cell subsets could represent a future treatment. From our studies we know that the NOD mouse spleen contained NK cells or precursors with the ability to home to the pancreas of NOD mice lacking T and B cells (figure 7, **paper I**).

9.1.6 Conclusions and future directions

During the past years NK cell subsets have been described in several organs, including the gut [43-45], the uterus [123, 125] and the liver [217, 218]. We describe pancreatic NK cells as a distinct subset. Their phenotype and function imply that they may be involved in the pathogenesis of T1D. The properties of pancreatic NK cells differ from all other tissue specific NK cell subsets described so far, suggesting that it accounts for a unique subset. Our work, together with other recent advances in the field, suggests a wider distribution and function of NK cells than previously estimated. One interesting possibility is that early NK cell precursors reside in the pancreas and can develop in this tissue, as has been shown for the lymph nodes and thymus [81, 82]. If NK cells do not mature in the pancreas they must be recruited from the circulation. Therefore, another relevant question is which chemokines and chemokine receptors that influence migration to the pancreas.

Based on the distinct properties of pancreatic NK cells we hypothesised that they would be involved in the pathogenesis of T1D. To test this hypothesis we developed a protocol to deplete NK cells in pancreas, PLN and spleen of NOD mice (**paper II**).

9.2 PREVENTION OF T1D BY TMB1 DEPLETION

In study II we sought to modulate development to T1D by lymphocyte subset depletion. Based on our findings in paper I and other recent work both in humans and mice, we believed that NK cells could play a pathogenic role in diabetes development [151, 180, 185]. We therefore primarily aimed at depleting NK cells from NOD mice. The most NK cell specific depleting antibody available to date for use in the NOD mouse is directed against the β subunit of the IL-2/15 receptor (TMβ1) [33]. All NK cells have a high expression of this marker (called CD122) and previous studies have established this treatment as an effective way to remove NK cells from the circulation [207]. CD122 is also expressed on a small subset of $CD8^+$ and $CD4^+$ memory and regulatory T cells [34], but the depletion of these subsets is not reported to affect adaptive immune responses in any great deal [33, 207].

Long-term treatment with 200 μg TMβ1 i.p. once weekly completely protected from T1D (figure 1, **paper II**). TMβ1 treatment protected even when initiated late, close to when T1D usually occurs in NOD mice (figure 2, **paper II**) (**figure 7**). This indicated that the effect of the treatment was on later stages in the pathogenesis, subsequent to the development of insulitis. Lymphocytes infiltrated the pancreas despite TMβ1 treatment, further arguing that late events of the pathogenesis were targeted by the TMβ1 treatment.

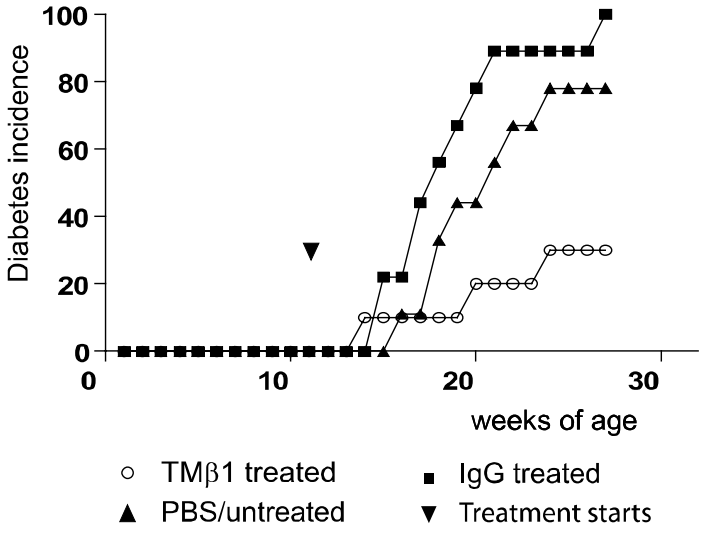

Figure 7. *Diabetes incidence in NOD mice treated with TMβ1 (200 μg i.p. once weekly), PBS or rat IgG from 12 weeks onwards. There was a statistical difference ($p<0.01$) between the rat IgG treated and the TMβ1 treated group at the end of the experiment.*

9.2.1 Effects on NK cells and T cells

As expected from the high expression of CD122 on NK cells, TMβ1 treatment completely depleted the NK cells from the pancreas, PLN and spleen and abolished NK cell function (figure 3-5, **paper II**).

In addition, a fraction of $CD8^+$ T cells in the spleen, but not in the pancreas, expresses CD122 and approximately 75% of these were depleted by the TMβ1 treatment (figure 4, **paper II**). The spleen $CD8^+$ T cell population as a whole was however not

significantly decreased after TMβ1 treatment, probably reflecting the relatively small size of the removed population. Despite the depletion of a subset of $CD8^+$ T cells, the gross $CD8^+$ T cell response against a non-diabetogenic antigen was unaltered and T cell priming in the PLN was not affected (figure 5, **paper II**). Although the pancreatic $CD8^+$ T cells lacked CD122 expression, this subset surprisingly decreased after TMβ1 treatment (figure 3-4, **paper II**). One possible explanation for this is that pancreatic $CD8^+$ T cells are dependent on cytokines produced by the NK cells for their survival. Thereby, depletion of NK cells would indirectly cause a reduction of the $CD8^+$ T cells. An alternative possible explanation to this paradoxical finding could be a high influx of $CD122^+$ $CD8^+$ T cells into the pancreas and a rapid downregulation of CD122 at the pancreatic site. A peripheral depletion of $CD122^+$ $CD8^+$ T cells would then interfere with the availability and entry of $CD8^+$ T cells, leading to a decreased total population of $CD8^+$ T cells in the pancreas.

TMβ1 treatment affected both the NK cells and $CD8^+$ T cells. As of now we can not firmly conclude the relative contribution of these lymphocyte subsets to the pathogenesis of T1D and it is possible that both cell types participate in the beta cell cytotoxicity.

9.2.2 Role of $CD122^+$ $CD8^+$ T cells in T1D

A subset of activated $CD8^+$ $NKG2D^+$ $CD122^+$ T cells could be detected in the periphery of NOD mice (figure 3 and S1, **paper II**). This is intriguing since NKG2D blockade in NOD mice protects from T1D in a very similar way as we see with TMβ1 treatment [140] (figure 1-2, **paper II**). One speculation is therefore that this subset of T cells could mediate important pathogenic effects. Since all NK cells express NKG2D, the disease protection by NKG2D blockade could however also be dependent on NK cells.

9.2.3 Role of NK cells in T1D

Although a subset of T cells was depleted by the TMβ1 treatment, we did not observe any general T cell suppression, at least not with respect to $CD8^+$ T cell responses against a non-diabetogenic antigen, T cell priming in the PLN or the T cell activation status in the pancreas. This in combination with the complete abolishment of NK cell numbers and function make us believe that the depletion of NK cells is at least in part responsible for the protection from T1D in our system. The literature on the role of NK cells in T1D is contradictory. A protective role for NK cells has been suggested in NOD mice treated with complete Freund's adjuvant (CFA). This treatment usually protects from T1D but in the absence of NK cells these animals develop disease [219]. On the contrary, a pathogenic role was shown BDC2.5/NOD mice treated with anti-CTLA-4 antibody or in NOD mice with transgenic expression of IFN-β in the beta cells [184, 185]. Further supporting a pathogenic role is recent data showing that mice lacking the NKp46 receptor are more resistant to streptozotocin induced T1D [216]. Activation of NK cell within the pancreas was also responsible for the rapid precipitation of T1D following Treg cell ablation in BDC2.5/NOD mice [173].

The contradictory results on the role of NK cells in T1D could be explained by a dual role for NK cells, acting both as cytotoxic and immune regulatory cells [39, 151, 220] (**figure 8**). NK cells could promote T1D for example by beta cell cytotoxicity or activation of DCs. NK cells could provide an early source of for example IFN-γ in the pancreas or the PLN and thereby influence the adaptive immune response [221]. In addition, although NK cells are important producers of IFN-γ they also have the capacity to produce Th2 inducing cytokines such as IL-10, IL-5 and IL-13, which could protect from T1D [222-224].

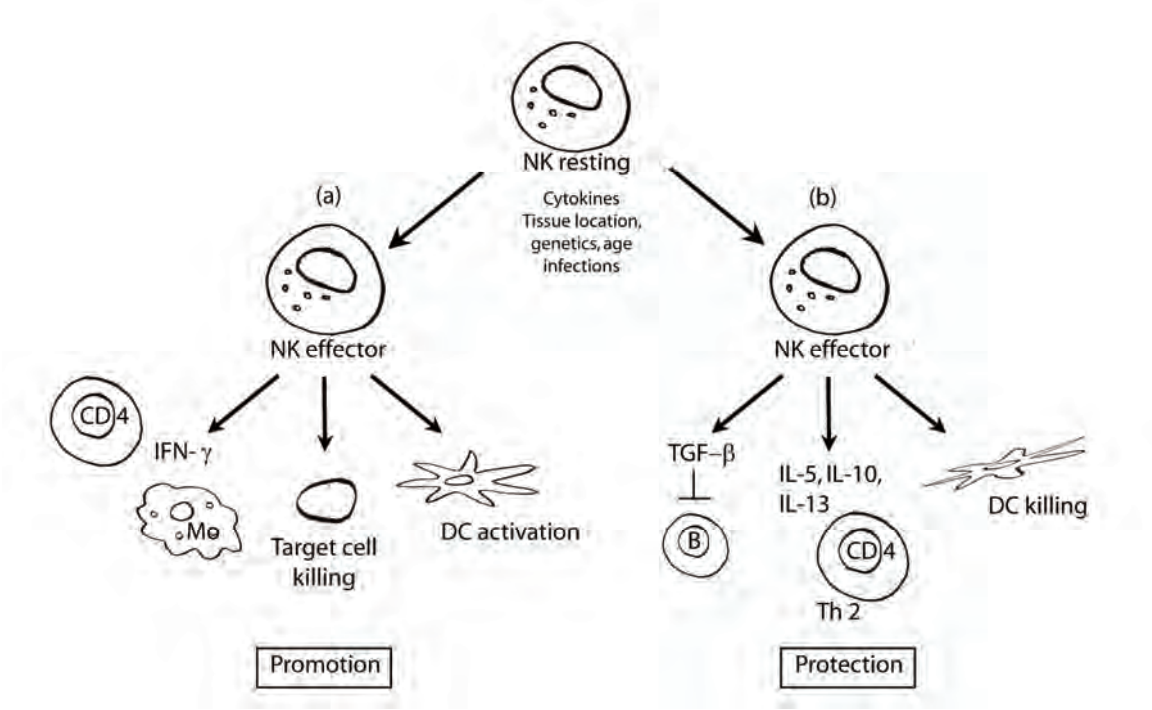

Figure 8. *Proposed dual roles for NK cells during the pathogenesis in autoimmune disease: both promoting* ***(a)*** *and protecting from* ***(b)*** *disease.*
(Adapted from Johansson et al. TRENDS in immunology 2005)

9.2.4 Conclusions and future directions

Depletion of CD122 positive cells by treatment with a monoclonal antibody protected from T1D in NOD mice. NK cells and a subset of $CD8^+$ T cells were depleted by this treatment. Although the exact mechanisms behind the disease preventive effect by TMβ1 depletion were not established, the results are promising from a clinical point of view. TMβ1 treatment represents an immunological treatment of T1D in the NOD mouse which is effective late in the disease process and only affects the immune response in a relatively limited fashion. In future studies it will be important to address the relative contribution of the NK cells and $CD8^+$ T cells in the T1D protection in this model, for example by transfer studies with different subpopulations of immune cells sorted away.

9.3 DAP12 IN T1D PATHOGENESIS

The adaptor molecule DAP12 is required for the signalling of multiple activating receptors on NK cells, monocytes, macrophages, dendritic cells, mast cells and some T, NKT and B cells [100].

9.3.1 DAP12 in autoimmune disease

DAP12 mutations in humans affect the CNS and DAP12 deficiency in mice is paired with CNS hypo-myelinisation [225]. Most studies on the functional role of DAP12 have therefore focused on diseases in the CNS, such as multiple sclerosis (MS) and the MOG peptide induced experimental autoimmune encephalomyelitis (EAE). The role of DAP12 in EAE is controversial [105, 226, 227]. Initial studies showed that DAP12 deficient mice were resistant to EAE. Inefficient priming of the autoreactive T cells was suggested to be responsible for the protection, since the T cells in the draining lymph node displayed lower proliferation and IFN-γ production [105]. Contrary to these results, DAP12 signalling was associated with prevention of disease in later studies. Blockade of one of the DAP12 associated receptors, TREM2, led to aggravated EAE [227] and TREM2-transducted myeloid precursor cells conferred protection from EAE when adoptively transferred at the onset of disease [226]. One proposed mechanism was that that TREM2-expressing macrophages or microglia are important in facilitating phagocytosis and clearance of apoptotic cells in the CNS, thus limiting the accumulation of antigen for priming of autoreactive T cells [226, 227]. The TREM2-transduced myeloid cells also produced anti-inflammatory cytokines, suggesting a possible involvement in limiting the immune response and tissue repair [226]. The opposing results on the role of DAP12 in EAE may be explained by the different animal models, with a complete abolishment of DAP12 signalling in the first study but deficiency of only one of the DAP12 associated receptors, TREM2, in the latter studies. Taken together, DAP12 could influence EAE at several steps in the pathogenesis, possibly via receptors and cells with both protective and aggravating functions [228].

A potential role of DAP12 in human autoimmunity still remains uncertain. Variations in the genes for DAP12 or TREM2 are unlikely to play a role in the susceptibility to MS [229]. Altered levels of DAP12 have however been measured in patients with autoimmune disease and studies have shown unaltered or decreased DAP12 expression in the NK cells of patients with SLE [99, 230]. Another disease with autoimmune manifestations is *l*arge *g*ranular *l*ymphocyte *l*eukaemia (LGLL), characterised by clonal expansion of $CD8^+$ T cells or NK cells [231]. These patients suffer from neutropenia, anaemia and autoimmune manifestations, such as rheumatoid arthritis (RA) and pulmonary artery hypertension (PAH) and have elevated DAP12 levels compared to healthy controls [232]. Up- or downregulated DAP12 expression in lymphocytes is however hard to interpret and could be the consequence, rather than the cause, of the disease. In conclusion, the possible role of DAP12 in human autoimmunity is not clear and merits further studies.

9.3.2 Possible mechanisms of DAP12 involvement in T1D

Prior to our studies, DAP12 deficiency had only been shown to cause resistance to MOG induced EAE, as described above [105]. Since both EAE and T1D are organ-specific autoimmune diseases, we hypothesised that DAP12 deficiency would lead to prevention also of T1D. To assess this, we introduced the DAP12 loss of function mutation [106] onto BDC2.5/$B6^{g7}$ mice, a TCR transgenic model of accelerated T1D

[21]. Surprisingly however, absence of DAP12 led to increased incidence of T1D (figure 1, **paper III**) (**figure 9**).

There are several possible scenarios for how DAP12 could influence T1D pathogenesis. For example, impaired phagocytotic clearing of apoptotic beta cells by DAP12 deficient macrophages could increase the availability of autoantigens [226], leading to amplified activation of autoreactive T and B cells. Altered DAP12 signalling in APCs could also lead to decreased activation and maturation after antigen encounter, affecting migration to the PLN or altering APC interactions with T cells. Together, such changes could affect the priming of autoreactive or regulatory T cells in draining lymph nodes or in tissues. An intrinsic effect of DAP12 on autoreactive T cells in T1D is also possible, since DAP12 is expressed on a subset of T cells.

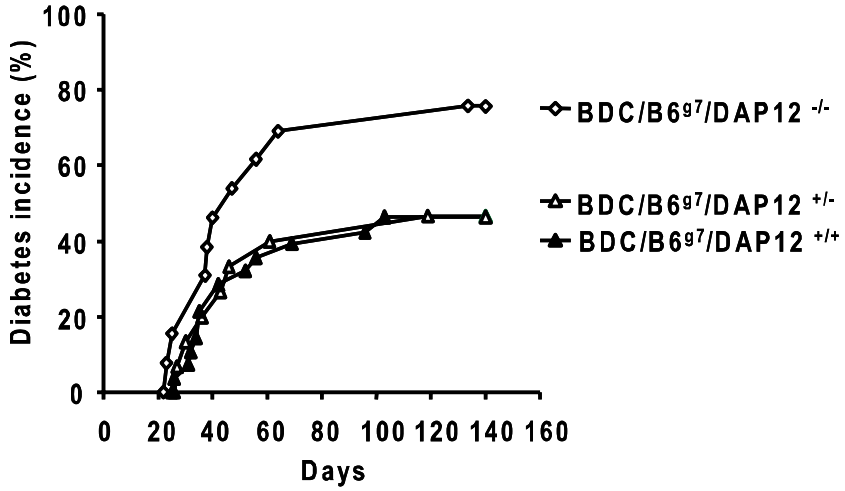

Figure 9. *Effect of DAP12 signalling on diabetes development in BDC2.5/B6^{g7} mice. The graph shows cumulative diabetes incidence in BDC2.5/B6^{g7}/DAP12$^{-/-}$, BDC2.5/B6^{g7}/DAP12$^{+/-}$ and BDC2.5/B6^{g7}/DAP12$^{+/+}$ mice.*

Based on our data from paper III, we propose a model where DAP12 deficiency results in impaired activation of Tregs in the PLN, which in turn leads to insufficient suppression of autoreactive T cells at this site. This allows for enhanced priming of BDC2.5 T cells in the PLN and accelerated disease. Although this hypothesis is not conclusively proven by the results in paper III, we base our model on the following observations: Autoreactive BDC2.5 T cells had a higher expression of the activation marker CD69 and proliferated more in the PLN in absence of DAP12 (figure 2 and 5, **paper III**), indicating that they had been more efficiently primed. Although DAP12 is expressed in some T cells [100], we do not believe that the effects are directly on the autoreactive T cells in our system. We conclude this since the adoptively transferred T cells were obtained from mice with normal DAP12 signalling and the defective function of DAP12 was thus present only in the host cells.

Instead, the increased priming of autoreactive T cells in the PLN could either be due to enhanced presentation of antigen by APCs or defective suppression by for example Tregs. Since DCs are important APCs in the pathogenesis of T1D we investigated if any alterations could be noted among these cells. We found that in the absence of DAP12 there was an increased frequency of plasmacytoid DCs in the PLN (figure 4, **paper III**) which is in line with earlier observations [233]. We speculated that the

phenotypic changes possibly reflected impaired priming ability of the DCs. However, when we tested the capacity of the PLN resident DCs to prime BDC2.5 T cells *in vitro*, wild type and DAP12 deficient DCs primed T cells equally well, indicating that the absence of DAP12 in DCs likely does not affect T cell priming. An alternative possibility would be that Treg suppression of T cell priming in the PLN would be altered [171], causing the accelerated T1D development in DAP12 deficient mice. To assess this we performed adoptive transfer experiments. Spleen cells, or Treg depleted spleen cells, were transferred to wild type $B6^{g7}$or DAP12 deficient $B6^{g7}$ mice. As expected, the presence of Tregs in the inoculum diminished BDC2.5 T cell proliferation in the PLN after transfer into $B6^{g7}$ mice. Interestingly, this was not the case in the DAP12 deficient host, where the co-transfer of Tregs failed to inhibit proliferation of the BDC2.5 T cells (figure 6, **paper III**). This suggests that insufficient Treg suppression of autoreacive T cells is responsible for the increase in T1D pathogenesis in DAP12 deficient mice, possibly as a result of altered antigen presentation by DC. Our study is the first to suggest a link between DAP12 and Tregs in the PLN in autoimmunity.

9.3.3 Conclusions and future directions

The abolishment of DAP12 signalling leads to increased diabetes in the BDC2.5/$B6^{g7}$ model for T1D. Our data do not fully explain the mechanism by which DAP12 acts to protect from disease, but suggest that it may be due to insufficient suppression by Tregs on the priming of autoreactive T cells in the PLN. In future studies the relative contribution of the DAP12 associated receptors and immune cells in the PLN will have to be investigated. One exciting possibility would be to closer investigate the interactions in the PLN between DC, Tregs and T cells *in vitro*. Assessment of the cytokines produced in T cells primed by DAP12 deficient cells could further provide important insights to the mechanisms involved in this system. In light of our studies on pancreatic NK cells and the role of NK cells in T1D (paper I and II), closer investigations on the NK cells in these mice would similarly be interesting.

9.4 NUCLEOBINDIN-2 IN THE PANCREAS

At the initiation of the studies in paper IV, nucleobindin-2 had recently been identified as a protein with anorexogenic effects and had mainly been described within the central nervous system (CNS) [8]. In **paper IV** we investigated nucleobindin-2 in the endocrine pancreas of humans and rats and in relation to T2D and glycaemic state. The distribution and function of this protein outside CNS attracted the interest of several research groups, and our work is accompanied by other studies of high relevance to our findings [234, 235].

9.4.1 Nucleobindin-2 in the pancreas

The protein nucleobindin-2 modulates energy homeostasis by effects in the hypothalamus [8]. This made us speculate that it could influence also glucose homeostasis in the pancreas and we decided to study nucleobindin-2 in the pancreas. Indeed, we detected nucleobindin-2 in the pancreatic islets in humans and rats (figure 1-4, **paper IV**) (**figure 10**). The presence of nucleobindin-2 in the pancreas is in line with two recent studies in rats and mice [234, 235]. When further analysing the cellular distribution of nucleobindin-2 in the pancreas we found it selectively expressed in the beta cells. No nucleobindin-2 could be detected in the cells containing somatostatin, glucagon or pancreatic polypeptide (PP). Our finding that nucleobindin-2 can be detected also in human pancreatic islets open up for a possible clinical significance of studying this protein in the pancreas. In addition, nucleobindin-2 was recently identified in human CNS [236] and plasma [237].

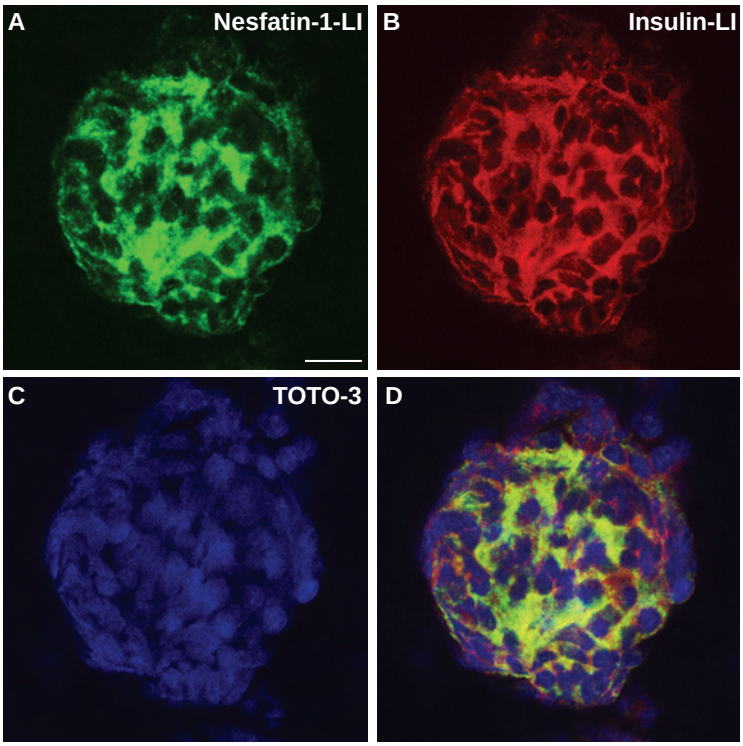

Figure 10. *Confocal micrographs from human islet processed for immunofluorescence triple-staining for nucleobindin-2 (A), insulin (B) and the nuclear marker TOTO-3 (C). Merged image shown in (D).LI=like immunofluorescence. Scale bar = 20µm.*

Intriguingly, the islets retrieved from the Goto-Kakizaki (GK) rats, an animal model of T2D, had lower nucleobindin-2 content than control rats (figure 4, **paper IV**). Following overnight fast however, the nucleobindin-2 levels in the GK rat islets increased to levels similar to control islets. For the interpretation of these data it is

important to note that the beta cell mass in the colony of GK rats used for our studies have been shown not to differ from that of control rats [30]. This confirms that decreased nucleobindin-2 levels are not simply a consequence of fewer beta cells.

Nucleobindin-2 has been described also in the endocrine-cell-rich part of the gastric mucosa [234]. In relation to our results it is important to note that mRNA levels of nucleobindin-2 in these cells decrease following fasting. The discrepancy between these results and ours may be due to the different techniques used to detect nucleobindin-2, possibly reflecting kinetic differences in RNA and protein synthesis. Another interesting possibility is that the regulation of nucleobindin-2 differs between different endocrine cells.

9.4.2 Regulation of nucleobindin-2 by glucose

The co-distribution of nucleobindin-2 and insulin in the beta cells led us to hypothesise that nucleobindin-2 similar to insulin is released from the beta cells. Therefore, we investigated whether nucleobindin-2 is released from pancreatic islets and if so, whether glucose is a regulator. Isolated islets from GK and control rats where incubated with 3.3 mM *vs* 16.7 mM glucose and a slight increase in nucleobindin-2 release was detected from the islets incubated with high concentration of glucose (figure 5, **paper IV**), as measured by EIA. A similar trend was observed in islets from both GK and control rats, but reached statistical significance only in the latter. There was, however, no significant difference between the strains. The amount of secreted nucleobindin-2 was also markedly lower than the amount of insulin.

In line with the glucose induced nucleobindin-2 release from islets *in vitro,* we speculated that serum levels of nucleobindin-2 would increase following intraperitoneal glucose tolerance test (ipGTT). Surprisingly, the serum concentration of nucleobindin-2 after ipGTT paradoxically decreased in both GK and control rats (figure 6, **paper IV**). We interpret this as if serum nucleobindin-2 most likely does not primarily originate from the pancreatic beta cells. A recent human study showed no significant changes of serum nucleobindin-2 after oral glucose ingestion and T2D patients had slightly decreased levels [237]. Clearly, the regulation of serum nucleobindin-2 merits further studies.

Of interest for T2D is also the recent finding that an oral anti-diabetic drug, troglitazone, markedly stabilizes nucleobindin-2 mRNA [238]. This provides a novel mode of action for this kind of drugs, the thiazolidinediones, and emphasises the possibility that nucleobindin-2 is involved in T2D pathogenesis.

9.4.3 Conclusions and future directions

We have for the first time detected the anorexogenic protein nucleobindin-2 in the human pancreatic beta cells. Although nucleobindin-2 was released from pancreatic islets in response to glucose *in vitro*, the existence and possible role of *in vivo* secretion is still uncertain. Further explorations of the role of nucleobindin-2 specifically in the pancreatic islets could reveal its role within the beta cells and potentially be of importance also for the understanding of T2D. Nucleobindin-2 has recently been shown to be released from adipose tissue following stimulation with glucose, IL-6 or TNF-α *in vitro* [239]. One interesting possibility to explore further is therefore that cytokines modulate nucleobindin-2 also in the pancreas.

10 CONCLUDING REMARKS

The development of new treatments for diabetes represents a major health challenge and requires better understanding of the pathogenic processes.

In the present investigation novel approaches have been applied to gain knowledge in the field of T1D and T2D. In our studies of the pathogenesis of T1D we have focused on the pancreatic immune response, the still controversial role of NK cells and the adaptor protein DAP12. Taken together our results stress the importance of focusing future studies on the interactions between the immune response and non-immune cells. This scientific approach would be relevant not only in organ-specific autoimmune diseases but also solid tumour malignancies and possibly physiologic events. Our results also support the belief that NK cells are indeed pathogenic in T1D. Modulations of NK cells or subsets thereof could therefore possibly represent an attractive future treatment of T1D, with only limited effects on the immune system. Future studies will have to explore this field further and the implementation of such immune therapy is also critically dependent on the definition of better biomarkers to detect individuals at risk. We further conclude that the novel anorexogenic protein nucleobindin-2 is expressed in human pancreatic islets and may be an important mediator to further explore in diabetes. Generally, the interactions between the immune, endocrine and nervous system in diabetes is an emerging and fascinating field which merits further investigations.

11 SAMMANFATTNING PÅ SVENSKA

Diabetes drabbar ca 5% av världens befolkning och skapar lidande, allvarliga senkomplikationer och stora samhällsekonomiska kostnader. Typ 1 diabetes (T1D) beror framför allt på en T cells-medierad autoimmun destruktion av de insulin-producerande beta-cellerna i pankreas. Typ 2 diabetes (T2D) är istället en metabol sjukdom där otillräcklig insulinfrisättning i kombination med sänkt insulinkänslighet i lever, muskel och fett-celler leder till hyperglykemi. Båda sjukdomarna uppstår till följd av multipla genetik- och miljö-faktorer och de exakta patogena mekanismerna är inte helt klarlagda.

Vi har framför allt studerat immunpatogenesen vid T1D i den non-obesa diabetiska (NOD)-musen. NOD-musen utvecklar spontant en autoimmun sjukdom som liknar människans T1D. Vi har undersökt rollen för natural killer (NK) celler i utvecklandet av T1D och identifierat en unik subgrupp av NK celler i pankreas (bukspottkörteln) hos NOD-möss. De pankreatiska NK cellernas egenskaper tyder på att de är aktiverade, mogna och eventuellt utmattade (**artikel I**). En möjlig hypotes är att de är effektorceller i den autoimmuna processen. Pankreatiska NK celler deltar då i patogenesen genom att bilda proinflammatoriska cytokiner samt orsaka beta-cell-skada, varefter de tröttas ut. Något som stärker våra misstankar att NK cellerna är patogena vid T1D är att när NK cellerna tas bort från NOD-musen *in vivo* skyddar det mot T1D (**artikel II**). Vi har även studerat rollen för signalleringsproteinet DAP12 i utvecklingen av T1D. DAP12 uttrycks brett i immunsystemet och associerar till aktiverande receptorer framför allt i NK celler och myeloida celler såsom dendrit-celler och makrofager. Avsaknad av DAP12 ökar insjuknandet i T1D i en förenklad musmodell för T1D (**artikel III**). Våra resultat tyder på att det beror på en minskad effekt av de T regulatoriska cellerna, vilket leder till en ökad aktivering av autoreaktiva T celler i de pankreatiska lymfknutorna och kraftfullare immundestruktion av de pankreatiska beta-cellerna.

I likhet med T1D är processer i beta-cellerna mycket viktiga för utvecklingen av T2D, men de patogena mekanismerna skiljer sig åt. Flera av de proteiner och peptider som kontrollerar energibalansen i det centrala nervsystemet (CNS) återfinns också i pankreas. Nucleobindin-2 är ett protein som uttrycks i stora delar av CNS och som nyligen visats reglera energibalansen i kroppen genom effekter i CNS. Vi har visat att nucleobindin-2 finns i de pankreatiska beta-cellerna hos människa och råtta (**artikel IV**). Nivåerna av nucleobindin-2 påverkas av glukos, men rollen för nucleobindin-2 i beta-cellerna måste utredas vidare.

Sammanfattningsvis har vi studerat flera möjliga patogena mekanismer vid diabetes. För T1D har vi identifierat de pankreatiska NK cellerna som en viktig subgrupp av NK celler och sett att NK celler troligen är patogena vid T1D. Dessutom visar vi att avsaknad av signalleringsproteinet DAP12 leder till accelererad T1D. Vi har också för första gången identifierat neuropeptiden nucleobindin-2 i humana beta-celler och undersökt hur den påverkas av glukos och T2D. Vi hoppas att våra resultat ska bidra till en ökade grundläggande förståelse för diabetes-patogenesen, som är så viktigt för utvecklandet av framtida behandlingar.

12 ACKNOWLEDGEMENTS

The work with this thesis has been a fantastic experience. Thank you all who have contributed to my work during these years! I would especially like to mention

My supervisors **Petter Höglund** and **Klas Kärre**. I have really enjoyed learning from your broad and deep knowledge on NK cell biology and immunology, your profound passion for science and your eagerness to discuss new data and literature. Petter, I am especially grateful for your positive attitude and your open scientific mind. Klas, thanks for your curiosity towards new knowledge and for bringing the scientific discussions to new dimensions.

All members of the Höglund and Kärre groups. Especially my collaborators on various projects: **Louise Berg** for your great "let's do it" attitude and for always seeing the humoristic side of things, **Maria Johansson** for your highly appreciated cookie supply and for always being so happy to discuss science, **Susanne Johansson** for being so kind, fun and smart and for our tradition to bring candy surprises during late night experiments, **Arnika Wagner** and **Stina Wickström** for great collaborations and marvellous cakes. **Sofia Berglund, Petter Brodin, Jens Gertow, Mantas Okas, Sadia Salam**, **Jonas Sundbäck, Kanth Tadepally, Rossana Tallerico, Michael Uhlin.** Thank you all for being such nice company during days and nights!

Previous members of the group, especially **Marjet Elemans** for past and present collaborations and great company in the office and on trips, **Håkan Hall, Sofia Johansson, Sara Lemos** and **Hanna Sjölin** for good collaborations on the studies in this thesis, **André Ortlieb, Danika Schepis** and **Eleftheria Rosmaraki** for your fantastic sense of humour.

Björn Önfelt and his group, especially **Karolin Guldevall** and **Bruno Vanherberghen**, for being the microscopy wizards.

Thanks also to the other research groups sharing labs with us: A joke or a helping hand is never far away!

Prefects of the department of Microbiology, Tumor and Cell Biology (MTC) for creating a good working atmosphere with inspiring seminars and great facilities. Colleagues at the department for good company and for sharing reagents and methods. Special thanks to my fellow PhD students at the department, in particular **Karin Blomqvist** for our frequent pep talks in the coffee room and for always being so optimistic and **Emilie Flaberg** for your devotion to science and our fun dance sessions at the MTC pubs.

Colleagues and friends at the Karolinska Institute for making it a perfect place to work. Especially **Tobias Alfvén** for being the ultimate collaborator on various projects, my external mentor **Elias Arnér** for inspiring talks, **Niklas Björkström** for fun excursions on conferences, **Christian Broberger** for a lot of laughter and for making me feel so welcome in your lab. A special thanks also to the members of the Broberger lab,

especially **Kylie Foo**, for fun discussions on arts and for helping me out with various practical things in the lab. **Gustaf Edgren** for lunch company and for delineating the "Brauner-Brauner-Brauner" study, **Malin Flodström-Tullberg** for exciting discussions on the pathogenesis of diabetes and more, **Tomas Hökfelt** for showing me how fascinating science can be and the art of growing orange trees, **Eva Klein** for wonderful discussions on science and life on the late evening bus home from work, **Stina Salomonsson** for being an enthusiastic project work supervisor during my first steps in research, **Ola Winqvist** and **Annika Scheynius** and everybody at the clinical immunology research unit for collaborations, generously letting me use equipment and inviting me for coffees and celebrations. Special thanks to **Mikael Karlsson, Emma Lindh** and **Lisa Westerberg** for great company in the lab, at courses and on conferences.

James Di Santo and group members at the Pasteur institute, Paris, for generously welcoming me during a three month research visit. I very much enjoyed working in your excellent scientific environment!

All co-authors for exciting and fruitful collaborations!

Colleagues and friends at the Karolinska Institutet Development Council (Framtidsrådet), Karolinska Institutet Language Centre (KI Språkcentrum) and the Swedish Society of Medicine (Läkaresällskapet) for adding new and inspiring aspects during these years.

My friends from outside the Karolinska Institutet for being the greatest, funniest and most supportive. Thanks for persistently inviting me to coffees and dinners despite numerous late cancellations when experiments were running slowly.

My relatives, including my grandparents in beloved memory. Especially my wise, warm and strong grandmother **Fannie** for always showing such interest in my studies. My extraordinary parents for love and full support in any matter and for running the best restaurant and hotel thirty seconds from KI. Highly appreciated during late nights in the lab! **Mamma** for always seeing and creating possibilities and proposing exciting wild ideas. **Pappa** for a unique capacity to see things in a broader perspective and for your subtle sense of humour.

Nånnis for being the most fantastic sister and my best friend. I'm looking forward to our future collaborations. It was so much fun when we were doing experiments sitting next to each other in the lab hood – true sisterhood!

My love **Lukas** for enriching me tremendously with your passion for life. You are surely the most NK cell knowledgeable architect by now. You are amazing!

I'm grateful for financial support from the Karolinska Institutet MD PhD programme, Fernström's Foundation, the Swedish Society of Medicine and Karolinska Institutet.

13 REFERENCES

1. Anderson, M.S. and J.A. Bluestone, *The NOD mouse: a model of immune dysregulation.* Annu Rev Immunol, 2005. **23**: p. 447-85.
2. Hoglund, P., et al., *Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes.* J Exp Med, 1999. **189**(2): p. 331-9.
3. Stadinski, B., J. Kappler, and G.S. Eisenbarth, *Molecular targeting of islet autoantigens.* Immunity. **32**(4): p. 446-56.
4. Di Santo, J.P., *Natural killer cells: diversity in search of a niche.* Nat Immunol, 2008. **9**(5): p. 473-5.
5. Defronzo, R.A., *Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus.* Diabetes, 2009. **58**(4): p. 773-95.
6. Donath, M.Y., et al., *Cytokine production by islets in health and diabetes: cellular origin, regulation and function.* Trends Endocrinol Metab. **21**(5): p. 261-7.
7. Dhillo, W.S., *Appetite regulation: an overview.* Thyroid, 2007. **17**(5): p. 433-45.
8. Oh, I.S., et al., *Identification of nesfatin-1 as a satiety molecule in the hypothalamus.* Nature, 2006. **443**(7112): p. 709-12.
9. Parton, L.E., et al., *Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity.* Nature, 2007. **449**(7159): p. 228-32.
10. Ahren, B., H. Martensson, and B. Falck, *Effects of neuropeptide Y on insulin and glucagon secretion in the pig.* Neuropeptides, 1991. **20**(1): p. 49-55.
11. Dunning, B.E., et al., *Galanin: a novel pancreatic neuropeptide.* Am J Physiol, 1986. **251**(1 Pt 1): p. E127-33.
12. Ludvigsson, J., *Why diabetes incidence increases--a unifying theory.* Ann N Y Acad Sci, 2006. **1079**: p. 374-82.
13. Wild, S., et al., *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.* Diabetes Care, 2004. **27**(5): p. 1047-53.
14. Todd, J.A., *Etiology of type 1 diabetes.* Immunity. **32**(4): p. 457-67.
15. Gregersen, P.K. and L.M. Olsson, *Recent advances in the genetics of autoimmune disease.* Annu Rev Immunol, 2009. **27**: p. 363-91.
16. Santamaria, P., *The long and winding road to understanding and conquering type 1 diabetes.* Immunity. **32**(4): p. 437-45.
17. Makino, S., et al., *Breeding of a non-obese, diabetic strain of mice.* Jikken Dobutsu, 1980. **29**(1): p. 1-13.
18. Andre, I., et al., *Checkpoints in the progression of autoimmune disease: lessons from diabetes models.* Proc Natl Acad Sci U S A, 1996. **93**(6): p. 2260-3.
19. Many, M.C., S. Maniratunga, and J.F. Denef, *The non-obese diabetic (NOD) mouse: an animal model for autoimmune thyroiditis.* Exp Clin Endocrinol Diabetes, 1996. **104 Suppl 3**: p. 17-20.
20. Haskins, K. and D. Wegmann, *Diabetogenic T-cell clones.* Diabetes, 1996. **45**(10): p. 1299-305.
21. Gonzalez, A., et al., *Genetic control of diabetes progression.* Immunity, 1997. **7**(6): p. 873-83.
22. Katz, J.D., et al., *Following a diabetogenic T cell from genesis through pathogenesis.* Cell, 1993. **74**(6): p. 1089-100.
23. Lam-Tse, W.K., A. Lernmark, and H.A. Drexhage, *Animal models of endocrine/organ-specific autoimmune diseases: do they really help us to understand human autoimmunity?* Springer Semin Immunopathol, 2002. **24**(3): p. 297-321.
24. Sjoholm, A. and T. Nystrom, *Inflammation and the etiology of type 2 diabetes.* Diabetes Metab Res Rev, 2006. **22**(1): p. 4-10.
25. Donath, M.Y., et al., *Islet inflammation impairs the pancreatic beta-cell in type 2 diabetes.* Physiology (Bethesda), 2009. **24**: p. 325-31.

26. Ostenson, C.G., et al., *Impaired gene and protein expression of exocytotic soluble N-ethylmaleimide attachment protein receptor complex proteins in pancreatic islets of type 2 diabetic patients.* Diabetes, 2006. **55**(2): p. 435-40.
27. Perry, J.R. and T.M. Frayling, *New gene variants alter type 2 diabetes risk predominantly through reduced beta-cell function.* Curr Opin Clin Nutr Metab Care, 2008. **11**(4): p. 371-7.
28. Del Prato, S., G. Penno, and R. Miccoli, *Changing the treatment paradigm for type 2 diabetes.* Diabetes Care, 2009. **32 Suppl 2**: p. S217-22.
29. Goto, Y., M. Kakizaki, and N. Masaki, *Production of spontaneous diabetic rats by repetition of selective breeding.* Tohoku J Exp Med, 1976. **119**(1): p. 85-90.
30. Ostenson, C.G. and S. Efendic, *Islet gene expression and function in type 2 diabetes; studies in the Goto-Kakizaki rat and humans.* Diabetes Obes Metab, 2007. **9 Suppl 2**: p. 180-6.
31. Guidotti, L.G. and F.V. Chisari, *Cytokine-mediated control of viral infections.* Virology, 2000. **273**(2): p. 221-7.
32. Becknell, B. and M.A. Caligiuri, *Interleukin-2, interleukin-15, and their roles in human natural killer cells.* Adv Immunol, 2005. **86**: p. 209-39.
33. Ehl, S., et al., *A comparison of efficacy and specificity of three NK depleting antibodies.* J Immunol Methods, 1996. **199**(2): p. 149-53.
34. Nelson, B.H., *IL-2, regulatory T cells, and tolerance.* J Immunol, 2004. **172**(7): p. 3983-8.
35. Suzuki, H., et al., *Are CD8+CD122+ cells regulatory T cells or memory T cells?* Hum Immunol, 2008. **69**(11): p. 751-4.
36. Rodgers, J.R. and R.G. Cook, *MHC class Ib molecules bridge innate and acquired immunity.* Nat Rev Immunol, 2005. **5**(6): p. 459-71.
37. Biron, C.A., et al., *Natural killer cells in antiviral defense: function and regulation by innate cytokines.* Annu Rev Immunol, 1999. **17**: p. 189-220.
38. Setoguchi, R., et al., *Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization.* J Exp Med, 2005. **201**(5): p. 723-35.
39. Johansson, S., et al., *NK cells: elusive players in autoimmunity.* Trends Immunol, 2005. **26**(11): p. 613-8.
40. Kimura, M.Y. and T. Nakayama, *Differentiation of NK1 and NK2 cells.* Crit Rev Immunol, 2005. **25**(5): p. 361-74.
41. Fehniger, T.A. and M.A. Caligiuri, *Interleukin 15: biology and relevance to human disease.* Blood, 2001. **97**(1): p. 14-32.
42. Luci, C., et al., *Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin.* Nat Immunol, 2009. **10**(1): p. 75-82.
43. Cella, M., et al., *A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity.* Nature, 2009. **457**(7230): p. 722-5.
44. Sanos, S.L., et al., *RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells.* Nat Immunol, 2009. **10**(1): p. 83-91.
45. Satoh-Takayama, N., et al., *Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense.* Immunity, 2008. **29**(6): p. 958-70.
46. Schepers, K., R. Arens, and T.N. Schumacher, *Dissection of cytotoxic and helper T cell responses.* Cell Mol Life Sci, 2005. **62**(23): p. 2695-710.
47. Szabo, S.J., et al., *Molecular mechanisms regulating Th1 immune responses.* Annu Rev Immunol, 2003. **21**: p. 713-58.
48. Weaver, C.T., et al., *Th17: an effector CD4 T cell lineage with regulatory T cell ties.* Immunity, 2006. **24**(6): p. 677-88.
49. Mellanby, R.J., D.C. Thomas, and J. Lamb, *Role of regulatory T-cells in autoimmunity.* Clin Sci (Lond), 2009. **116**(8): p. 639-49.
50. Sakaguchi, S., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self.* Nat Immunol, 2005. **6**(4): p. 345-52.
51. Hsieh, C.S., et al., *Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors.* Immunity, 2004. **21**(2): p. 267-77.

52. von Boehmer, H., *Mechanisms of suppression by suppressor T cells.* Nat Immunol, 2005. **6**(4): p. 338-44.
53. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
54. Nakamura, K., A. Kitani, and W. Strober, *Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta.* J Exp Med, 2001. **194**(5): p. 629-44.
55. Grossman, W.J., et al., *Human T regulatory cells can use the perforin pathway to cause autologous target cell death.* Immunity, 2004. **21**(4): p. 589-601.
56. Sgouroudis, E. and C.A. Piccirillo, *Control of type 1 diabetes by CD4+Foxp3+ regulatory T cells: lessons from mouse models and implications for human disease.* Diabetes Metab Res Rev, 2009. **25**(3): p. 208-18.
57. Horak, I., et al., *Interleukin-2 deficient mice: a new model to study autoimmunity and self-tolerance.* Immunol Rev, 1995. **148**: p. 35-44.
58. Kramer, S., A. Schimpl, and T. Hunig, *Immunopathology of interleukin (IL) 2-deficient mice: thymus dependence and suppression by thymus-dependent cells with an intact IL-2 gene.* J Exp Med, 1995. **182**(6): p. 1769-76.
59. Willerford, D.M., et al., *Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment.* Immunity, 1995. **3**(4): p. 521-30.
60. Suzuki, H., et al., *Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta.* Science, 1995. **268**(5216): p. 1472-6.
61. Bendelac, A., P.B. Savage, and L. Teyton, *The biology of NKT cells.* Annu Rev Immunol, 2007. **25**: p. 297-336.
62. Fletcher, M.T. and A.G. Baxter, *Clinical application of NKT cell biology in type I (autoimmune) diabetes mellitus.* Immunol Cell Biol, 2009. **87**(4): p. 315-23.
63. Beetz, S., et al., *Innate immune functions of human gammadelta T cells.* Immunobiology, 2008. **213**(3-4): p. 173-82.
64. Kiessling, R., et al., *"Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell.* Eur J Immunol, 1975. **5**(2): p. 117-21.
65. Kiessling, R., E. Klein, and H. Wigzell, *"Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype.* Eur J Immunol, 1975. **5**(2): p. 112-7.
66. Herberman, R.B., et al., *Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells.* Int J Cancer, 1975. **16**(2): p. 230-9.
67. Herberman, R.B., M.E. Nunn, and D.H. Lavrin, *Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity.* Int J Cancer, 1975. **16**(2): p. 216-29.
68. Screpanti, V., et al., *Impact of FASL-induced apoptosis in the elimination of tumor cells by NK cells.* Mol Immunol, 2005. **42**(4): p. 495-9.
69. Vivier, E., et al., *Functions of natural killer cells.* Nat Immunol, 2008. **9**(5): p. 503-10.
70. Cooper, M.A., et al., *Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset.* Blood, 2001. **97**(10): p. 3146-51.
71. Caligiuri, M.A., *Human natural killer cells.* Blood, 2008. **112**(3): p. 461-9.
72. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009. **457**(7229): p. 557-61.
73. O'Leary, J.G., et al., *T cell- and B cell-independent adaptive immunity mediated by natural killer cells.* Nat Immunol, 2006. **7**(5): p. 507-16.
74. Kim, S., et al., *In vivo developmental stages in murine natural killer cell maturation.* Nat Immunol, 2002. **3**(6): p. 523-8.
75. Freud, A.G., et al., *Evidence for discrete stages of human natural killer cell differentiation in vivo.* J Exp Med, 2006. **203**(4): p. 1033-43.

76. Rosmaraki, E.E., et al., *Identification of committed NK cell progenitors in adult murine bone marrow.* Eur J Immunol, 2001. **31**(6): p. 1900-9.
77. Mrozek, E., P. Anderson, and M.A. Caligiuri, *Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells.* Blood, 1996. **87**(7): p. 2632-40.
78. Freud, A.G. and M.A. Caligiuri, *Human natural killer cell development.* Immunol Rev, 2006. **214**: p. 56-72.
79. Di Santo, J.P., *A defining factor for natural killer cell development.* Nat Immunol, 2009. **10**(10): p. 1051-2.
80. Gascoyne, D.M., et al., *The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development.* Nat Immunol, 2009. **10**(10): p. 1118-24.
81. Vosshenrich, C.A., et al., *A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127.* Nat Immunol, 2006. **7**(11): p. 1217-24.
82. Freud, A.G., et al., *A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells.* Immunity, 2005. **22**(3): p. 295-304.
83. Huntington, N.D., C.A. Vosshenrich, and J.P. Di Santo, *Developmental pathways that generate natural-killer-cell diversity in mice and humans.* Nat Rev Immunol, 2007. **7**(9): p. 703-14.
84. Karre, K., et al., *Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy.* Nature, 1986. **319**(6055): p. 675-8.
85. Karre, K., *Natural killer cell recognition of missing self.* Nat Immunol, 2008. **9**(5): p. 477-80.
86. Raulet, D.H. and R.E. Vance, *Self-tolerance of natural killer cells.* Nat Rev Immunol, 2006. **6**(7): p. 520-31.
87. Lanier, L.L., *NK cell recognition.* Annu Rev Immunol, 2005. **23**: p. 225-74.
88. Raulet, D.H., *Roles of the NKG2D immunoreceptor and its ligands.* Nat Rev Immunol, 2003. **3**(10): p. 781-90.
89. Diefenbach, A., et al., *Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages.* Nat Immunol, 2000. **1**(2): p. 119-26.
90. Groh, V., et al., *Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation.* Nature, 2002. **419**(6908): p. 734-8.
91. Bryceson, Y.T., et al., *Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion.* Blood, 2006. **107**(1): p. 159-66.
92. Alter, G., J.M. Malenfant, and M. Altfeld, *CD107a as a functional marker for the identification of natural killer cell activity.* J Immunol Methods, 2004. **294**(1-2): p. 15-22.
93. Fukuda, M., *Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking.* J Biol Chem, 1991. **266**(32): p. 21327-30.
94. Huntington, N.D., et al., *NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation.* J Immunol, 2007. **178**(8): p. 4764-70.
95. Robbins, S.H., et al., *Cutting edge: inhibitory functions of the killer cell lectin-like receptor G1 molecule during the activation of mouse NK cells.* J Immunol, 2002. **168**(6): p. 2585-9.
96. Robbins, S.H., et al., *Expansion and contraction of the NK cell compartment in response to murine cytomegalovirus infection.* J Immunol, 2004. **173**(1): p. 259-66.
97. Olcese, L., et al., *Human killer cell activatory receptors for MHC class I molecules are included in a multimeric complex expressed by natural killer cells.* J Immunol, 1997. **158**(11): p. 5083-6.
98. Lanier, L.L., et al., *Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells.* Nature, 1998. **391**(6668): p. 703-7.
99. Schleinitz, N., et al., *Pattern of DAP12 expression in leukocytes from both healthy and systemic lupus erythematosus patients.* PLoS One, 2009. **4**(7): p. e6264.

100. Lanier, L.L., *DAP10- and DAP12-associated receptors in innate immunity.* Immunol Rev, 2009. **227**(1): p. 150-60.
101. Colonna, M., *TREMs in the immune system and beyond.* Nat Rev Immunol, 2003. **3**(6): p. 445-53.
102. Hamerman, J.A., et al., *Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12.* Nat Immunol, 2005. **6**(6): p. 579-86.
103. Hakola, H.P., *Neuropsychiatric and genetic aspects of a new hereditary disease characterized by progressive dementia and lipomembranous polycystic osteodysplasia.* Acta Psychiatr Scand Suppl, 1972. **232**: p. 1-173.
104. Verloes, A., et al., *Nasu-Hakola syndrome: polycystic lipomembranous osteodysplasia with sclerosing leucoencephalopathy and presenile dementia.* J Med Genet, 1997. **34**(9): p. 753-7.
105. Bakker, A.B., et al., *DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming.* Immunity, 2000. **13**(3): p. 345-53.
106. Tomasello, E., et al., *Combined natural killer cell and dendritic cell functional deficiency in KARAP/DAP12 loss-of-function mutant mice.* Immunity, 2000. **13**(3): p. 355-64.
107. Tomasello, E., et al., *Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12.* J Biol Chem, 1998. **273**(51): p. 34115-9.
108. Leonard, W.J. and R. Spolski, *Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation.* Nat Rev Immunol, 2005. **5**(9): p. 688-98.
109. Gidlund, M., et al., *Enhanced NK cell activity in mice injected with interferon and interferon inducers.* Nature, 1978. **273**(5665): p. 759-61.
110. Garcia-Sastre, A. and C.A. Biron, *Type 1 interferons and the virus-host relationship: a lesson in detente.* Science, 2006. **312**(5775): p. 879-82.
111. Biron, C.A., G. Sonnenfeld, and R.M. Welsh, *Interferon induces natural killer cell blastogenesis in vivo.* J Leukoc Biol, 1984. **35**(1): p. 31-7.
112. Huntington, N.D., et al., *IL-15 trans-presentation promotes human NK cell development and differentiation in vivo.* J Exp Med, 2009. **206**(1): p. 25-34.
113. Nguyen, K.B., et al., *Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection.* J Immunol, 2002. **169**(8): p. 4279-87.
114. Lucas, M., et al., *Dendritic cells prime natural killer cells by trans-presenting interleukin 15.* Immunity, 2007. **26**(4): p. 503-17.
115. Orange, J.S. and C.A. Biron, *Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection.* J Immunol, 1996. **156**(12): p. 4746-56.
116. Orange, J.S. and C.A. Biron, *An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections.* J Immunol, 1996. **156**(3): p. 1138-42.
117. Monteiro, J.M., C. Harvey, and G. Trinchieri, *Role of interleukin-12 in primary influenza virus infection.* J Virol, 1998. **72**(6): p. 4825-31.
118. Farag, S.S. and M.A. Caligiuri, *Human natural killer cell development and biology.* Blood Rev, 2006. **20**(3): p. 123-37.
119. Hayakawa, Y. and M.J. Smyth, *CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity.* J Immunol, 2006. **176**(3): p. 1517-24.
120. Chiossone, L., et al., *Maturation of mouse NK cells is a 4-stage developmental program.* Blood, 2009. **113**(22): p. 5488-96.
121. de Matos, C.T., et al., *Activating and inhibitory receptors on synovial fluid natural killer cells of arthritis patients: role of CD94/NKG2A in control of cytokine secretion.* Immunology, 2007. **122**(2): p. 291-301.
122. Hayakawa, Y., et al., *Functional subsets of mouse natural killer cells.* Immunol Rev, 2006. **214**: p. 47-55.
123. Anne Croy, B., et al., *Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones.* Immunol Rev, 2006. **214**: p. 161-85.

124. Ashkar, A.A., J.P. Di Santo, and B.A. Croy, *Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy.* J Exp Med, 2000. **192**(2): p. 259-70.
125. Yadi, H., et al., *Unique receptor repertoire in mouse uterine NK cells.* J Immunol, 2008. **181**(9): p. 6140-7.
126. Trowsdale, J. and A. Moffett, *NK receptor interactions with MHC class I molecules in pregnancy.* Semin Immunol, 2008. **20**(6): p. 317-20.
127. Koopman, L.A., et al., *Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential.* J Exp Med, 2003. **198**(8): p. 1201-12.
128. Fernando, M.M., et al., *Defining the role of the MHC in autoimmunity: a review and pooled analysis.* PLoS Genet, 2008. **4**(4): p. e1000024.
129. Suri, A., M.G. Levisetti, and E.R. Unanue, *Do the peptide-binding properties of diabetogenic class II molecules explain autoreactivity?* Curr Opin Immunol, 2008. **20**(1): p. 105-10.
130. Lettre, G. and J.D. Rioux, *Autoimmune diseases: insights from genome-wide association studies.* Hum Mol Genet, 2008. **17**(R2): p. R116-21.
131. Rahman, A. and D.A. Isenberg, *Systemic lupus erythematosus.* N Engl J Med, 2008. **358**(9): p. 929-39.
132. Wardemann, H., et al., *Predominant autoantibody production by early human B cell precursors.* Science, 2003. **301**(5638): p. 1374-7.
133. Ohashi, P.S. and A.L. DeFranco, *Making and breaking tolerance.* Curr Opin Immunol, 2002. **14**(6): p. 744-59.
134. You, S. and L. Chatenoud, *Proinsulin: a unique autoantigen triggering autoimmune diabetes.* J Clin Invest, 2006. **116**(12): p. 3108-10.
135. Guilherme, L., J. Kalil, and M. Cunningham, *Molecular mimicry in the autoimmune pathogenesis of rheumatic heart disease.* Autoimmunity, 2006. **39**(1): p. 31-9.
136. Albani, S., *Infection and molecular mimicry in autoimmune diseases of childhood.* Clin Exp Rheumatol, 1994. **12 Suppl 10**: p. S35-41.
137. Hill, N.J., et al., *The target tissue in autoimmunity--an influential niche.* Eur J Immunol, 2007. **37**(3): p. 589-97.
138. Frigerio, S., et al., *Beta cells are responsible for CXCR3-mediated T-cell infiltration in insulitis.* Nat Med, 2002. **8**(12): p. 1414-20.
139. Marquardt, N., et al., *Murine CXCR3+CD27bright NK cells resemble the human CD56bright NK-cell population.* Eur J Immunol. **40**(5): p. 1428-39.
140. Ogasawara, K., et al., *NKG2D blockade prevents autoimmune diabetes in NOD mice.* Immunity, 2004. **20**(6): p. 757-67.
141. Ansari, M.J., et al., *The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice.* J Exp Med, 2003. **198**(1): p. 63-9.
142. Lieberman, S.M. and T.P. DiLorenzo, *A comprehensive guide to antibody and T-cell responses in type 1 diabetes.* Tissue Antigens, 2003. **62**(5): p. 359-77.
143. Di Lorenzo, T.P., M. Peakman, and B.O. Roep, *Translational mini-review series on type 1 diabetes: Systematic analysis of T cell epitopes in autoimmune diabetes.* Clin Exp Immunol, 2007. **148**(1): p. 1-16.
144. Krishnamurthy, B., et al., *Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP.* J Clin Invest, 2006. **116**(12): p. 3258-65.
145. Nakayama, M., et al., *Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice.* Nature, 2005. **435**(7039): p. 220-3.
146. Moriyama, H., et al., *Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse.* Proc Natl Acad Sci U S A, 2003. **100**(18): p. 10376-81.
147. Wicker, L.S., et al., *Type 1 diabetes genes and pathways shared by humans and NOD mice.* J Autoimmun, 2005. **25 Suppl**: p. 29-33.
148. Kurts, C., et al., *Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells.* J Exp Med, 1997. **186**(2): p. 239-45.

149. Hugues, S., et al., *Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells.* Immunity, 2002. **16**(2): p. 169-81.
150. Turley, S.J., et al., *Endocrine self and gut non-self intersect in the pancreatic lymph nodes.* Proc Natl Acad Sci U S A, 2005. **102**(49): p. 17729-33.
151. Dotta, F., et al., *Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients.* Proc Natl Acad Sci U S A, 2007. **104**(12): p. 5115-20.
152. Turley, S., et al., *Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model.* J Exp Med, 2003. **198**(10): p. 1527-37.
153. Chatenoud, L., et al., *Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice.* Proc Natl Acad Sci U S A, 1994. **91**(1): p. 123-7.
154. Wicker, L.S., B.J. Miller, and Y. Mullen, *Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice.* Diabetes, 1986. **35**(8): p. 855-60.
155. Bendelac, A., et al., *Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells.* J Exp Med, 1987. **166**(4): p. 823-32.
156. Yagi, H., et al., *Analysis of the roles of CD4+ and CD8+ T cells in autoimmune diabetes of NOD mice using transfer to NOD athymic nude mice.* Eur J Immunol, 1992. **22**(9): p. 2387-93.
157. Wong, F.S., et al., *CD8 T cell clones from young nonobese diabetic (NOD) islets can transfer rapid onset of diabetes in NOD mice in the absence of CD4 cells.* J Exp Med, 1996. **183**(1): p. 67-76.
158. Hanninen, A., et al., *Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus.* J Clin Invest, 1992. **90**(5): p. 1901-10.
159. Bottazzo, G.F., et al., *In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis.* N Engl J Med, 1985. **313**(6): p. 353-60.
160. Tsai, S., A. Shameli, and P. Santamaria, *CD8+ T cells in type 1 diabetes.* Adv Immunol, 2008. **100**: p. 79-124.
161. Herold, K.C., et al., *A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes.* Diabetes, 2005. **54**(6): p. 1763-9.
162. Herold, K.C., et al., *Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus.* N Engl J Med, 2002. **346**(22): p. 1692-8.
163. Nejentsev, S., et al., *Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A.* Nature, 2007. **450**(7171): p. 887-92.
164. Bennett, C.L., et al., *The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3.* Nat Genet, 2001. **27**(1): p. 20-1.
165. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
166. Brunkow, M.E., et al., *Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse.* Nat Genet, 2001. **27**(1): p. 68-73.
167. Tritt, M., et al., *Functional waning of naturally occurring CD4+ regulatory T-cells contributes to the onset of autoimmune diabetes.* Diabetes, 2008. **57**(1): p. 113-23.
168. Tang, Q., et al., *Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells.* J Immunol, 2003. **171**(7): p. 3348-52.
169. Salomon, B., et al., *B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes.* Immunity, 2000. **12**(4): p. 431-40.

170. Pop, S.M., et al., *Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes.* J Exp Med, 2005. **201**(8): p. 1333-46.
171. Tang, Q., et al., *Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice.* Nat Immunol, 2006. **7**(1): p. 83-92.
172. Serra, P., et al., *CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells.* Immunity, 2003. **19**(6): p. 877-89.
173. Feuerer, M., et al., *How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets.* Immunity, 2009. **31**(4): p. 654-64.
174. Shi, F.D., et al., *Germ line deletion of the CD1 locus exacerbates diabetes in the NOD mouse.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6777-82.
175. Novak, J., et al., *Prevention of type 1 diabetes by invariant NKT cells is independent of peripheral CD1d expression.* J Immunol, 2007. **178**(3): p. 1332-40.
176. Gombert, J.M., et al., *Early quantitative and functional deficiency of NK1+-like thymocytes in the NOD mouse.* Eur J Immunol, 1996. **26**(12): p. 2989-98.
177. Naumov, Y.N., et al., *Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13838-43.
178. Chen, Y.G., et al., *Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes.* J Immunol, 2005. **174**(3): p. 1196-204.
179. Griseri, T., et al., *Invariant NKT cells exacerbate type 1 diabetes induced by CD8 T cells.* J Immunol, 2005. **175**(4): p. 2091-101.
180. Rodacki, M., et al., *Altered natural killer cells in type 1 diabetic patients.* Diabetes, 2007. **56**(1): p. 177-85.
181. Poulton, L.D., et al., *Cytometric and functional analyses of NK and NKT cell deficiencies in NOD mice.* Int Immunol, 2001. **13**(7): p. 887-96.
182. Johansson, S.E., et al., *Broadly impaired NK cell function in non-obese diabetic mice is partially restored by NK cell activation in vivo and by IL-12/IL-18 in vitro.* Int Immunol, 2004. **16**(1): p. 1-11.
183. Lee, I.F., et al., *Critical role for IFN-gamma in natural killer cell-mediated protection from diabetes.* Eur J Immunol, 2008. **38**(1): p. 82-9.
184. Alba, A., et al., *Natural killer cells are required for accelerated type 1 diabetes driven by interferon-beta.* Clin Exp Immunol, 2008. **151**(3): p. 467-75.
185. Poirot, L., C. Benoist, and D. Mathis, *Natural killer cells distinguish innocuous and destructive forms of pancreatic islet autoimmunity.* Proc Natl Acad Sci U S A, 2004. **101**(21): p. 8102-7.
186. Stiller, C.R., et al., *Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset.* Science, 1984. **223**(4643): p. 1362-7.
187. Dupre, J., et al., *Clinical trials of cyclosporin in IDDM.* Diabetes Care, 1988. **11 Suppl 1**: p. 37-44.
188. Luo, X., K.C. Herold, and S.D. Miller, *Immunotherapy of type 1 diabetes: where are we and where should we be going?* Immunity. **32**(4): p. 488-99.
189. You, S., et al., *Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment.* Proc Natl Acad Sci U S A, 2007. **104**(15): p. 6335-40.
190. Pescovitz, M.D., et al., *Rituximab, B-lymphocyte depletion, and preservation of beta-cell function.* N Engl J Med, 2009. **361**(22): p. 2143-52.
191. Mandrup-Poulsen, T., L. Pickersgill, and M.Y. Donath, *Blockade of interleukin 1 in type 1 diabetes mellitus.* Nat Rev Endocrinol. **6**(3): p. 158-66.
192. Maedler, K., et al., *Interleukin-1 beta targeted therapy for type 2 diabetes.* Expert Opin Biol Ther, 2009. **9**(9): p. 1177-88.
193. Ludvigsson, J., *Therapy with GAD in diabetes.* Diabetes Metab Res Rev, 2009. **25**(4): p. 307-15.
194. Zhou, L., et al., *Serotonin 2C receptor agonists improve type 2 diabetes via melanocortin-4 receptor signaling pathways.* Cell Metab, 2007. **6**(5): p. 398-405.

195. Brailoiu, G.C., et al., *Nesfatin-1: distribution and interaction with a G protein-coupled receptor in the rat brain.* Endocrinology, 2007. **148**(10): p. 5088-94.
196. Foo, K.S., H. Brismar, and C. Broberger, *Distribution and neuropeptide coexistence of nucleobindin-2 mRNA/nesfatin-like immunoreactivity in the rat CNS.* Neuroscience, 2008. **156**(3): p. 563-79.
197. Barnikol-Watanabe, S., et al., *Human protein NEFA, a novel DNA binding/EF-hand/leucine zipper protein. Molecular cloning and sequence analysis of the cDNA, isolation and characterization of the protein.* Biol Chem Hoppe Seyler, 1994. **375**(8): p. 497-512.
198. Tung, J.W., et al., *New approaches to fluorescence compensation and visualization of FACS data.* Clin Immunol, 2004. **110**(3): p. 277-83.
199. Hulspas, R., et al., *Flow cytometry and the stability of phycoerythrin-tandem dye conjugates.* Cytometry A, 2009. **75**(11): p. 966-72.
200. Bryceson, Y.T., et al., *Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells.* J Exp Med, 2005. **202**(7): p. 1001-12.
201. Asquith, B., et al., *Lymphocyte kinetics: the interpretation of labelling data.* Trends Immunol, 2002. **23**(12): p. 596-601.
202. Ganusov, V.V., et al., *Quantifying cell turnover using CFSE data.* J Immunol Methods, 2005. **298**(1-2): p. 183-200.
203. Zamboni, I. and C. De Martino, *Buffered picric acid formaldehyde. A new rapid fixative for electron microscopy.* J Cell Biol, 1967. **35**: p. 148A.
204. Bobrow, M.N., et al., *Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays.* J Immunol Methods, 1989. **125**(1-2): p. 279-85.
205. Seaman, W.E., et al., *Depletion of natural killer cells in mice by monoclonal antibody to NK-1.1. Reduction in host defense against malignancy without loss of cellular or humoral immunity.* J Immunol, 1987. **138**(12): p. 4539-44.
206. Kasai, M., et al., *A glycolipid on the surface of mouse natural killer cells.* Eur J Immunol, 1980. **10**(3): p. 175-80.
207. Tanaka, T., et al., *Selective long-term elimination of natural killer cells in vivo by an anti-interleukin 2 receptor beta chain monoclonal antibody in mice.* J Exp Med, 1993. **178**(3): p. 1103-7.
208. Belanger, S., et al., *Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome.* Genes Immun, 2008. **9**(6): p. 509-21.
209. Parham, P., *MHC class I molecules and KIRs in human history, health and survival.* Nat Rev Immunol, 2005. **5**(3): p. 201-14.
210. Satoh-Takayama, N., et al., *The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium.* J Immunol, 2009. **183**(10): p. 6579-87.
211. Zamai, L., et al., *Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells.* J Exp Med, 1998. **188**(12): p. 2375-80.
212. Fernandez, N.C., et al., *A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules.* Blood, 2005. **105**(11): p. 4416-23.
213. Corral, L., et al., *NK cell expression of the killer cell lectin-like receptor G1 (KLRG1), the mouse homolog of MAFA, is modulated by MHC class I molecules.* Eur J Immunol, 2000. **30**(3): p. 920-30.
214. Barber, D.L., et al., *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature, 2006. **439**(7077): p. 682-7.
215. Suwanai, H., et al., *A defective Il15 allele underlies the deficiency in natural killer cell activity in nonobese diabetic mice.* Proc Natl Acad Sci U S A.
216. Gur, C., et al., *The activating receptor NKp46 is essential for the development of type 1 diabetes.* Nat Immunol, 2010. **11**(2): p. 121-8.
217. Takeda, K., et al., *TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver.* Blood, 2005. **105**(5): p. 2082-9.

218. Smyth, M.J., et al., *Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis.* J Exp Med, 2001. **193**(6): p. 661-70.
219. Lee, I.F., et al., *Regulation of autoimmune diabetes by complete Freund's adjuvant is mediated by NK cells.* J Immunol, 2004. **172**(2): p. 937-42.
220. Flodstrom, M., et al., *The natural killer cell -- friend or foe in autoimmune disease?* Scand J Immunol, 2002. **55**(5): p. 432-41.
221. Martin-Fontecha, A., et al., *Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming.* Nat Immunol, 2004. **5**(12): p. 1260-5.
222. Loza, M.J., et al., *Expression of type 1 (interferon gamma) and type 2 (interleukin-13, interleukin-5) cytokines at distinct stages of natural killer cell differentiation from progenitor cells.* Blood, 2002. **99**(4): p. 1273-81.
223. Lauwerys, B.R., et al., *Cytokine production and killer activity of NK/T-NK cells derived with IL-2, IL-15, or the combination of IL-12 and IL-18.* J Immunol, 2000. **165**(4): p. 1847-53.
224. Chakir, H., A.M. Lemay, and J.R. Webb, *Cytokine expression by murine DX5+ cells in response to IL-12, IL-18, or the combination of IL-12 and IL-18.* Cell Immunol, 2001. **212**(1): p. 71-81.
225. Kaifu, T., et al., *Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice.* J Clin Invest, 2003. **111**(3): p. 323-32.
226. Takahashi, K., et al., *TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis.* PLoS Med, 2007. **4**(4): p. e124.
227. Piccio, L., et al., *Blockade of TREM-2 exacerbates experimental autoimmune encephalomyelitis.* Eur J Immunol, 2007. **37**(5): p. 1290-301.
228. Takaki, R., S.R. Watson, and L.L. Lanier, *DAP12: an adapter protein with dual functionality.* Immunol Rev, 2006. **214**: p. 118-29.
229. Sulonen, A.M., et al., *No evidence for shared etiology in two demyelinative disorders, MS and PLOSL.* J Neuroimmunol, 2009. **206**(1-2): p. 86-90.
230. Toyabe, S., U. Kaneko, and M. Uchiyama, *Decreased DAP12 expression in natural killer lymphocytes from patients with systemic lupus erythematosus is associated with increased transcript mutations.* J Autoimmun, 2004. **23**(4): p. 371-8.
231. Lamy, T. and T.P. Loughran, Jr., *Current concepts: large granular lymphocyte leukemia.* Blood Rev, 1999. **13**(4): p. 230-40.
232. Chen, X., et al., *A critical role for DAP10 and DAP12 in CD8+ T cell-mediated tissue damage in large granular lymphocyte leukemia.* Blood, 2009. **113**(14): p. 3226-34.
233. Sjolin, H., et al., *DAP12 signaling regulates plasmacytoid dendritic cell homeostasis and down-modulates their function during viral infection.* J Immunol, 2006. **177**(5): p. 2908-16.
234. Stengel, A., et al., *Identification and characterization of nesfatin-1 immunoreactivity in endocrine cell types of the rat gastric oxyntic mucosa.* Endocrinology, 2009. **150**(1): p. 232-8.
235. Gonzalez, R., A. Tiwari, and S. Unniappan, *Pancreatic beta cells colocalize insulin and pronesfatin immunoreactivity in rodents.* Biochem Biophys Res Commun, 2009. **381**(4): p. 643-8.
236. Aydin, S., et al., *Nesfatin-1 and ghrelin levels in serum and saliva of epileptic patients: hormonal changes can have a major effect on seizure disorders.* Mol Cell Biochem, 2009. **328**(1-2): p. 49-56.
237. Li, Q.C., et al., *Fasting plasma levels of nesfatin-1 in patients with type 1 and type 2 diabetes mellitus and the nutrient-related fluctuation of nesfatin-1 level in normal humans.* Regul Pept, 2010. **159**(1-3): p. 72-7.
238. Yamada, M., et al., *Troglitazone, a Ligand of Peroxisome Proliferator-Activated Receptor-{gamma}, Stabilizes NUCB2 (Nesfatin) mRNA by Activating the ERK1/2 Pathway: Isolation and Characterization of the Human NUCB2 Gene.* Endocrinology.
239. Ramanjaneya, M., et al., *Identification of Nesfatin-1 in Human and Murine Adipose Tissue: A Novel Depot-Specific Adipokine with Increased Levels in Obesity.* Endocrinology.