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INTERFERONS IN ANTIVIRAL DEFENSE AND AUTOIMMUNITY: FOCUS ON TYPE 1 DIABETES

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All human actions have one or more of these seven causes:
chance, nature, compulsion, habit, reason, passion, and desire.

Aristotle

ABSTRACT

Type 1 diabetes (T1D) is a disease characterized by the loss of insulin producing β-cells in the pancreatic islets of Langerhans. Interferons (IFNs), produced by immune cells and infected parenchymal cells, may be protective or damaging in the pathogenesis of T1D. An intact β-cell response to IFNs is critical for β-cell survival and protection from diabetes during a Coxsackievirus B (CVB) infection, a virus associated with T1D in humans. It has also been suggested that IFNs may protect from natural killer (NK) cell dependent destruction. Importantly, while being protective during infection with CVB, the pancreatic β-cell response to cytokines is crucial for the development of type 1 diabetes (T1D) in the non-obese diabetic (NOD) mouse. NOD mice overexpressing the Suppressor of Cytokine Signaling 1 (SOCS-1) specifically in the β-cells are protected from spontaneous diabetes. The work in this thesis focuses on the mechanisms behind the protective and damaging effects of IFNs in the pathogenesis of T1D. Moreover, it identifies a possible source of IFNγ during CVB infection.

This thesis shows that IFNs trigger an antiviral state in mouse and human islets. Both the RNase L and the dsRNA-dependent protein kinase (PKR) pathways are induced by IFNs in mouse islets and play important roles in providing unique and complementary antiviral activities that regulate the outcome of CVB infection. Moreover, this thesis shows that human islet cells also respond to IFNs by expressing signature genes of antiviral defense. It further demonstrates that human islets express three intracellular sensors for viral RNA, the toll like receptor 3 (TLR3) gene, the retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene-5 (MDA-5), which contribute to the production of type I IFN in infected cells. These observations suggest that human islet cells have the possibility to detect an invading virus and to produce type I IFNs during an infection. The work presented in this thesis identifies the NK cell as a possible contributor of IFNγ during CVB infection: it shows that CVB interferes with the expression of NK cell receptor ligands on infected cells and that IFNγ production, rather than cytotoxicity, marks the early human NK cell response to CVB infection. Finally, this thesis gives new insights into how IFNs, by acting directly on β-cells, contribute to disease development in the NOD mouse. It demonstrates that the β-cell, by responding to the pro-inflammatory pancreas milieu, strongly influences the percentage of self-reactive CD8 T-cells in the pancreas.

In conclusion, this thesis supports the notion that cytokine-exposed islet cells affect islet infection and inflammation, highlighting an important role for the β-cell in the local regulation of the diabetogenic process. By providing a basic understanding for how β-cells respond to IFNs, and how this relates to their defense against CVB and to the accumulation of pathogenic cells in the pancreas, it may contribute to a future unraveling of the mechanisms underlying β-cell loss in T1D.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

I. Flodström-Tullberg M, Hultcrantz M, Stotland A, Maday A, Tsai D, Fine C, Williams B, Silverman R, and Sarvetnick N. 2005. RNase L and Double-Stranded RNA-Dependent Protein Kinase Exert Complementary Roles in Islet Cell Defense during Coxsackievirus Infection. *Journal of Immunology 174 (3), 1171-1177*.

II. Hultcrantz M, Hühn M.H, Wolf M, Olsson A, Jacobson S, Williams B.R, Korsgren O, Flodström-Tullberg M. 2007. Interferons Induce an Antiviral State in Human Pancreatic Islet Cells. *Virology 367 (1), 92-101*.

III. Hühn M.H, Hultcrantz M, Lind K, Ljunggren H.G, Malmberg K.J, Flodström-Tullberg M. 2007. IFN-γ Production Dominates the Early Human Natural Killer Cell Response to Coxsackievirus Infection. *Cellular Microbiology, accepted for publication.*

IV. Hultcrantz M, Jacobson S, Santamaria P, Flodström-Tullberg M. The Target Cell Response to Cytokines Governs the Autoreactive T-cell Repertoire in the Pancreas. *Manuscript submitted.*

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LIST OF ABBREVIATIONS

CV	Coxsackievirus
CVB	Coxsackievirus, serotype B
DC	Dendritic cell
DMK	Dystrophia myotonica kinase
dsRNA	Double-stranded RNA
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
IRF9	Interferon-regulatory factor-9
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
JAK	Janus tyrosine kinase
MDA-5	Melanoma differentiation-associated gene 5
NK cells	Natural killer cells
NOD	Non-obese diabetic
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PKR	Double-stranded RNA activated serine/threonine protein kinase
PRR	Pattern-recognition receptor
RIG-I	Retinoic acid-inducible gene I
RT-PCR	Real-time PCR
SNP	Short nucleotide polymorphism
SOCS-1	Suppressor of cytokine signaling 1
STAT	Signal transducer and activators of transcription
T1D	Type 1 diabetes
tg	Transgenic
TLR	Toll-like receptor
WB	Western blot

INTRODUCTION

TYPE 1 DIABETES

Diabetes is a heterogeneous group of clinical disorders resulting from deficiencies in synthesis, secretion, and signaling of the hormone insulin. Insulin is produced by the β-cells within the islets of Langerhans in the pancreas. The two most common forms of diabetes have been classified as type 1 and type 2 diabetes (1).

Type 2 diabetes is due to a gradual decrease in insulin sensitivity in tissues, leading to hyperglycemia and β-cell failure. Type 1 diabetes (T1D) is a disease characterized by β-cell destruction or dysfunction leading to the loss of insulin production. With the discovery of insulin in the early 1920s (2), it is possible to live with this disease. However, besides the need for life-long treatment with insulin, patients with T1D develop long-term complications including vascular disease, eye problems, kidney disease and nerve damage. Today, there is no cure for T1D (3; 4).

Incidence

The incidence of T1D varies dramatically between different countries in the world. A worldwide study of the diabetes incidence in children under 15 years of age between the years 1990-1994 showed a more than 350-fold variation between different regions (5). The lowest incidence was found in China and Venezuela with 0.1/100.000 new cases per year and the highest was observed in Sardinia (36.8/100.000 per year) and Finland (36.5/100.000 per year). The overall incidence of T1D is increasing in the world with a shift toward onset at a younger age (3; 6). In Sweden, for example, the incidence increased from 21.1/100.000 children in 1978 to 31.9/100.000 in 1997 (7) and reached 44.9/100.000 children in 2006 (Dr. G. Dahlquist, coordinator for *the Swedish Childhood Diabetes Register*, personal communication).

Islets of Langerhans and Pathophysiology

The islets of Langerhans are compact clusters of endocrine cells scattered throughout the pancreas. The adult human pancreas contains approximately one million islets, constituting 1-1.5% of the total pancreas mass. The islets are composed of four major cell types. Besides the insulin producing β-cells, there are α-cells producing glucagon, δ-cells that produce somatostatin and PP cells, responsible for the production of pancreatic polypeptide (1).

When clinical manifestations of T1D appear, the majority of islets have often lost their β-cells, and an inflammation (insulitis) consisting of CD8 and CD4 T-cells as well as B-cells, macrophages and natural killer (NK) cells is present in and around the islets (8).

Clinical diabetes is often preceded by a period of asymptomatic disease that can vary from months to years. What happens during this period is unclear, although autoantibodies to insulin and other islet specific proteins usually appear. The presence of these antibodies are commonly used to predict disease onset, however, it is generally believed that it is T-cells rather than antibodies that mediate β-cell destruction (9; 10).

Etiology of T1D

T1D is a multi-factorial disease with both genetic and environmental factors involved in its etiology. At least six genetic loci have been associated with disease susceptibility. The strongest linkage is to MHC class II, with some alleles providing susceptibility and others protection (11). Other genes associated with T1D are the insulin gene (12; 13), genes involved in T-cell activation (CTLA-4 (14; 15), PTPN22 (16; 17) and CD25 (18)), and most recently, a SNP variant in the gene for the viral sensing protein MDA-5 (melanoma differentiation-associated gene 5) (19).

Although genetic factors are important in regulating susceptibility to T1D, they only confer a risk of 21–72% as seen in concordance studies with monozygotic twins (20; 21). This, together with the varying incidence between countries and the rapid increase in T1D among young people, indicate that environmental factors also play an important role in the etiology. There are several environmental factors that have been suggested to play a role in disease development such as dietary factors (e.g., insulin from cow milk, gluten or vitamin D), and infections (22). Infections with enteroviruses, especially those with coxsackieviruses serotype B (CVB) have been linked to T1D (23; 24). This will be discussed further throughout this thesis.

INTERFERONS

Interferons (IFNs) are a group of cytokines originally discovered in 1957 through their ability to interfere with viral replication (25). IFNs can be divided into three functional groups based on their sequences and recognition by specific receptors; type I IFNs (IFNα, -β, -ε, -κ, -ω and -τ), type II IFN (IFNγ) and the newly described type III IFNs (IFNλ1, IFNλ2 and IFNλ3) (26; 27).

IFNs are produced early after viral infection. Type I IFNs are produced by cells at the local site of infection, including infected parenchymal cells and plasmacytoid dendritic cells (pDC), while type II IFN (IFNγ) can be produced by NK cells and later on by T-cells. The expression pattern of IFNλ seems to follow that of type I IFNs, but as of today, this has not been extensively studied. IFNs act on immune cells by activating NK cells, macrophages and T-cells, but also act in an auto- and paracrine manner to upregulate the intracellular antiviral defense in surrounding cells.

IFN signaling

The three different groups of IFNs signal through distinct receptors. The type I IFNs signal through the IFNα/β receptor, a heterodimer composed of IFNα-receptor subunit 1 and 2 (IFNAR1 and 2). Type II IFN (IFNγ) binds as a dimer to a tetrameric receptor composed of two IFNGR1 and two IFNGR2 subunits. Type III IFNs signal via a receptor consisting of an IL-28 Rα chain and the IL-10Rβ chain (which is also part of the receptors for IL-10, IL-22 and IL-26).

All three types of IFNs share the basic pattern of signaling, ultimately leading to the transcription of IFN stimulated genes (ISGs). When the ligand binds, Janus tyrosine kinases (JAKs) associated with the receptors are activated and in turn recruit and phosphorylate the signal transducers and activators of transcription (STATs) which will dimerize and induce the transcription of specific genes. The different receptors signal through both distinct and common JAKs and STATs. After signaling through the type I receptor, the JAKs, Tyk2 and JAK1 activate STAT 1 and 2, which will dimerize, associate with IFN-regulatory factor-9 (IRF9) forming the transcription factor IFN-stimulated gene factor 3 (ISGF3). Type I IFNs can also signal through STAT 1 or STAT 4 homodimers. According to present knowledge, type III IFN signaling is similar to that of type I IFNs. The type II IFN activates JAK1 and 2 and the formation of STAT1

dimers and can also activate ISGF3- and STAT3-dependent pathways (26-28).

The JAK/STAT signaling pathway is negatively regulated by proteins belonging to the suppressor of cytokine signaling (SOCS) family. This family includes eight intracellular proteins of which several have been shown to regulate responses to cytokines. One of the best characterized SOCS proteins is SOCS-1 which inhibits JAK1 and JAK2. SOCS-1 knockout mice succumb before three weeks of age from multi-organ inflammation, but will survive if they also lack the gene for IFNγ, indicating that SOCS-1 is critical for regulating IFNγ signaling. However, SOCS-1 has been shown to inhibit several signal transduction pathways including those triggered by IFNα, IL-4 and IL-6 (29; 30).

Viral detection and induction of IFN production

How viruses are recognized and an immune response is elicited, is an area of intense research since this could have important implications for treatments and vaccination. The innate immune system recognizes pathogens through the pattern-recognition receptors (PRRs). The main targets for recognition of viruses are nucleic acids, which can have chemical modifications or specific structural features that make them differ from the nucleic acid of the host.

The toll-like receptors (TLRs) are a family of transmembrane proteins that play a central role in sensing pathogens (31). They are primarily expressed by cells in the innate immune system e.g. macrophages and dendritic cells. The TLRs that could be relevant for detecting CVB are TLR3, recognizing viral double-stranded (ds)RNA, as well as TLR7 and TLR8 recognizing single-stranded RNA. TLR4 has also been implicated in the response to viruses by the recognition of protein components of the viral particle. Two additional PRRs for viral RNA are the recently described cytosolic proteins MDA-5 and retinoic acid-inducible gene I (RIG-I). They are ubiquitously expressed and recognize viral RNA in the cytoplasm of an infected cell thereby inducing the expression of type I IFNs (32-35). Little is yet known about which PRRs are important for the recognition of CVB, although TLR4, TLR7 and TLR8 have been suggested to play a role (36; 37).

IFNs and the antiviral state

The ability of IFNs to interfere with viral replication in the absence of immune cells has been termed the antiviral state. IFNs can induce the expression of more than 100 genes with diverse antiviral activities (38). Many of these gene products are involved in the intracellular antiviral defense aiming to block viral replication, such as the PKR (double-

stranded RNA activated serine/threonine protein kinase) (39) and the RNaseL (40) pathways which have been extensively studied, whereas many of the newly described proteins still have undefined functions.

There are IFN regulated genes with a broad and relatively unspecific antiviral activity, others are specific for distinct classes of viruses (38). The group of known ISGs that target viral replication is constantly growing, now also including non-protein gene products namely the cellular microRNAs that target viral transcripts (41). The studies on the SOCS-1-transgenic (tg) mouse (see below) as well as studies from other groups have shown that the IFN induced antiviral state can determine enterovirus pathogenicity and tissue tropism (42-44).

IFNs in T1D—The SOCS-1-tg mouse

Experimental models have demonstrated that IFNs may play a dual role in the pathogenesis of T1D. For example, β-cell dysfunction and death is induced when islets are exposed to IFNγ in conjunction with interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α) *in vitro* (reviewed in (45)). Ectopic expression of either IFNγ or IFNα in β-cells induces diabetes in normal mouse strains (46; 47), but in contrast to these observations, ingested IFNα prevents diabetes development in an animal model for T1D (48).

Our group previously created a transgenic mouse overexpressing SOCS-1 specifically in the β-cells, with the purpose of studying the effects of IFN signaling during viral infection and autoimmunity (42). At that time, it was not known whether the IFNs exerted their effects by affecting the immune system or the β-cells directly. The SOCS-1-tg mice turned out to have two interesting phenotypes that supported a hypothesis that IFNs regulate diabetes development by directly affecting the β-cell:

1. **The SOCS-1-tg mice developed diabetes after an infection with CVB** (42), indicating that IFN signaling in β-cells is critical for β-cell survival during a CVB infection. Under the section *IFNs in the defense against virus induced diabetes* I will discuss this further together with a background on the host response to CVB infection.
2. **The SOCS-1-tg mice were protected from spontaneously developing diabetes** (49), indicating that the cytokine signaling in the β-cells also can enhance disease development. This will be discussed further together with a background on the mouse model under the section *IFNs in the autoimmune process of T1D*.

ENTEROVIRUSES AND T1D

Enteroviruses are small, non-enveloped single-stranded RNA viruses belonging to the picornavirus family. Enteroviruses are common, frequently causing infections in all age groups. Most infections are asymptomatic or give only mild respiratory symptoms, however, some infections have serious outcomes such as aseptic meningitis, myocarditis or pancreatitis. Enteroviruses are distinguished from other picornaviruses by certain physical properties. One characteristic is that they are stable in low pH and can resist the acidity in the stomach, thereof their fecal-oral route of infection. Enteroviruses are divided into polioviruses, coxsackie A and B viruses, echoviruses and newer enteroviruses. These groups of viruses are further divided into serotypes, CVB for example has six different serotypes (50; 51).

Coxsackievirus infection in the etiology of T1D

There have been numerous reports connecting CVB with T1D (23; 24). The first reports came in 1969, by Gamble *et al* showing that CVB antibodies were more frequent in T1D patients than in healthy controls, and that the onset of T1D parallels the seasonality of enterovirus infections (52; 53). Since then, various epidemiological studies and case reports have supported this connection. For example, there are studies showing that increased levels of islet cell autoantibodies parallel enterovirus infections in prediabetic individuals (54; 55). Enteroviruses can infect islet cells *in vitro* (56; 57) as well as *in vivo*, and there are case reports were CVB have been found in β-cells from T1D patients or individuals that have succumbed from a CVB infection (56; 57). In a recent study by Dotta *et al*, enterovirus was found in the islets of three out of six newly diagnosed T1D patients and in at least one patient the virus was typed to CVB (58).

Mechanisms by which CVB may cause T1D

There are different hypotheses as to how enteroviruses may cause T1D. One hypothesis is that autoimmunity against the β-cells can be induced without the β-cells themselves necessarily being infected. This could be explained by molecular mimicry, where shared epotopes between microbes and self proteins could lead to cross-reactivity (59). Molecular mimicry has been discussed in the context of many autoimmune diseases, but there is no direct evidence that it is involved in T1D. There is sequence homology between a CVB protein and an islet autoantigen, GAD65, but no cross-reactivity of T-cells has been found (60). Another

proposed mechanism is bystander activation, where potentially autoreactive T-cells would be activated by the inflammatory milieu during viral infection (61).

The fact that β-cells actually can be infected by CVB *in vivo* has put an emphasis on direct effects on the islets. Most CVBs are lytic viruses and could therefore cause substantial damage. There are also reports suggesting that CVB can cause persistent infection in islets (62; 63), which could render them dysfunctional. An interesting observation in the study by Dotta *et al* was that two out of six recently-onset diabetics displayed intact islets with insulin-positive β-cells. It is possible that their reduced function had something to do with the fact that they were infected with enteroviruses (58). The presence of a large number of β-cells in these patients gives hope for future treatments of recent onset T1D patients to regain their β-cell function.

A CVB infection could of course also activate the immune system towards the islets in an attempt to eradicate infected cells which could cause β-cell damage and perhaps lead to epitope spreading, with the activation of potentially autoreactive T-cells (59).

Can CVB infections explain the increasing incidence of T1D?

A study comparing enterovirus antibodies in children between countries with high and low diabetes incidence, suggested an inverse correlation between T1D and enterovirus infections in the background population (64). Although T1D is increasing, the frequency of CVB infections have decreased in developed countries, such as Sweden and Finland (65).

There could be several reasons for these observations. One could be that there is no connection between CVB infections and T1D. Another explanation could be that there are differences in the diabetogenicity between different strains of CVB. However, it could also be explained by the fact that repeated infections are needed to prevent autoimmunity. This has been described in two, not mutually exclusive hypothesis. The *hygiene hypothesis* states that our immune system needs to fight infections to keep a good balance between activating and regulatory mechanisms (66), without this balance allergy or autoimmunity can develop.

The *polio hypothesis* states that fewer infections and infections later in life can cause more severe disease outcomes. This was true for infections with another enterovirus, the poliovirus, at the beginning of the last century. As poliovirus infections decreased due to improved hygiene, the complications after developing an infection increased. This was due to lower levels of protective antibodies conferred by mothers to their children and to the fact that children had their initial infection later in life

when maternal antibodies could not protect them. The same scenario could be true today for other enteroviruses (67).

IFNs IN THE DEFENSE AGAINST VIRUS INDUCED DIABETES

Host defense against CVB with an emphasis on NK cells

Both innate and adaptive immune responses are important in fighting a virus infection. They block infection, inhibit viral replication and eliminate infected cells. The innate immune response holds the infection in check before the adaptive immune system becomes activated. The adaptive immune response is responsible for viral clearance and for the immunological memory.

Very little is known about the human immune response to CVB. Type I IFNs are of critical importance for controlling a CVB infection in mice as IFN receptor knockout mice succumb quickly to low doses of CVB (42). B-cells play an important role in the life-long protection against enteroviruses by producing neutralizing antibodies after infection or vaccination. This is emphasized by increased susceptibility to enterovirus infections in agammaglobulinemics as well as neonates and infants. T-cell responses are not well studied, however T-cells directed against CVB have been isolated (50; 68).

Studies in mice have suggested a role for NK cells in the immune response to CVB infection, for example by limiting virus replication (69; 70). NK cells are lymphocytes derived from the bone marrow belonging to the innate immune system. They can play an important role in the early response to certain viruses by killing virus infected cells or by producing cytokines. NK cells are one of the main producers of IFNγ, which can act directly on cells to induce an antiviral state or by activating the adaptive immune response. The activity of NK cells is regulated by activating and inhibitory ligands on target cells, with an important inhibitory ligand being MHC class I (71). Many viruses, including CVB (72; 73 and Paper III), down regulate MHC class I—presumably to avoid recognition by CD8 T cells—and can thereby theoretically become targets for NK cells. In a recent publication, islets from three newly diagnosed patients with T1D stained positive for enterovirus. NK cells were found as the main infiltrate surrounding these islets, raising the questions of whether they could play a role in viral clearance and/or immunopathology (58).

The β-cell antiviral response to CVB

CVB can infect β-cells *in vitro,* often leading to β-cell dysfunction and destruction (62; 74), however most infections *in vivo* are cleared without the development of T1D in the host. The SOCS-1-tg mouse model has

helped to resolve this paradox by demonstrating that IFN signaling in β-cells is critical for β-cells to survive a CVB infection (42; 75).

As mentioned above, type I IFNs are quickly produced at the local site of infection and reach distal organs such as the pancreas ahead of the virus. The IFNs induce an antiviral state, which lower permissiveness to infection. Tissue sections showed that there was a selective loss of the insulin producing cells in the SOCS-1-tg islets after infection, while the islets from non-tg mice were unaffected (42; 75). This clearly suggested that β-cells are dependent on IFN signals in order to survive a CVB infection *in vivo*. Additional studies demonstrated that pre-treatment with IFNs protected mouse islet cells from CVB replication *in vitro,* further supporting the role for IFNs in regulating β-cell permissiveness to CVB (42; 76). Taken together, the findings suggested that a reduced IFN response leads to enhanced β-cell permissiveness to CVB replication. The studies on the SOCS-1-tg mouse also showed that the destruction of the β-cells after infection was in part dependent on the presence of NK cells (42).

The role for IFNs in protecting *human* pancreatic islets from CVB infection (77) and the way IFNs provided protection from CVB remained unclear. Moreover, if and how NK cells contribute in the host antiviral response to CVB in humans had not been studied. With this as background, the focus of Papers I-III has been on the innate immune response during CVB infection.

IFNs IN THE AUTOIMMUNE PROCESS OF T1D

The work cited above establishes a clear basis for considering IFN signaling in β-cells to be of critical importance during a systemic virus infection. However, as mentioned previously, IFNs may also have detrimental effects in the pathogenesis of T1D. Prior to the studies on the SOCS-1-tg mouse, it was unknown whether IFNs exerted these negative effects by acting on cells in the immune system or by acting directly on the β-cells. With the goal of studying this issue, the SOCS-1-tg mouse was generated on the non-obese diabetic (NOD) mouse background.

The non-obese diabetic (NOD) mouse

The NOD mouse was discovered in Japan in 1980 during the selection of a cataract-prone strain (78). This mouse develops diabetes spontaneously at around 12-14 weeks of age as a result of a chronic autoimmune process against the β-cells. Today the NOD mouse is a commonly used animal model for T1D (79). Several of the susceptibility genes in humans have been shown to play a role in the pathogenic process in the NOD mouse (80). Environmental factors such as infections, will also influence the disease incidence (81).

As in many humans with T1D, there is a long asymptomatic period preceding disease onset in the NOD mouse. Autoantibodies are present and lymphocytes start infiltrating the pancreas already as early as three weeks of age. T-cells, both CD8 and CD4, are necessary for disease development (79). Islet reactive T-cells are initially primed in the pancreatic lymph nodes (PLNs) and removal of the PLNs before three weeks of age will completely protect the NOD mouse from diabetes development (82). Three pathogenic CD8 T-cell populations isolated from inflamed islets have been described, recognizing insulin, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and dystrophia myotonica kinase (DMK) (83). Many of these CD8 cells express the activating receptor NKG2D and blocking this receptor decreases diabetes incidence and insulitis (84). Moreover, the accumulation and function of the effector T-cells in the pancreas have been suggested to be regulated by FoxP3 expressing regulatory T-cells (85), but this regulation is not sufficient to inhibit disease onset in the majority of mice. Other immune cells involved in the disease process, presumably as antigen presenting cells (APCs), are macrophages, dendritic cells and B-cells (79).

Protection from spontaneous diabetes

When the SOCS-1-tg mouse was generated, it became apparent that the β-cell response to cytokines influenced the autoimmune process in the NOD mouse. The SOCS-1-tg mouse had a reduced incidence of diabetes compared to the non-tg mouse and was therefore a useful model in studying the role of cytokine signaling in β-cells during the development of autoimmunity (49).

The SOCS-1 expression in β-cells did not affect central tolerance in the mice and the early recruitment of lymphocytes to the pancreas did not differ from the recruitment seen in the non-tg mice. However, the T-cells that infiltrated the pancreas of 18-week-old non-diabetic SOCS-1-tg mice had a reduced capacity to trigger diabetes if adoptively transferred into NODscid recipients. This suggested that β-cells have the capacity, through their response to cytokines, to affect the surrounding T-cells. However, it remained unclear how the β-cell response to IFNs affected the T-cell repertoire in the pancreas. This was the focus of Paper IV.

OBJECTIVE

The objective of this thesis was to give a better understanding of the β-cell's role in regulating its own fate by responding to IFNs during infections and autoimmune processes.

SPECIFIC AIMS

I. To investigate whether two proteins involved in antiviral defense, namely the PKR and the RNase L pathways are involved in the defense against CVB.

II. To study genes expressed in human islets after IFN treatment, and to determine whether IFNs induce an antiviral state in human islets.

III. To examine the expression of activating and inhibitory ligands on CVB infected cells as well as the human NK cell response to CVB infected cells, with regards to cytotoxicity and IFNγ production.

IV. To analyze how the β-cell response to cytokines affects the T-cell repertoire in the pancreas during autoimmunity.

MATERIALS AND METHODS

The work in this thesis has included studies on different mouse models to characterize critical events during infection and autoimmunity (Papers I and IV). I have also had the opportunity and privilege to work with human islets provided by the Nordic Network for Clinical Islet Transplantation (Paper II). This has provided the unique possibility to confirm findings from studies on mice in a human setting. All experiments were conducted in accordance with institutional guidelines and approved by the local ethic committees. For further details on material and methods please see the original papers.

ANIMAL AND HUMAN CELLS AND TISSUE

Mouse islets were isolated by injection of collagenase into the bile duct, removal of the pancreas and subsequent hand picking of the islets from the total pancreatic digest. The islets were cultured in RPMI 1640 (supplemented with 10% heat-inactivated FCS, L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin) and media was changed every other day. The islets were cultured for at least 6 days in order to remove remaining exocrine tissue and immune cells. To isolate *pancreatic-infiltrating lymphocytes*, circulating blood was removed by perfusion with PBS prior to pancreas retrieval. Total lymphocytes were isolated from collagenase-digested pancreata using Lymphoprep or total T-cells were isolated using MACS separation with positive selection of CD90. Single-cell suspensions of *PLNs and spleens* were prepared by homogenizing the organs between two frosted slides.

Human islets from ten donors (five female and five male) were purified from human cadaver donors at the Uppsala University Hospital (The Nordic Network for Clinical Islet Transplantation) (86). After arrival at Karolinska Institutet, the islets were cultured in RPMI 1640 (supplemented with 10mM HEPES buffer, 0.25µg/ml fungizone, 50µg/ml gentamycin, 2mM L-glutamine, 10µg/ml ciprofloxacin and 10% heat-inactivated human serum) and media was changed every other day. The average donor age was 57 ± 12 years (range 36 to 74 years), and the cold ischaemia time was 8.24 ± 4.35 hours (range 4.34 to 20.04 hours). The islets had a purity of 83 ± 12% (range 60-95%), as determined by

dithizone staining, and were further purified by hand picking. The quality of the islets was evaluated by insulin release in response to low and high glucose concentrations. For eight donors, a dynamic perfusion system was used as previously described (87). The islets responded with a stimulation index of 14.5 ± 10.7 (range 3.6 to 32.3). For two donors, glucose-stimulated insulin release was measured after static incubation and the simulation index for these islets were 3.2 and 3.8 respectively.

HeLa and HepG2 cells (a kind gift from Dr. L. Poellinger, Karolinska Institutet), were grown in RPMI 1640 (supplemented with 10% heat-inactivated FCS, L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin). Cells were passaged two-three times per week.

Pooled *HUVEC (Human Umbilical Vein Endothelial Cells)*, a kind gift of Drs. H. Domeij and J. Frostegård, Karolinska Institutet, were cultured in EBM® supplemented with EGM™ SingleQuots® at 37°C and 5% CO_2. Media was changed every other day and cells were passaged when reaching ≥ 80% confluency.

PBMCs (Peripheral Blood Mononuclear Cells) were isolated from healthy human donors by density gradient centrifugation. In brief, blood was diluted with PBS (1:1), pipetted onto Lymphoprep and centrifuged for 20 minutes at room temperature. Lymphocytes were carefully removed and washed three times in PBS before being resuspended in RPMI 1640 (supplemented with 10% heat-inactivated FCS, L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin) at a concentration of 10^7 cells/ml. All PBMC isolates were cultured overnight in RPMI before they were used for experiments. In order to obtain activated cells, PBMC were cultured over night in the presence of 250U/ml rIL-2.

IN VIVO EXPERIMENTS

Mice were bred in specific pathogen-free environments at The Scripps Research Institutet (TSRI) and Karolinska Institutet. Screening for transgenic mice was performed with PCR using DNA from tail biopsies. Diabetes monitoring was performed by measuring blood glucose levels in blood drawn from non-fasting animals. Animals were considered diabetic if they had a blood glucose value of 13.0 mM or above for two consecutive measurements. In Paper I, animals were infected with one intraperitoneal injection of CVB4 (100 PFU diluted in 200 µl Hank's balanced salt solution, HBSS) and sacrificed on days 3 or 4 post infection. Alternatively, the mice were monitored for survival for a 24-day study period. Adoptive transfers of autoreactive T-cells (Paper IV) were performed by intravenous injections in the tail vein.

MICROARRAY ANALYSIS

To obtain an overview of the gene expression after IFN treatment in human and mouse islets, microarray analyses were performed (Papers II and IV). The purpose was not to define the complete response to IFNs, but rather to find candidate genes of interest, and to confirm the expression of these genes with other methods. The quantity and quality of the RNA was determined using an Agilent Bioanalyzer (performed by the Bio-informatics and Expression Analysis core facility at Karolinska Institutet).

Human islets from four donors were exposed for six hours to IFNα2b (1000U/ml) or IFNγ1b (1000U/ml). The RNA was converted into cDNA and analyzed by the Genomics Core at the Lerner Institute, Cleveland, Ohio, on a microarray, described in (88), representing 2.178 genes involved in innate immune responses (consisting of 950 genes containing adenylate/uridylate (AU)-rich elements, 855 ISGs, 288 dsRNA responsive genes and 85 housekeeping genes).

cDNA from mouse islets cultured with or without IFNα (1000U/ml) or IFNγ (1000U/ml) for 6h were run on affymetrix Mouse Genome 430 2.0 arrays, an array comprising the whole mouse genome with more then 39.000 transcripts. The arrays were run and analyzed by the Bio-informatics and Expression Analysis core facility at KI.

VIRUS STOCKS AND INFECTIONS

The CVB4 Edwards strain 2 (E2) (89) (Paper I) was originally obtained from C. Gauntt (University of Texas, San Antonio, Texas) and the stock of CVB3 Nancy (Papers II-III) was obtained from Dr. G. Frisk, Uppsala University.

In vitro infections of mouse and human islets were performed as previously described (42). Briefly, islets were treated for 24h with IFNα or IFNγ (1000U/ml) or left untreated. Islets from each condition were washed with serum free media and infected for 1.5h with virus in serum free media. Thereafter, the islets were washed and placed in Millicell culture plate inserts containing fresh culture medium, with or without IFNs. The islets were incubated at 37°C and the media was changed every second day until day six post infection. Viral titers of the culture media were determined by a standard plaque assay using HeLa cells (61).

Infections of HeLa cells, HepG2 cells and HUVEC were performed in serum-free media for one hour. Thereafter, the virus was washed away

and the cells incubated at 37°C. For co-culture experiments cells were trypsinized after infection, washed with culture medium and placed in 96 well plates together with PBMC.

PCR

PCR is a powerful tool to determine gene expression and has been used to various degrees in all four papers. In Paper I, semi-quantitative PCR was used to study the expression levels of 2-5AS after IFN treatment. Preliminary experiments were performed to establish reaction conditions that allowed reproducible and reliable amplifications. To determine the amounts, PCR products were run on agarose gels containing ethidium bromide. The bands were visualized by UV light, photographed and analyzed using NIH Image 1.63. The intensities of the 2-5AS bands were expressed in arbitrary density units and normalized to the actin density units. In Papers II-IV real-time (RT) PCR was used to quantify gene expression, using either TaqMan® Gene Expression assays or RT^2 Real-Time™ SYBR Green/ROX PCR master mix. All samples were run in triplicate and the mRNA expression level for each gene was normalized against mRNA for the housekeeping gene GAPDH.

WESTERN BLOT (WB) ANALYSIS

To study the protein expression in mouse and human islets, WB analysis was performed on selected proteins (Papers I and II). Islet cells were lysed and the total protein concentrations determined using the Bradford method or a BCA Protein Assay Kit. Equal amounts of total protein extracts from each sample were loaded on SDS polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes. After protein detection, membranes were stripped and relabeled with antibodies against actin. Band densities for the different proteins were analyzed by NIH Image 1.63 and normalized against the densities for actin.

FACS ANALYSIS

In Papers III and IV, FACS analysis was the main technique used for characterizing cells. Stainings for cell surface markers, intracellular proteins and various functional markers, such as CD107a degranulation and viability measurements were performed. Data were collected using a

FACSCalibur Instrument or CyAn and analyzed using CellQuest or FlowJo software.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Organs fixed in formalin and embedded in paraffin were cut in 4 μm thick sections and stained with a primary antibody against CXCL10. To detect bound primary antibody, a secondary biotinylated antibody was used in conjunction with Elite Vectastain ABC kit and Peroxidase Substrate kit. Slides were counterstained in Mayer's Hematoxylin.

STATISTICAL ANALYSIS

For the specific statistical methods used for each experiment, the reader is referred to the original papers. In general, non-parametric tests were used when a normal distribution could not be presumed.

RESULTS AND DISCUSSION

PAPER I

RNase L and Double-Stranded RNA-Dependent Protein Kinase Exert Complementary Roles in Islet Cell Defense during Coxsackievirus Infection

The studies on the SOCS-1-tg mouse had indicated that the islet response to IFNs was critical for β-cell survival during CVB infection (42), but little was known regarding the IFN-induced islet defense against CVB. In Paper I the hypothesis that two known pathways, the 2-5AS/RNase L and PKR pathways, mediate the antiviral activities of IFNs in CVB-infected pancreatic islets was tested.

The 2-5ASs are a group of enzymes that become activated by dsRNA, thereafter synthesizing 2-5 oligoadenylates (2-5As), which in turn activate the intracellular endonuclease RNaseL. The expression of 2-5ASs can be regulated by IFNs and their level of expression is often the rate-limiting step in the 2-5AS/RNaseL pathway (90). RNaseL cleaves within single stranded regions of RNA and has been shown to protect cells from viral replication in several ways, including cleavage of viral RNA and cellular RNA required for viral replication. In a recent study, the RNaseL pathway was shown to amplify the type I IFN response to viruses, supporting a broader antiviral state in the organism (91). The 2-5ASs provide protection from a number of RNA viruses (40) including some picornaviruses (92), however their potential role in regulating pancreatic islet cell permissiveness to CVB had not previously been tested.

PKR is a serine/threonine protein kinase that becomes activated by dsRNA. Activated PKR has a number of cellular functions, however best characterized is its ability to inactivate the ribosomal protein eiF2a resulting in blocking of protein translation and inhibition of viral replication (39). PKR is an IFN-regulated gene and is important for protection against a broad range of viruses. It has been suggested that the levels of endogenous PKR differ between cell types and could influence tissue tropism (93). The role of PKR in the islet defense against CVBs had not previously been studied.

By infecting knock-out mice, we discovered that both the 2-5AS/RNase L and PKR pathways were critical for the defense against CVB. Mice lacking either RNaseL or PKR succumbed to a dose of CVB that was not lethal to non-tg mice (Figure 2 in Paper I). We found that 2-5AS, RNAseL and PKR were expressed in mouse islets and that the expression levels of 2-5AS and PKR were regulated by IFNs (Figure 1 in Paper I). *In vitro* infections of islets from RNaseL or PKR knock-out mice revealed that RNase L is required for an intact IFNα-induced defense against CVB, while PKR is indispensable for an efficient IFNγ-induced islet anti-CVB defense, suggesting that the 2-5AS/RNase L and PKR pathways contribute with exclusive and complementary anti-CVB signals following exposure to IFNα and IFNγ, respectively (Figure 3 in Paper I).

Considering that IFNγ also increased the expression of 2-5AS mRNA, it was surprising to find that the 2-5AS/RNase L pathway does not play an important role in the IFN-γ-mediated protection from CVB. The same was true for PKR, which is upregulated by IFNα, but does not seem to play an important role in the IFNα-mediated protection. This indicates that the defense against CVB is complex and that there could be other factors induced by IFNα and IFNγ, respectively, that could influence the activity of these pathways.

Another interesting observation was that although the anti-CVB action of IFNα and IFNγ was clearly impaired in islet cells lacking RNase L and PKR, respectively, it was not completely lost. Weak residual antiviral activities were also observed in IFN-treated islets from double knockout (DKO) mice, indicating that other proteins may contribute to the regulation of islet permissiveness to CVB. Many antiviral defense proteins have been described that potentially could play a role in the islet defense against CVB. One candidate gene that we found interesting was the exonuclease ISG20 due to its differential expression in non-tg compared to SOCS-1-tg islets (Hultcrantz and Flodström-Tullberg, unpublished observation). ISG20 has been shown to confer resistance to RNA viruses such as vesicular stomatitis virus *in vitro* (94), but it was not known whether ISG20 played a role in the protection against CVB. However, overexpression of ISG20 in HeLa cells did not reduce the degree of infection (Hultcrantz and Flodström-Tullberg, unpublished observation), indicating that this particular protein does not play a major role in anti-CVB defense. Finding other proteins involved in the islet defense against CVB is a challenge for the future.

PAPER II

Interferons Induce an Antiviral State in Human Pancreatic Islet Cells

After having studied the IFN induced anti-CVB defense in mouse islets, the natural question was whether this information was relevant also in the human context. It was still unknown whether IFNs would up-regulate the expression of genes involved in antiviral defense and protect human islets from CVB replication. To study the human islet antiviral response to IFNs, islets were treated for six hours with IFNα or IFNγ. By using a custom microarray comprising genes involved in innate immune responses (88), as well as RT-PCR and WB analysis, we could show that human pancreatic islet cells respond to type I (IFNα) and to a certain extent also type II (IFNγ) IFNs by upregulating the expression of several genes involved in antiviral defense and viral detection.

As we showed in Paper I, the *2-5ASs* play an important role in the IFNα mediated protection from CVB in mouse islets. The expression of *2-5AS* in human islets was found to be regulated by IFNα and to a lower degree also IFNγ (Figure 1B in Paper II), indicating that the regulation of these enzymes seen in mouse islets also stands true for human islets. Whether their role is as prominent in the protection from CVB in human islets is still unknown. If that is the case, it could be of importance since attempts are being made to induce this pathway with new therapeutic tools (95).

Another gene found to be upregulated in our array, was the antiviral protein Viperin (*v*irus *i*nhibitory *p*rotein *e*ndoplasmic *r*eticulum-associated, *in*terferon inducible). Previously, Viperin has been shown to be expressed in human islets following *in vitro* infection with CVB, and after stimulation with IL-1 + IFNγ (96). In this study, we demonstrated that Viperin mRNA and protein are expressed by human islets following either IFNα or IFNγ stimulation alone (Figures 1A and 3A in Paper II). Viperin has an antiviral effect against human cytomegalovirus (CMV) and hepatitis C virus *in vitro* (97; 98), and it is expressed in human cell lines after IFN-treatment and during infection with both RNA and DNA viruses (97; 98). In a recent study, Viperin was shown to block influenza A virus release from the plasma membrane by affecting the formation of lipid rafts, which are the sites of influenza virus budding (99). It is still unknown whether Viperin contributes to human islet anti-CVB defense. However, if the effect on lipid rafts is the sole mechanism for Viperin mediated antiviral defense, this would be unlikely since CVB does not have a lipid envelope. Of course, this does not rule out that Viperin might play a role in the islet defense against other viruses, such as CMV.

CXCL10 is a chemokine that attracts CXCR3 expressing T-cells to sites of infection and inflammation (100, and references therein). Others have shown that human islets express CXCL10 following treatment with IFNγ in combination with other cytokines, or after CVB infection (96; 101; 102). In this study we showed that CXCL10 mRNA expression is significantly increased following IFNα treatment alone (Figure 1C in Paper II).

Studies in an animal model have shown that islet production of CXCL10 during infection leads to a recruitment of virus-specific CXCR3+ T-cells to the islets (103), suggesting that IFN-induced human islet expression of CXCL10 is central in the host defense against viruses with a tropism for pancreatic islet cells. It is of note that CXCL10 expression may also recruit islet-reactive T-cells to the pancreas in individuals predisposed to develop T1D (103; 104).

Todd and colleagues recently identified a new T1D susceptibility locus (19). A candidate within this locus is the gene encoding the cytosolic viral RNA sensor MDA-5. We analyzed the expression of MDA-5 as well as two other known intracellular sensors for viral RNA, namely RIG-I and TLR3. RIG-I recognizes 5'triphosphate RNA present in many RNA viruses (33; 35), and acts via the same signaling pathway as MDA-5 to induce type I IFN production in virus-infected cells (32; 34). TLR3 located in the endosomes induces type I IFN after detection of phagocytosed dsRNA (105). Interestingly, mRNA for all three genes were expressed in human islets and their expression levels increased significantly following IFNα-treatment (Figure 2 in Paper II). The expression of MDA-5 and RIG-I was also confirmed on the protein level with WB analysis (Figures 3B-C in Paper II). Chehadeh *et al* have previously shown that human islets produce type I IFN in response to CVB infection *in vitro* (62). This together with our findings, suggests that human islet cells could contribute to the regulation of their own permissiveness to certain virus infections by TLR3, MDA-5- or RIG-I-induced endogenous type I IFN production.

Knowing that human islets respond to IFNs by upregulating genes involved in the defense against viruses, it was interesting to study whether this would have an effect on islet permissiveness to CVB replication. Human islet cells are permissive to CVBs (56; 57), but it had not previously been shown whether IFNs regulate their permissiveness to CVB infection. By pretreating islets with IFNs and infecting them *in vitro*, we found a substantial block of CVB replication in islets treated with IFNs: IFNα led to a nearly complete protection from CVB replication while IFNγ prevented CVB replication to a somewhat lesser degree (Figure 4 in Paper II). This correlates with the fact that IFNγ was

less potent in inducing the expression of genes involved in intracellular antiviral defense (Figure 1 and table 1 in Paper II).

It remains to be elucidated whether the genes studied here or other antiviral pathways are important for the islet defense against CVB. Future experiments will also determine whether the islets can detect an infecting CVB through MDA-5, RIG-I or TLR3 recognition and thereby produce type I IFNs. Although these questions remain to be answered, this study clearly supports the notion that human islet cells, by responding to IFNs, regulate islet infection and inflammation (Figure 1 (106)).

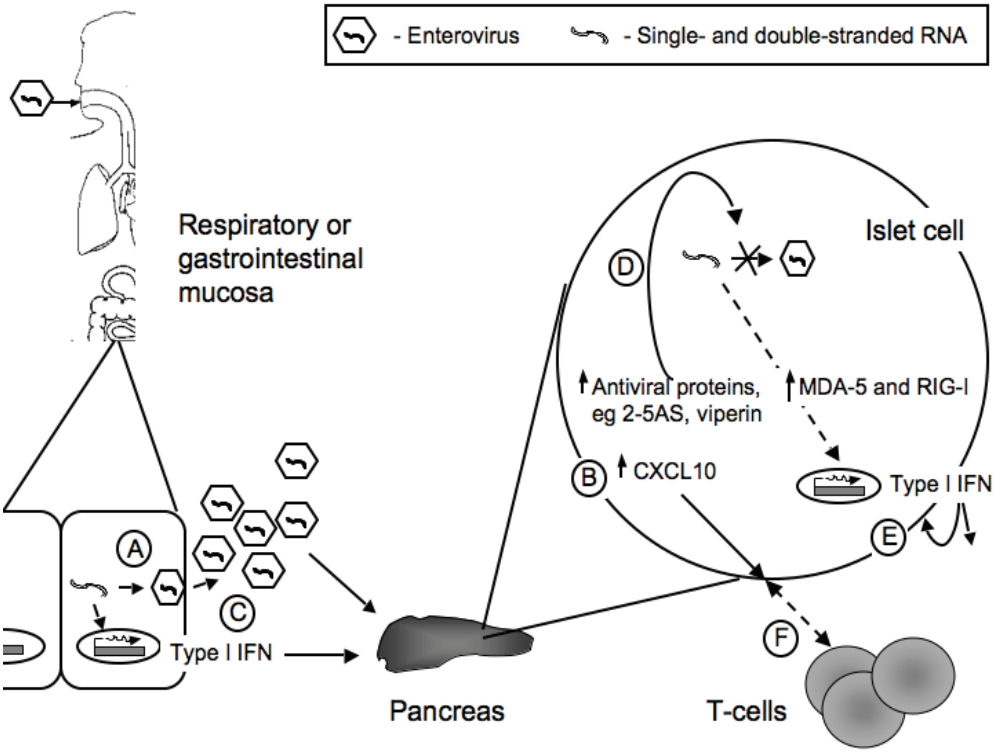

Figure 1: Schematic model for how IFNs acting directly on islet cells may contribute to the regulation of infection, inflammation and autoimmunity. (A) At the local site of infection, here exemplified by CVB infecting the gastrointestinal or lung mucosa, infected cells rapidly produce type I IFNs that spread systemically, reaching distal cells and tissues such as the pancreas. (B) Within the pancreas, the IFNs act on the islet cell by upregulating genes involved in virus detection and antiviral defense. (C) Once the virus has completed its replication cycle, new virus particles are released which eventually reach the pancreas. (D) By then, the islets have entered an IFN-induced antiviral state, which prevents virus replication and may facilitate type I IFN production in infected cells. (E) The release of type I IFNs by islet cells may further enhance the intra-islet antiviral defense, contributing to the prevention of excessive tissue damage. (F) In parallel, the IFN-induced local expression of genes involved in lymphocyte chemotaxis, such as CXCL10, may attract virus specific T-cells to the pancreas. In genetically predisposed individuals, the pro-inflammatory milieu may result in bystander activation of autoreactive T-cells (61), contributing to cell destruction and development of T1D.

PAPER III

IFNγ Production Dominates the Early Human Natural Killer Cell Response to Coxsackievirus Infection

The studies with the SOCS-1-tg mouse had indicated that NK cells were involved in the islet destruction during CVB infection. However the mechanism(s) by which NK cells contribute to β-cell damage was not known. NK cells have potent antiviral activity, both by killing virus infected cells and by the production of cytokines. The balance of activating and inhibitory signals relayed from the cell surface controls the induction of NK cell activity (107). Virus infections can lead to the upregulation of activating NK cell ligands (108; 109). One of the most powerful NK cell activating receptors is NKG2D (110), with the ligands MICA, MICB and ULBP1-4. Many viruses can also downregulate the inhibiting receptor MHC class I, presumably to avoid the recognition of CD8 T-cells. Prior to this study, it was unknown if and how NK cells are involved in the defense against CVB in humans.

Since very little was known in general about NK cell responses to CVB and since there is a limited availability of human islets, we believed it would be unethical to begin studying the NK cell response to human islets before some initial questions were answered. For this reason, we carried out a basic study, analyzing the effect of CVB on human cell lines and the human NK cell response to CVB infection *in vitro*.

By setting up stainings for the intracellular viral protein VP1, we could distinguish between infected and uninfected cells within the same culture (Figure 1 in Paper III). This enabled studies on the cell surface expression of NK cell ligands after infection. We could show that CVB infection led to a markedly lower cell surface expression of the inhibitory NK cell receptor ligands HLA class I on HeLa and HepG2 cells as well as HUVEC (Figure 2 in Paper III). CVB expresses proteins that can interfere with intracellular protein transport and up-regulate endocytosis in host cells, which could result in the lower HLA expression seen here (72; 111-113). Surprisingly, we found a decrease in the surface expression of the activating receptor MICA as well (Figure 2 in Paper III). The regulation of the NK cell activation was therefore unpredictable. We studied degranulation of NK cells as well as caspase activity and viability of infected HeLa cells after co-culture with PBMCs and showed that infection did not lead to increased NK cell killing of infected cells (Figure 3 in Paper III).

Another important function of NK cells is their production of IFNγ, which can act on cells by upregulating their intracellular antiviral defense, as well as activate the adaptive immune response. We studied the IFNγ expression in NK cells, after encounter of infected cells, by intracellular FACS analysis and ELISA and could show that NK cells respond to infected cell lines by producing IFNγ (Figure 4 in Paper III). NK cells have recently been demonstrated to surround enterovirus-positive pancreatic islet cells in patients with recent onset T1D (114), however what role the NK cells are playing there is still unclear. As was shown in Paper II, a milieu containing IFNs could rescue human islet from viral replication. On the other hand, IFNγ can have detrimental effects on the islets if it is combined with other proinflammatory cytokines (115-117). In an animal model where the β-cells were ectopically expressing IFNγ, the mice were protected from the damaging effects of a CVB infection (118). In contrast to this, the SOCS-1-tg mouse is protected from CVB induced diabetes when NK cells are depleted, indicating that NK cells increase islet damage during infection (42). This reflects the delicate balance in fighting an infection without causing excessive tissue damage. For the pancreatic islets, with low capacity for regeneration, this balance is of course of critical importance. Further studies on the interaction between infected human islets and NK cells is now warranted.

PAPER IV

The Target Cell Response to Cytokines Governs the Autoreactive T-cell Repertoire in the Pancreas

Previous studies had shown that overexpression of SOCS-1 in the β-cells of NOD mice reduced the incidence of diabetes (49). These studies also indicated that there was no difference in numbers of lymphocytes infiltrating the pancreas, but rather in the pathogenicity of these cells. Our overall aim with this study was to understand more about the mechanisms by which β-cell cytokine signaling increases the incidence of diabetes in the NOD mouse. We approached this question in two ways: by studying the genes expressed in the islets after IFN stimulation as well as by studying the T-cells infiltrating the pancreas.

Affymetrix Mouse Genome 430 2.0 arrays were used to study the gene expression in IFN-stimulated SOCS-1-tg and non-tg islets. Our hope was to find candidate genes that we could study further and perhaps link to T-cell activation or homing. The array comprised the whole mouse genome with more than 39.000 transcripts. Naturally this generated a substantial amount of information. Our initial thought was to find candidate genes that were regulated in non-tg islets, but not in SOCS-1-tg islets—however many genes were regulated in both, albeit to a lower degree in the SOCS-1-tg islets (Table 1). A possible explanation for the fact that gene regulation was also observed in the islets of the SOCS-1-tg mice, could be expression in the other cell types within the islets which should respond normally to IFNs (i.e., α, δ and PP cells). Another reason could be that the SOCS-1 expression is not enough to completely block the signaling pathway induced by IFNs.

	IFNα vs control		IFNγ vs control	
	increased	decreased	increased	decreased
Regulated genes in non-tg islets	238	43	503	168
Regulated genes in SOCS-1-tg islets	189	5	216	42

Table 1. An illustration of the numbers of genes regulated by IFN-treatment in SOCS-1-tg and non-tg NOD islets analyzed on affymetrix Mouse Genome 430 2.0 arrays.

One gene found to be expressed at higher levels in non-tg vs. SOCS-1-tg islets was the chemokine CXCL10 (discussed under Paper II). A difference in the expression of this gene was also seen using RT-PCR (Figure 1B in Paper IV). Immunohistochemical stainings showed that islet cells adjacent to the infiltrating immune cells express strong levels of CXCL10 (Figure 2). However, it is still unclear which comes first: Is CXCL10 expressed initially recruiting immune cells to the islets or is it that CXCL10 is upregulated in the islet cells after the arrival of IFN-producing immune cells? Perhaps both mechanisms are involved and the interaction between islets and immune cells leads to an acceleration of the diabetogenic process.

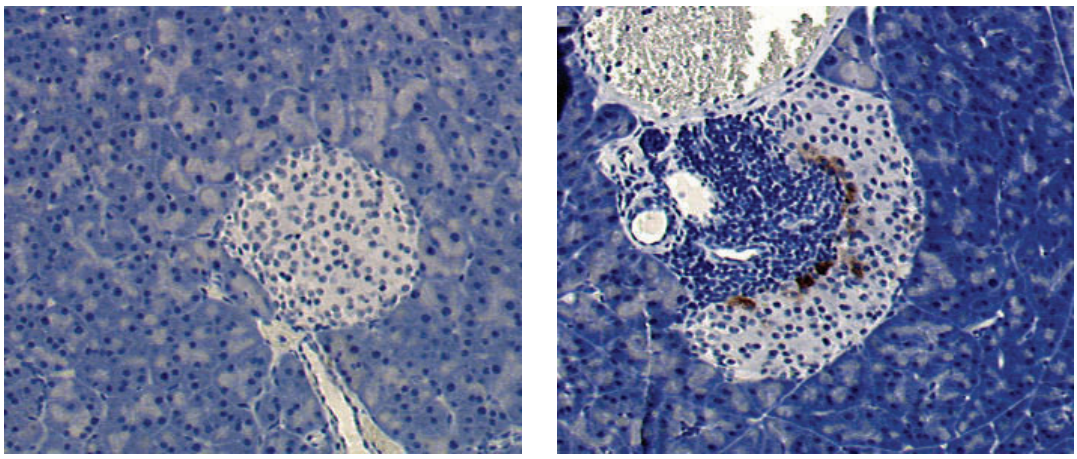

Figure 2. Islet cells adjacent to infiltrating immune cells express CXCL10. The picture shows islets from 8-week old mice. To the left is an islet with no insulitis and to the right an islet with invading lymphocytes. CXCL10 staining is shown in brown. Original magnification x10.

In parallel, the T-cell repertoire in the pancreas of pre-diabetic SOCS-1-tg and non-tg mice was studied to see whether there were differences that could be correlated to pathogenicity. We characterized the composition of the infiltrating T-cells by isolating T-cells from the pancreata, staining them for various markers, and analyzing them with FACS analysis. No differences in the major T-cell populations were found (Figure 2 in Paper IV), but the percentage of pathogenic IGRP-specific CD8 T-cells, known to be predictive for disease development (119), was significantly lower in the SOCS-1-tg pancreas as well as PLNs compared to the same organs in the non-tg mice (Figure 3A in Paper IV).

IGRP-specific CD8 T-cells are important for disease development in NOD mice, and have been shown to infiltrate the pancreas of the NOD mouse to varying degrees (120; 121). Moreover, studies by Chong *et al*

show that 8.3 TCR-tg NOD mice harboring T-cells recognizing an epitope within the IGRP protein, are protected from diabetes if crossed to the SOCS-1-tg mice (122), further illustrating the relevance of SOCS-1 in preventing β-cell damage caused by these specific T-cells.

Although the mechanisms remain to be elucidated, this study suggests that the β-cell response to cytokines is essential for the accumulation of pathogenic T-cells and the progression from insulitis to diabetes in the NOD mouse. By adoptive transfer studies using CFSE labeled splenocytes from 8.3 TCR-tg NOD, we showed that there was no difference in proliferation in the PLN (Figure 3C in Paper IV), suggesting that T-cell priming in the PLN occurs normally in the SOCS-1-tg mouse. Neither was the proliferation of transferred cells isolated from the pancreas lower in SOCS-1-tg mice (Hultcrantz and Flodström-Tullberg, *unpublished observation*), indicating that there was no change in the restimulation of islet-specific T-cell clones upon their arrival in the pancreas.

One possible explanation for the lower accumulation of IGRP specific T-cells in the pancreas of SOCS-1-tg mice could be a reduced expression of MHC class I, since it has been shown that SOCS-1 prevents the cytokine-induced expression of MHC class I on β-cells. Interestingly, a similar phenotype was seen when MHC class I expression was blocked in β-cells by the expression of the adenovirus transgene E19. In these mice, the accumulation of autoreactive CD8 T-cells in islets was impaired, however no effect was seen on the priming in the PLNs (123). Another explanation to the change in the frequency of IGRP specific T-cells in the pancreas of the SOCS-1-tg mice could be the differential expression of IFN inducible chemokines (e.g. CXCL10), something that remains to be studied.

In summary, this study shows that the cytokine signaling in β-cells affects the accumulation of pathogenic T-cells in the pancreas of NOD mice. This highlights an additional level of regulation that is present in non-lymphoid tissue and is dependent on the target cell itself. These findings are of importance by showing that modulation of β-cell cytokine responses can affect the local regulation of the autoimmune process (compare Figure 1).

CONCLUDING REMARKS

This thesis describes some of the positive and negative effects of IFNs during CVB infection and autoimmunity. It shows that the 2-5AS/RNaseL and the PKR pathways are important for the IFN induced islet defense against CVB in mice, and verifies that IFNs have similar effects on human islets by upregulating antiviral defense genes and blocking viral replication. On the other hand, the IFN response in islets can have detrimental effects in individuals susceptible to T1D by increasing the accumulation of pathogenic T-cells in the pancreas. Furthermore, in this thesis a possible source of IFN has been identified in the islet vicinity during CVB infection: NK cells respond to CVB infected cells primarily by IFNγ production and not killing. Taken together, these findings suggest that the β-cell is an active player in disease development, involved in antiviral defense and in an intricate communication with the immune system.

Individual variations in the β-cell specific response to cytokines and the host defense against virus infections could possibly influence susceptibility to T1D. Studies of the responses to IFNs and infection in healthy individuals as well as individuals with T1D will therefore be an interesting prospect for the future. For example, it will be of importance to see whether the SNP in the viral-sensing gene MDA-5 that has been associated with an increased risk for T1D confers a difference in the IFN response to viruses such as CVB. If an increased susceptibility to T1D can be linked to certain variations in the defense against viruses, it would increase our understanding of the factors and mechanisms that trigger β-cell death. This knowledge could also be a tool for finding individuals at risk for developing viral induced T1D, individuals who might benefit from a future vaccine to CVB.

SVENSK SAMMANFATTNING

Typ 1 Diabetes (T1D), tidigare kallad ungdomsdiabetes, är en sjukdom som beror på att man inte längre kan producera insulin, vilket är nödvändigt för att transportera glukos från blodet in i cellerna. Anledningen till att inget insulin produceras är att de insulinproducerande β-cellerna, som finns i de så kallade Langerhanska öarna i bukspottkörteln, har blivit förstörda. Patienter med T1D behöver därför insulinbehandling livet ut.

T1D beskrivs ofta som en autoimmun sjukdom, dvs. en sjukdom som orsakas av att det egna immunförsvaret attackerar organ i kroppen. Varför detta sker är fortfarande oklart. Genom studier på enäggstvillingar har man kommit fram till att både gener och miljöfaktorer spelar in. Det finns flera argument för varför miljöfaktorer förmodligen spelar roll. Till exempel varierar incidensen av T1D dramatiskt mellan vissa länder, något som inte går att förklara med genetiska skillnader. Incidensen ökar kraftigt för närvarande i hela världen och sjukdomen debuterar i tidigare åldrar än förr. Denna utveckling sker mycket snabbare än fortplantningen av gener för T1D.

Både kostvanor och infektioner har föreslagits som möjliga miljöfaktorer i uppkomsten av T1D. Vad beträffar kosten har till exempel intag av mjölk, gluten och D-vitamin diskuterats. En grupp av virus som tycks vara involverade i uppkomsten av T1D är Coxsackievirus (CV). Detta är mycket vanliga virus, vilka oftast inte orsakar några symptom alls eller endast lättare förkylningssymptom. I enstaka fall kan de emellertid ge allvarliga sjukdomar som inflammation i hjärnhinnorna, hjärtmuskeln eller bukspottkörteln.

Många iakttagelser stöder hypotesen att CV kan vara involverat i uppkomsten av T1D. Den första studien kom 1969 där man visade att det var mer vanligt med antikroppar mot CV hos patienter med T1D än hos friska kontroller. Samma forskargrupp visade även att det var vanligare att drabbas av T1D på hösten och vintern, samma årstider som CV infektionerna var mest frekventa. Sedan dess har många studier bekräftat detta samband. Man har till exempel studerat β-cellerna och visat att CV kan infektera och förstöra dem. Vid flera tillfällen har man även hittat virus i β-cellerna från patienter som avlidit med T1D.

Det finns flera olika hypoteser om hur CV infektioner leder till T1D. Om β-cellerna blir infekterade skulle viruset kunna förstöra dem. β-cellerna skulle även kunna bli förstörda av kroppens eget immunförsvar när detta försöker bekämpa viruset genom att angripa de infekterade cellerna. Även om CV infektioner är mycket vanliga är det trots allt relativt ovanligt att utveckla T1D. En orsak till att β-cellerna i normala fall inte blir infekterade av viruset kan vara att de är skickliga på att försvara sig mot en sådan infektion.

Alla celler i kroppen har en viss förmåga att försvara sig mot virusangrepp. När de första cellerna blivit infekterade, till exempel i tarmslemhinnan, skickar de ut signalsubstanser, så kallade interferoner (från engelskans interfere = hindra) som signalerar till kroppens övriga celler att de ska stärka sitt försvar då det är ett virus i antågande. Celler som fått denna varningssignal börjar producera olika protein som kan hindra viruset från att föröka sig i dem. Det finns mer än 100 sådana försvarsproteiner, en del som kan verka mot många olika virus och en del som enbart kan bekämpa vissa typer av virus. Man vet fortfarande inte hur de flesta av dessa proteiner fungerar. Vår forskargrupp har tidigare visat att om man försämrar β-cellernas förmåga att reagera på interferoner, blir de lättare infekterade av CV. Möss vars β-celler inte kan reagera på interferoner utvecklar därför diabetes efter en CV-infektion. För att kunna förstå varför vissa individer skulle kunna vara mer känsliga för att utveckla T1D efter CV-infektioner, behöver man veta mer om hur β-celler fungerar i friska individer och hur ett normalt försvar mot CV ser ut.

Den här avhandlingen visar att friska Langerhanska öar har ett effektivt försvar mot CV-infektioner om de behandlas med interferoner. Det antivirala försvaret inducerat av interferoner studerades i Langerhanska öar från möss och det kunde visas att två proteiner, de så kallade PKR- och RNaseL-proteinerna, är viktiga för skyddet mot CV. Innan denna studie var det inte känt hur celler kunde skydda sig mot just CV-infektioner. Kunskapen om vilka protein som är involverade i försvaret mot CV kan vara betydelsefull både för förståelsen av sjukdomsutveckling och för möjligheten att hitta personer med högre risk att utveckla diabetes efter en infektion. En framtida konsekvens av detta skulle kunna vara att personer som producerar mindre mängd av dessa försvarsproteiner i stället vaccineras mot CV infektioner.

Under avhandlingsarbetet fanns den unika möjligheten att studera humana Langerhanska öar och det kunde visas att även dessa började producera en rad olika protein efter interferonbehandling, av vilka flera är involverade i försvaret mot virus. Skillnaden mellan mus och människa kan i många fall vara stor, därför var det betydelsefullt att bekräfta att även humana Langerhanska öar skyddas mot CV om de

behandlas med interferoner. Det blir intressant att i framtiden studera om detta antivirala försvar skiljer sig mellan patienter med T1D och friska kontrollpersoner.

Interferoner är helt nödvändiga i skyddet mot virusinfektioner, men det har även visat sig att interferon kan ha negativa effekter på β-celler om cellerna exponeras för höga koncentrationer under lång tid. Interferoner produceras inte enbart av infekterade β-celler utan även av celler i immunförsvaret, till exempel av de så kallade NK cellerna (natural killer cells) och de cytotoxiska T-cellerna. Man har funnit att dessa immunceller omger de Langerhanska öarna hos individer som har T1D.

NK celler är specialiserade på att bekämpa olika virusinfektioner genom att döda infekterade celler och/eller producera interferoner. Det var tidigare inte känt om NK celler är involverade i försvaret mot just CV. Avhandlingen visar att NK celler reagerar på CV infekterade celler genom att producera stora mängder interferoner, men utan att döda dem. Detta innebär att NK celler skulle kunna vara både viktiga i försvaret mot CV, men även kunna ha skadliga effekter på β-cellerna om öarna omringas av interferonproducerande NK celler under en alltför lång tid.

Cytotoxiska T-celler är en grupp av immunceller som reagerar på förändringar i andra celler, till exempel vid infektioner. Under en virusinfektion kan de döda infekterade celler och även producera interferon. Från tidigare studier fanns indikationer om att β-cellerna kan, genom att reagera på interferoner, påverka vilka T-celler som kommer till bukspottkörteln. För att studera detta användes i denna avhandling möss vars β-celler inte kunde svara på interferoner. Studien visade att β-cellernas reaktion på interferon ökade mängden av en viss T-cell som man sedan tidigare vet är skadlig och leder till diabetesutveckling i musmodeller. Betydligt mindre av dessa T-celler rekryterades till öarna i de möss vars β-celler inte svarade på interferoner. Detta visar att β-cellernas reaktion på interferoner inte enbart påverkar den egna cellens förmåga att försvara sig mot virus, utan även miljön runt omkring genom rekryteringen av cytotoxiska T-celler.

Denna avhandling ger ökad kunskap om vårt försvar mot CV och de Langerhanska öarnas egen del i detta försvar. Detta kan vara betydelsefullt för att hitta nya behandlingsmöjligheter och förebyggande åtgärder för T1D. β-cellernas svar på interferoner är ett tveeggat svärd och kunskap också om de negativa effekterna av interferoner är viktig för att kunna undvika denna påverkan på diabetesutvecklingen i framtiden. Sammantaget har avhandlingen visat att β-cellen tar en aktiv del både i försvaret mot infektioner och i utvecklingen av diabetes.

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