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INTERACTIONS BETWEEN ENTEROVIRUSES AND THE HOST – IMPLICATIONS FOR TYPE 1 DIABETES

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To Johanna and my beloved family

ABSTRACT

Type 1 diabetes (T1D) is a disease that results after a selective destruction of the insulin-producing β-cells in the pancreas. The lack of β-cells renders T1D patients completely unable to synthesize and secrete insulin, which leads to a life-long need of exogenous insulin for survival. The cause for this selective destruction is still debated, but both genetic and environmental factors have been shown to regulate susceptibility and development of T1D. Together with my colleagues, I have studied different aspects of the observed link between enterovirus (EV) infections, especially those by Coxsackievirus serotype B (CVB) and the development of T1D.

CVB infections may accelerate diabetes development in diabetes-prone mice. This acceleration can however be abrogated by activated iNKT cells. By studying how stimulated or non-stimulated iNKT cells differently regulated macrophages after a CVB4 infection, we have suggested a mechanism for this suppression. We showed that iNKT cells activated in the presence of CVB4 induced suppressive functions in islet-resident macrophages, which then inhibited diabetes development in diabetes-prone mice.

Recently, two multi-center studies suggested CVB1 infections to be diabetogenic, causing human β-cell autoimmunity and T1D development. The identification of a specific virus strain suggests a possibility to use a vaccine, in order to reduce diabetes development. We therefore developed a prototype CVB1 vaccine and tested the functions and safety profile in two different animal models. We showed that the vaccine was well tolerated and protected mice from CVB1 infection. Furthermore, we also observed that the vaccine was safe in an autoimmune setting where we showed no acceleration of diabetes development or triggered autoimmunity in vaccinated mice prone to develop autoimmune diabetes.

Despite the findings that CVB1 may be diabetogenic, an inverse correlation between the incidence of T1D and the number of recorded EV infections have been observed. This counterintuitive observation may be explained by the poliovirus hypothesis, stating that a low herd immunity due to a low frequency of virus infections in the population, may make children that lack maternally transferred antibodies more susceptible to diabetogenic infections. Hence, we tested if maternally transferred antibodies transferred could protect offspring from a diabetogenic CVB3 infection in a mouse model for virus-induced diabetes. Our results support a role for the poliovirus hypothesis in explaining the observed inverse correlation between T1D incidence and prevalence of EVs.

The primary site of replication for CVBs is in intestinal epithelial cells (IECs), but what regulates the viral spread to other targeted organs is not known. IFN-λs may regulate permissiveness to the infection. We therefore investigated how intestinal epithelial cells respond to IFN-λ stimulation and found that they could upregulate specific antiviral proteins. This suggested that IECs could be used in a model to study if IFN-λs can regulate IEC permissiveness to CVBs.

LIST OF PUBLICATIONS

I. Liana Ghazarian, Julien Diana, Lucie Beaudoin, **Pär G. Larsson**, Raj K. Puri, Nico van Rooijen, Malin Flodström-Tullberg, and Agnès Lehuen. Protection against type 1 diabetes upon Coxsackievirus B4 infection and iNKT cell stimulation: role of suppressive macrophages.
Diabetes. 2013 vol. 62 (11) pp. 3785-3796.

II. **Pär G. Larsson***, Lakshmikanth Tadepally*, Olli H. Laitinen, Renata Utorova, Stella Jacobson, Minni Koivunen, Nicolas Devard, Valerie Lecouturier, Mikael Knip, Heikki Hyöty and Malin Flodström-Tullberg. Preclinical evaluation of efficacy and safety of a new vaccine against Coxsackievirus B1 – Implications for type 1 diabetes development.
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III. **Pär G. Larsson**, Tadepally Lakshmikanth, Emma Svedin, Cecile King and Malin Flodström-Tullberg. Previous maternal infection protects offspring from enterovirus infection and prevents experimental diabetes development in mice.
Diabetologia. 2013 vol. 56 (4) pp. 867-874.

IV. **Pär Larsson***, Virginia Stone*, Erna Domsgen and Malin Flodström-Tullberg Human intestinal epithelial cells enter an antiviral state upon stimulation with interferon lambda.
** Contributed equally. Manuscript.*

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Prologue

"Type 1 diabetes is a chronic autoimmune disease characterized by the immune-mediated destruction of the insulin-producing β-cells in the pancreas". In various literary formulations, this message is classically delivered in the first sentence of most scientific articles about human type 1 diabetes (T1D). A following sentence usually suggests that "this or that" can cause, trigger or affect the autoimmune attack on the β-cells but the truth is; we don't know. We can't prevent the disease and we can't cure it, we can only treat patients with life-long supplementation of exogenous insulin. Despite years of research and billions of dollars spent, science still lacks an answer to the quite simple question, asked by every child developing T1D;
- Why did I get diabetes?

A simple message understood from all scientific articles written about T1D is that this is truly a complex disease. As indicated from both human and murine studies, genetics and environmental factors can both regulate susceptibility to T1D, but exactly how this is done is still unknown. The rise in T1D incidence observed world-wide cannot be explained by genetic factors alone, but rather points to an environmental change that exposes genetically susceptible individuals to a higher risk for developing T1D. Also, we may never isolate one single factor that can explain the etiology of all T1D cases, but rather find that the disease is heterogeneous in nature, with several different factors individually regulating destruction of the β-cells, all leading to the same result, namely T1D development.

However, one independent environmental factor suggested to be involved in T1D development in some patients is enterovirus (EV) infections, especially those of Coxsackievirus serotype B (CVBs). Numerous observations have linked EV infections to T1D and triggering or acceleration of β-cell autoimmunity. Recently, CVB1 infections were specifically suggested to trigger both the development of β-cell autoimmunity and T1D development. The identification of specific virus strains associated with T1D development suggests that one could potentially develop a vaccine targeted at specific viruses, in order to protect genetically susceptible individuals from developing virus-induced T1D.

The aim of this thesis was to further investigate if and how CVBs are involved in the development of T1D and to develop strategies to prevent CVB infections through the use of vaccines and novel vaccination strategies. I will explain how my results can be interpreted and further discuss what this means in broader terms. I will also give my own thoughts on future perspectives for T1D research and what I think future T1D research projects should focus on.

LIST OF ABBREVIATIONS

αGC	α-galactosylceramice
APC	Antigen-presenting cell
BCR	B-cell receptor
CAR	Coxsackie and Adenovirus Receptor
CD	Cluster of Differentiation
CVB	Coxsackievirus serotype B
DAF	Decay-Accelerating Factor
EV	Enterovirus
GAD	Glutamate decarboxylase
HLA	Human leukocyte antige
HPV	Human papillomavirus
IAA	Insulin-autoantibody
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
iNKT	invariant Natural Killer T-cell
ISG	Interferon stimulated gene
ISH	In situ hybridization
MDA5	Melanoma differentiated-associated 5
MHC	Major histocompatibility complex
NK	Natural killer
NOD	Non-obese diabetic
PAMP	Pathogen-associated molecular pattern
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
Socs-1 tg	Suppressor of cytokine signaling-1 transgenic
T1D	Type 1 diabetes
T2D	Type 2 diabetes
T_C	Cytotoxic T-cell
TCR	T-cell receptor

T_H	T-helper cell
TLR3	Toll-like receptor 3
TNF-α	Tumor necrosis factor α
T_{reg}	Regulatory T-cell

1 INTRODUCTION

Several different forms of diabetes exist, with the two most common forms denoted type 1 (T1D) and type 2 diabetes (T2D). Both forms are characterized by crucial deficiencies affecting production and function of insulin, a hormone involved in glucose metabolism. Insulin is produced by the beta (β) cells in the pancreas and is crucial for most cells to absorb and utilize glucose from the blood as an energy source.

In laymen terms, the cellular function of insulin can be depicted as a key unlocking and opening a door, where insulin is 'unlocking' and activating specific cellular functions involved in glucose uptake. The difference between T1D and T2D can also be simplified and explained using this analogy; T1D is the result of virtually no insulin being produced or secreted, resembling a lost key. T2D results from insulin resistance in combination with insufficient amounts of insulin, which can be compared to using a key that does not fit or a non-functional lock, in combination with too few or no keys. For both diseases, the cellular inability to absorb glucose results in high concentration of glucose in the blood (hyperglycemia), a fatal condition if not properly treated.

According to the World Health Organization (WHO) and the American Diabetes Association (ADA), about 90% of all diabetes cases are considered to be T2D and between 5-10% are considered to be T1D cases [1, 2]. However, these figures might not truly reflect the reality since numerous T2D cases actually require insulin treatment, a hallmark for T1D.

Development of T2D is hereditary, but also highly related to a sedentary or unhealthy life style and diet, and most patients that develop the disease are obese. Treatment normally requires a change of life style and regular exercise, in combination with anti-diabetic drugs to stimulate insulin sensitivity. Some patients may also require insulin treatments.

However, the more severe type of diabetes, T1D, is as we know today not dependent on life style, but rather a chronic disease with a complete lack of endogenous insulin production. Before the discovery of insulin [3], T1D was inevitably fatal, but today patients survive through life long treatment with exogenous insulin. The following chapters will focus only on T1D, the etiology of the disease and potential preventative therapies.

1.1 TYPE 1 DIABETES

Human T1D, previously called insulin-dependent, juvenile or childhood-onset diabetes mellitus, results from a selective destruction of the insulin-producing β-cells in the pancreas, rendering patients completely unable to synthesize insulin. The ADA have described three significant hallmarks of T1D, used to distinguish T1D from other forms of diabetes [2]:

1. Immune-mediated β-cell destruction
2. Insulin deficiency

3. Low or undetectable levels of C-peptide, a cleavage product formed when active insulin is synthesized.

T1D development seems to have two peaks, between 5-7 years of age and in adolescence in close proximity to puberty (reviewed in [4, 5]). T1D can however develop at any age, even in adults. Due to the previous classification of T1D as juvenile diabetes, it has been suggested that possibly 5-15% of all T2D cases are misdiagnosed cases of adult T1D, implicating that the percentage and number of T1D cases worldwide is considerably underestimated [5, 6]. Adult onset of T1D has previously been considered to be a unique form of diabetes, called Latent Autoimmune Diabetes in the Adults (LADA), but recent studies suggests that LADA actually falls under the T1D classification [7].

T1D patients require life-long treatments with exogenous insulin. Due to the acidic environment in the stomach, insulin cannot be delivered in tablet-form, hence insulin administration has to be done via subcutaneous injections prior to every meal. In addition to this, patients may also need injections with long-lasting insulin, in order to obtain a baseline effect of insulin needed to control the metabolism. The recent development of smart insulin pumps has greatly benefitted T1D patients, with a reduced number of daily injections and more stable blood glucose levels. T1D is however still a severe disease, and many patients still have difficulties with controlling and regulating their blood glucose levels, despite the recent development in diabetes care and equipment [4]. The lack of controlled and stable blood glucose levels can lead to severe short- and long-term complications. Short-term, life-threatening complications are ketoacidosis and hypoglycemia, whereas long-term diabetes-related complications include microvascular disease like nephropathy, neuropathy and retinopathy as well as cardiovascular and cerebrovascular disease [4].

1.2 THE HISTORY OF T1D

The first description of a disease thought to be T1D was found in a manuscript by the Egyptian physician Hesy-Ra from around 1500 BC, describing patients suffering from polyuria, one of the earliest signs of T1D development [8]. The word *diabetes* is derived from the Greek word *diabainein* – to pass through, and was first used by the Greek physician Arateus around 250 BC, when he described the disease as the "meltdown of flesh and limbs into urine" [8]. The addition of the Latin word *mellitus* – sweet as honey, was done in 1674 by Thomas Willis at the University of Oxford, after tasting "wonderfully sweet" urine from T1D patients [8]. The name *diabetes mellitus* hence refers to one of the first symptoms of disease development, polyuria, and the sweet the urine secreted by the patients. For years, the sweetness of urine was used as a diagnostic marker for T1D.

With the exception of somewhat functional diets and fasting cures, which at best resulted in a few additional years of survival, T1D was untreatable and inevitably fatal. It took until the end of the 19th century before some major medical breakthroughs resulted in the discovery and isolation of insulin, which facilitated the development of a functional treatment for T1D.

In 1869, the German medical student Paul Langerhans discovered the specific cell clusters in the pancreas that we know as “the islets of Langerhans” [9]. The French pathologist Gustave-Édouard Laguesse thereafter coined the name of these cell clusters “the islets of Langerhans” in 1891 [9]. The initial breakthrough was however made in 1889, when Joseph von Mering and Oskar Minkowksi at the University of Strasbourg found that a pancreatectomy of dogs resulted in them developing T1D ([8] and the original German paper [10]). In 1901, the American pathologist Eugene Opie discovered morphological differences in the islets of Langerhans of T1D patients, linking the islet function to T1D.

In 1920, spurred by these findings, Frederick Banting initiated collaboration with Professor James Macleod in Toronto. After being assigned Charles Best as a lab assistant, Banting and Best were the first to isolate functional insulin in 1921 and they managed to successfully treat pancreatectomized dogs with bovine pancreatic islets extracts for an extended period of time [3, 8]. In collaboration with Macleod and chemist James Collip, they attempted the first human trial in 1922, by injecting Leonard Thompson, a 14-year-old diabetic boy, with insulin purified from bovine pancreata [8]. With the help of the medical company Eli Lilly and Company, they improved the purification process of the islets extracts, and in 1923 insulin was made commercially available for patients in Canada and the United States [8]. Due to the extraordinary fact that Banting and Best made their patented discovery of insulin available free of charge, T1D patients everywhere could quickly benefit from the novel insulin treatments. Banting and Macleod were awarded the Nobel prize in Physiology or Medicine in 1923, a prize they immediately shared with Best and Collip [8].

After being limited to harvesting insulin form animal organs, the next major breakthrough was made in 1982, when the joined effort by Genentech and Eli Lilly launched the first recombinant human insulin [8]. Today there are several different forms of insulin available on the market, all produced through the use of recombinant technology in yeast or bacteria.

1.3 T1D INCIDENCE

The incidence of T1D has increased rapidly world wide during the last decades [11-14]. There are marked differences in the observed incidence in the age interval 0-14.9 years, with incidence figures ranging from <1 per 100.000 individuals in low incidence countries to >60 cases per 100.000 individuals in Finland, the country with the highest incidence of T1D [11-15]. The T1D incidence in Sweden was recently shown to be almost 44 cases per 100.000 individuals, which puts Sweden as having the second highest T1D incidence in the world [16].

The rapid increase in T1D incidence is alarming and, even though predictions should be taken with cautions, two prospective studies have predicted a 30-70% total increase in T1D incidence by the year 2020 [14, 15].

1.4 THE PANCREAS

The human pancreas is a unique gland with both exocrine and endocrine functions. The exocrine functions include production of several digestive enzymes involved in breaking down carbohydrates, proteins and lipids, and secreted through the pancreatic duct into the duodenum. However, of focus in this thesis are the functions, or rather the lack of functions, of the endocrine part of the pancreas.

The endocrine parts of the pancreas are spread throughout the organ as small cellular clusters, known as the islets of Langerhans. These clusters may only represent about 1-2% of the total pancreatic mass, but they are crucial for survival [17]. The islets consist of several types of cells, all with their own unique endocrine function. The most abundant cell type in the islets are the insulin-producing β-cells, representing about 70% of the functional islets cells [18]. The glucagon-producing alpha (α) cells account for about 10% and the somatostatin-producing delta (δ) cells, pancreatic polypeptide-producing (PP) cells and ghrelin-producing epsilon (ε) cells represents 3%, 19% and 1%, respectively (reviewed in [18]). Since the islets are vascularized, endothelial cells may also account for a part of the total islet mass.

2 BASIC IMMUNOLOGY

The immune system is protecting us against all kinds of infectious agents through a network of specialized immune cells, lymphoid organs and signaling cytokines [19]. The regulation of all these cells and their functions is delicately balanced; an inactive immune system may result in tumor formations and severe infections, whereas an overactive immune system may increase the risk for developing allergies and autoimmune diseases [19]. The immune system is commonly divided in two arms; the innate immune system and the adaptive immune system. However, the two-way signaling between them is crucial for a mounting a proper immune response. For example, T-cells would not be able to function properly without the stimulation by dendritic cells and other antigen-presenting cells (APCs) and activated T-cells can further potentiate macrophages to become more aggressive in response to infectious agents. The following sections will provide a simplified description of the immune system, divided into the innate and the adaptive immune system, and some important concepts needed to understand the relevance for the immune system in the light of antiviral responses and autoimmunity.

2.1 THE INNATE IMMUNE SYSTEM

The innate immune system refers to the cells and functions that provide the host with an immediate defense against pathogens. The cellular components of the innate immune system include macrophages, neutrophils, basophils, eosinophils, monocytes, dendritic cells, natural killer (NK) cells and invariant Natural Killer T-cells (iNKT) [20]. All the aforementioned cells can be rapidly activated upon infection and act together with the complement system, numerous cytokines and acute phase proteins, in order to clear the host from pathogens. The innate immune system can orchestrate a grand attack on any pathogen it can recognize through pathogen associated molecular pattern receptors (PAMPs) or the lipid antigen-presenting CD1d. However, the activation of the innate immune system can also lead to tissue damage due to the relative unspecific highly inflammatory milieu [20].

2.1.1 iNKT cells

Despite being closely related to the T-cells of the adaptive immune system, the iNKT cells are usually considered to be part of the innate immune system [21]. iNKT cells express a highly restricted αβ T-cell receptor (TCR) with the α-chain Vα24Jα18 in humans and Vα14Jα18 in mouse, specifically recognizing lipid-antigens presented by CD1d [22, 23]. Upon activation, iNKT cells can produce and secrete, for example, large amounts of IL-4 and IFN-γ to activate macrophages and also antiviral T-cells of the adaptive immune system [22, 24]. The synthetic ligand α-galatosylceramide (αGC) is commonly used to experimentally stimulate and activate iNKT cells. The iNKT cells have different roles as a regulatory cell in the immune system. iNKT cells can both promote antiviral responses but also regulate autoimmunity and dampen autoreactive T-cells [25, 26].

2.1.2 Macrophages

Macrophages are important phagocytic immune cells of the innate immune that also act as APCs [27]. Monocytes from the blood stream can migrate to inflamed and infected sites due to chemotaxis and once there, they can differentiate into macrophages [27]. After phagocytic uptake of pathogens, macrophages can present processed antigens to orchestrate and activate the adaptive immune activation [28]. Macrophages are found in most tissues in the human body [27].

Generally there are two main subtypes of macrophages. The M1 macrophages are involved in inflammatory responses by secreting for example IL-1β, IL-6, IL-12 and TNF-α [28]. Activated M1 macrophages are commonly referred to as classically activated macrophages. The other subtype is the M2 macrophages, which have been suggested to have an important role in tissue repair and immune suppressive functions [28]. M2 macrophages produce and secrete anti-inflammatory cytokines for example IL-10 and TGF-β [28].

2.2 INTERFERONS

The interferons (IFNs) are an important group of cytokines with functions closely related to the innate immune system. IFNs are involved in the induction of the cellular antiviral defense and they can indirectly interfere with virus replication through the induced upregulation and transcription of antiviral effector genes. IFNs can also signal systemically to orchestrate an immune response towards an infecting virus [29].

Upon infection, the cell starts to produce and release IFNs, to signal to other cells to enter an antiviral state and prepare for encountering viruses [29, 30]. The IFN-induced antiviral state is regulated by interferon-stimulated genes (ISGs), encoding numerous antiviral effector proteins (reviewed in [31, 32]). The aim of these proteins is to reduce permissiveness to infections. Secreted IFNs can act locally through autocrine and paracrine signaling pathways to signal to surrounding cells but also systemically in order to activate antiviral immune cells [29, 30]. In an antiviral state, the cell responds vigorously to pathogens resulting in early recognition of pathogens or potentially apoptosis, since the cell is also primed to undergo apoptosis if successfully infected. Altogether this leads to a reduced viral replication and spread. IFNs are considered to be crucial for controlling virus infections.

There are three different types of human IFNs;

- Type I IFNs, sometimes only referred to as IFN-α/β, consists of thirteen different IFN-α subtypes, a single isoform of IFN-β, and the somewhat less abundant IFN-κ, -ω and -ε. Most cells in the body can express and respond to type I IFNs in response to virus infections [29].
- The type II IFN group only consists of one single isoform, IFN-γ, which is mostly produced by activated NK-, iNKT-, T_H-cells and macrophages. IFN-γ expression can amplify the antiviral activity of type I IFNs and is also critical for antitumor responses and for controlling intracellular bacteria like mycobacterium tuberculosis [29].

- Type III IFNs or IFN-λs, include four isoforms; IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B) and the quite rare IFN-λ4 [29, 30]. The expression of the receptor for IFN-λs have been shown to be quite restricted and mostly expressed by epithelial cells, hence suggesting a specific local role for IFN-λs. IFN-λs are might be expressed by infected cells, mainly of epithelial origin, but monocyte-derived dendritic cells and plasmacytoid dendritic cells have also been shown to produce and secrete IFN-λs (reviewed in [30]).

2.3 THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune response is very potent and specific, but significantly slower in responding to pathogens [33]. The main components of the adaptive immune system are T- and B-cells, also called T- and B-lymphocytes [27]. These cells' respective receptors, the TCR and B-cell receptor (BCR), are highly diverse thanks to a very elegant rearrangement mechanism [33] and they are highly specific for their respective antigens. Antigens are presented to T- and B-cells through interactions of the TCR and BCR with peptides bound to MHC class II on APCs or MHC class I, which is expressed by virtually all nucleated cells [27]. Infected cells can upregulate expression of MHC class I, in order to facilitate recognition of viral peptides by the immune system [27].

2.3.1 T-cells

There are numerous different subpopulations of T-cells, but only four main subtypes and their functions are described in this simplified overview [27, 33];

1. The T-helper cells (usually denoted T_H-cells or $CD4^+$ T-cells due to their distinct expression of CD4) are mainly responsible for activating other immune cells through co-stimulatory molecules, for example activation and maturation of B-cells and activation of cytotoxic $CD8^+$ T-cells [27].
2. The cytotoxic T-cells (T_C-cells or $CD8^+$ T-cells, due to the expression of CD8 on the surface) are potent killers of virus-infected cells or tumor cells. These are crucial components in controlling a virus infection since they recognize viral peptides presented by MHC class I on infected cells. Upon engagement, T_C-cells can kill infected cells through the release of cytotoxins (e.g. perforin and granzyme B) and by direct cell-cell contacts [27].
3. Memory T-cells can be either $CD4^+$ or $CD8^+$ and once activated by their respective antigen, they can persist for a long time. Upon re-exposure to the antigen, these cells can quickly expand to activate other immune cells [27].
4. The regulatory T-cells (T_{reg} cells or suppressor T-cells) are key components in dampening for example other T-cell responses after a virus infection is cleared. Suppression is mainly mediated through the cytokines IL-10 and TGF-β [27]. T_{reg} cells are crucial for maintain immunological tolerance [27].

2.3.2 B-cells

B-cells are formed in the bone marrow and their main functions are to produce antibodies and act as APCs [33]. Upon activation thorough the BCR, antibodies are

produced against foreign antigens such as viral and bacterial proteins, and the antibodies can opsonize infectious agents or render them incapable of infecting host cells. Through genomic rearrangement and class switching, the B-cells can produce antibodies with virtually endless specificities [27]. The five main classes of human antibodies are IgA, IgD, IgE, IgG and IgM, all with different distinct functions [27, 33]. The two isoforms IgA and IgG also have subclasses, IgA 1&2 and IgG 1-4. Like memory T-cells, activated B-cells can also develop into long-lived memory B-cells that can be reactivated upon later re-exposure to its specific antigen.

2.4 AUTOIMMUNITY

Autoimmunity is defined as the immune system failing to recognize self-antigens as non-pathogenic, resulting in the development of an erroneous immune response to endogenous tissues and proteins [27]. The key cellular players in autoimmunity are T_H1-cells, T_C-cells, T_{reg} cells and B-cells, but also iNKT cells and macrophages. In the circulation of healthy individuals, there are normally autoreactive T-cells and autoantibodies present, without causing disease [34]. However, autoimmunity and an autoimmune disease may develop if these cells are activated or the suppressive control over them is lost. Such a lack of suppression might be due to a lack of T_{reg} cells or dysfunctional T_{reg} cells, which can lead to uncontrolled T-cell activation and autoimmune diseases (e.g. [35, 36]). Studies have also shown that iNKT cells can inhibit autoimmunity through impairment of diabetogenic CD4 and CD8 T-cells in a mouse model for T1D [37].

The development of autoimmunity is dependent on both genetic factors and environmental factors, such as infectious agents, chemicals, drugs and smoking. There is a strong link between development of autoimmune disease and virus infections (reviewed in [38, 39]. Despite this link, a lack of microbial stimuli has also been suggested to be associated with an increased risk for developing autoimmunity; a phenomenon termed the ‘hygiene hypothesis’ [40].

T1D is classically considered to be of autoimmune nature with the β-cells being destroyed by the immune system [41]. Supporting a role for autoimmunity in T1D development is the finding that autoantibodies towards β-cell antigens generally precedes actual T1D manifestation by several years [42]. It has also been demonstrated that autoreactive T-cells can be found in or around the islets of some, but not all, diabetic patients [43]. Although the involvement of autoimmunity and autoreactive T-cells have been studied, the reason for triggering autoimmunity is less understood.

2.5 VIRUSES

Viruses are small, opportunistic and parasitic infectious agents without energy metabolism. Their life cycle relies solely on the host cells’ internal molecular machinery [44]. Hence, viruses are not actually “alive” according to the definition of life based on reproduction or replication and metabolism [45]. Despite not being alive, the virus’ ability to self-assemble has implications for the broad question of the origin

of life, as it supports an idea that life, as we know it, may have evolved from self-assembling molecules.

Viruses can infect various living organisms, from animals and plants to bacteria and archaea [44] and some viruses have a broad host range, infecting several different species and organisms. There are multiple different viruses and among these there are numerous serotypes, which in total, make viruses the most abundant biological entity on our planet [44, 46]. Viruses can also transfer genomic material between different organisms, making viruses key players in the evolution. When examining the human genome, several pseudogenes and introns have been discovered which all are due to viral genomic transfers.

Antibiotics do not work on viruses and virus infections are generally not treatable. Some antiviral drugs exist, but these are used with caution and only for the most severe virus infections, to avoid viruses mutating to withstand the antiviral effects. Depending on which virus it is, these mutated functions may spread to other viruses through recombination. Most virus infections are cleared after being recognized by the adaptive immune system, through the activation of antiviral T-cells and production of neutralizing antibodies by activated B-cells. However, the only way to induce protective immunity against virus infections and reduce the risk of becoming infected is through the use of vaccines, as further discussed in chapter 4.

3 T1D ETIOLOGY

Although the pathology of the selective destruction of the β-cells has been known for over a century, the etiology of T1D is still poorly understood. Clearly both genetic and environmental factors are involved in the development of disease, but how the two factors together regulate susceptibility, is still unknown.

Several different models have been proposed to explain how, and why, the β-cells are destroyed. In a now classical model of T1D development and β-cell destruction, the most simplistic view of the disease is shown in Fig. 1A (modified from Eisenbarth [41]). As depicted, in genetically susceptible individuals a "precipitating event" initiates the β-cell destruction (linear), which then progresses until falling below a specific threshold of critical β-cell mass, when overt T1D is presented. This model has then been further refined, with added information regarding autoantibodies and a potential gradual dysregulation of autoreactive effector T-cells and regulatory T-cells, suggesting T1D to be a relapsing-remitting disease, as shown in Fig. 1B (modified from von Herrah et al.[35]). The β-cell mass in Fig. 1B is decreasing over time due to the suggested varying number of effector T-cells and regulatory T-cells, which at different points lead to an overt attack on the β-cells by autoreactive T-cells and development of autoantibodies. However the regulatory T-cells may thereafter recover control over the autoreactive T-cells, and the curve flattens out again. However, at a certain point, the disequilibrium reaches such a state that the regulatory T-cells completely loose their dampening effect over the autoreactive effector T-cells, resulting in T1D [35].

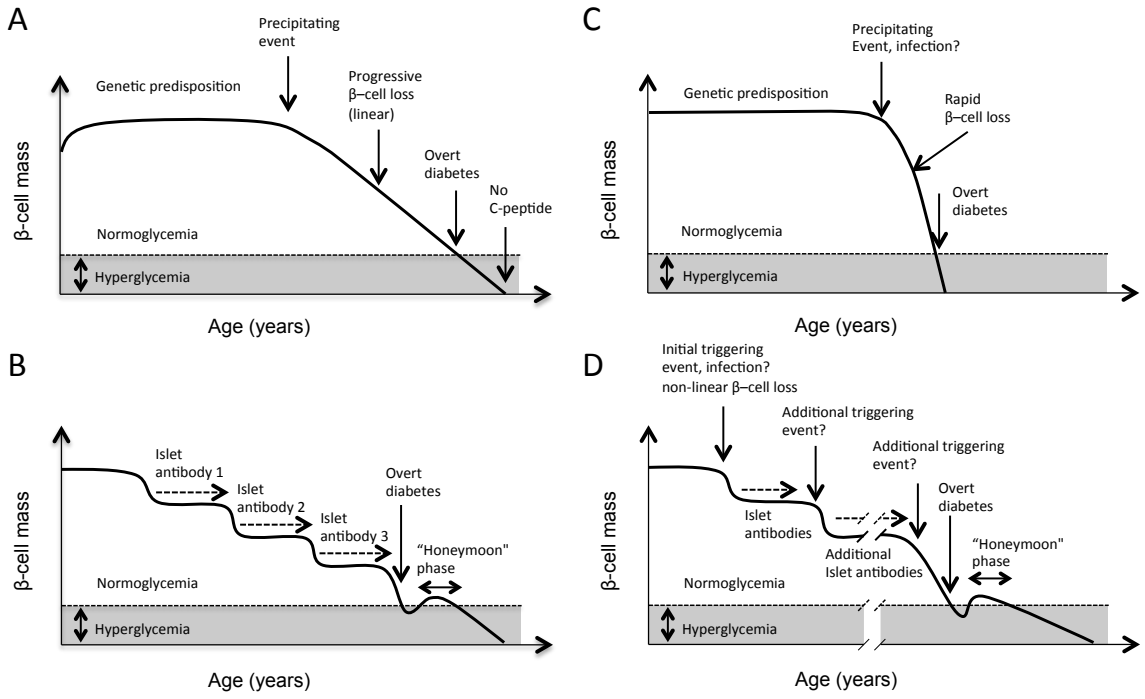

Figure 1. Different models for the development of T1D. A. The classical T1D model suggested by Eisenbarth in 1986 (modified from [41]). **B.** The relapsing-remitting model suggested by von Herrath et al. in 2007 (modified from [35]). **C.** A model representing acute onset of T1D or fulminant T1D. **D.** A combined model suggesting several triggering events, non-linear β-cell loss, induced autoimmunity and T1D development over time.

A third model for T1D development is shown in Fig. 1C, representing the quite rare form of T1D called fulminant diabetes which is characterized by a very rapid induction and destruction of the β-cells [47, 48]. It has been suggested that the development of fulminant diabetes could be the consequence of a virus infection in the β-cells which, upon recognition, rightfully should be killed by the immune system unfortunately also resulting in T1D development [47, 48].

However, these models alone are not enough to explain the complex etiology of T1D. A combined model may account for several different triggering events, autoimmunity and β-cell loss, hence the suggested combination model presented in Fig. 1D. The curve representing β-cell mass in Fig. 1D is not solely dependent on dysregulation of effector T-cells and regulatory T-cells, but also accounts for environmental triggering events. Potentially, the initial triggering event is an infection that establishes persistency within the β-cells and further triggering events are reactivations of the virus or an infection with a related virus strain, resulting in exacerbated β-cell autoimmunity.

3.1 GENETICS

Numerous genetic factors involved in the etiology of T1D have been identified (recently reviewed in [49, 50]). Functional studies of these genes have also suggested different pathways involved in the development of T1D. The genes most commonly associated with T1D development are specific variants of the Human Leukocyte Antigen (HLA) class II, which encode the MHC-proteins involved in antigen presentation. The HLA combinations contributing to the highest risk for T1D development are the haplotypes HLA-DR3/DQ2 (HLA-DRB1 *03/DQA1*05:01-DQB1*02:01) and HLA- DR4/DQ8 (HLA-DRB1*04/DQA1*03-DQB1*03:02) [49, 50]. The functions of other genes involved in regulating T1D susceptibility include regulation of immune responses, β-cell functions, insulin expression and recognition of virus infections [49, 50]. Interestingly, one gene identified through genome-wide association studies encodes for the MDA-5 protein, an intracellular sensor for viral RNA, potentially implicating a difference the response to certain viruses in people with T1D [51].

However, genetic factors cannot alone account the observed increase in incidence, as showed by the relatively low concordance rate for T1D development among monozygotic twins [52-54] and data suggesting that the increased T1D incidence is higher among individuals considered to have a low risk HLA genotype [55, 56]. Hence, the rise in T1D incidence reflects an environmental change that now, more than before, puts us at a higher risk for developing T1D.

3.2 ENVIRONMENT

In parallel with identified genetic factors, several environmental factors have also been suggested to regulate or trigger T1D development. Studies have suggested that dietary antigens [57-60], microbiotic alterations in the intestine [61-63] and virus infections [64-69] all can regulate susceptibility to T1D development.

Dietary antigens are basically anything we eat or drink. However, cow's milk [57, 58], which contains bovine insulin, and the cereal protein gliadin [59], involved in celiac disease, have attracted specific interest. Due to the presence of bovine insulin in milk, children may develop antibodies to the bovine insulin if exposed to cow's milk early in life [57, 58]. Induction of bovine-insulin immunity could then result in cross-reactive immunity toward human insulin and development of insulin-autoantibodies (IAAs), an early marker for the development of T1D [42]. Other studies indicated that dietary gluten could potentially trigger β-cell autoimmunity and diabetes development [59, 60]. Furthermore, dietary gluten/gliadin are antigens involved in celiac disease, an autoimmune disease that interestingly is more common among T1D patients than among healthy controls.

Differences in the composition of the intestinal microbiota in diabetes-prone mice compared to diabetes-resistant mice have been described, where certain bacterial compositions can be either protective or causative of disease [61]. Similar differences were recently described for humans, where both a lack of bacterial diversity and a changed bacterial composition was observed in T1D patients compared to healthy controls [62]. Combining these observations with the findings that dietary antigens can affect T1D development suggests that the intestine represents an important site for numerous interactions between genes and environment, some of which may be involved in the etiology of T1D.

Numerous studies have also linked virus infections to the development of T1D. Coxsackieviruses of serotype B (CVBs), members of the enterovirus (EV) genus, have specifically been implicated in T1D development (e.g. [64, 66-69]). However not investigated further in this thesis, rotavirus [65] and congenital rubella (reviewed in [70]) have also been suggested to have diabetogenic properties.

3.3 COXSACKIEVIRUS SEROTYPE B AND T1D

3.3.1 Background

CVBs are small, non-enveloped single-stranded RNA viruses of the EV genus. Commonly, CVB infections only cause subclinical flu-like symptoms but on rare occasions, they can lead to severe diseases like poliomyelitis, myocarditis, aseptic meningitis, hepatitis and pancreatitis [44]. CVBs spread through the fecal-oral route, mainly infecting humans through ingestion of contaminated food and water.

The primary site of replication for CVBs is in the intestinal mucosa [44], but what regulates virus spread from here is not known. CVBs can infect cells through the two main receptors Coxsackie and Adenovirus Receptor (CAR) [71] and Decay-Accelerating Factor (DAF) [72]. CAR is a component of tight junctions, a cellular adhesion complex that tightly link one cell to another, for example in cardiomyocytes [73] and epithelial cells [74, 75]. DAF is a protein involved in regulating complement attachment on the cell surface [72]. The finding that CAR is expressed in tight junctions in IECs, but not exposed on the luminal side of epithelial cells, may suggest that infection of IECs is further regulated by other factors [76].

Upon infection with CVBs, cells may recognize the infection and viral RNA through the pattern-recognition receptors MDA5 and TLR3 (reviewed in [77]). After binding to viral RNA, MDA5 initiates a signal cascade leading to the induction of type I IFN expression. This induced IFN production may thereafter activate several ISGs in the infected cell through paracrine signaling IFN signaling, but also signal to neighboring cells to enter an antiviral state [77]. The IFNs will also orchestrate and activate an immune response towards the virus. NK cells may be of importance in controlling the initial phase of infection, but upon activation of the adaptive immune response, T_C-cells and antibody-producing B-cells are the key mediators of immunity (recently reviewed in [78]).

3.3.2 CVBs and T1D

There are several reasons for appointing EVs and CVBs a diabetogenic role. The first suggestion of CVBs being involved in the etiology of T1D was made in 1969, when Gamble et al. found that recent onset T1D patients were positive for CVB antibodies more frequently than healthy controls [64]. In 1979, Yoon et al. isolated CVB4 from a diabetic patient, and showed that this virus could induce diabetes in a mouse model [79]. Since then, several epidemiological studies have supported a causal link between EV infections in the development of T1D [80-83] and the role for EVs in the etiology of T1D was also recently highlighted by several systematic literature reviews and meta-analyses [67-69]. Numerous studies have also detected EV RNA and/or EV proteins more frequently in pancreatic islets [84-87] and intestinal biopsies [88, 89] from T1D patients than in matching tissues from healthy individuals. It has also been suggested that EVs may cause a persistent infection in the intestinal mucosa, a finding that was suggested to be more common in T1D patients than in controls [88, 89]. A recent study further suggested that EV infections (non-sequenced) could accelerate the progression from islet autoimmunity to development of overt T1D [90]. However, most of the studies detecting EVs have not serotyped or sequenced their viral findings, hence it is difficult to know exactly which EV they actually have detected.

Among all EVs, the CVBs have historically been appointed to be the most diabetogenic EV serotypes, with a special focus on CVB4 [64, 79, 85, 91]. CVBs have also been shown to be pancreatropic and capable of infecting human islets in vitro [84, 92]. Recently, two multi-center studies indicated that infections with CVB1 were specifically associated with development of β-cell autoimmunity in some children [83] and that CVB1 infections were more common in children who developed T1D [93]. These studies clearly indicate a role for a specific virus in the etiology of T1D, however, they do not present a clear mechanism to explain how CVB1 infections are involved in T1D development.

3.3.3 The poliovirus hypothesis

There is a marked difference in T1D incidence world wide, with clear differences observed in different parts of the world, but also in neighboring countries [11-16]. Based on the increasing amount of circumstantial evidence for a link between EV infections and T1D development, the incidence of T1D should be high in countries with a high prevalence of observed EV infections, and vice versa. However, the

opposite has been observed in epidemiological studies conducted in different geographical regions of Europe, where the frequency of observed EV infections showed an inverse correlation to the incidence of T1D [13, 94, 95].

A parallel for this counter-intuitive observation has however been described earlier for poliovirus (PV) infections and the development of paralytic poliomyelitis [96]. Before the 20th century, PV infections were endemic but the number of poliomyelitis cases was low. With improved hygienic standards and public sanitation from the late 19th century, the prevalence of PV infections decreased, but surprisingly, the number of infant poliomyelitis cases increased dramatically. A suggested explanation for this phenomenon was the decline in herd immunity in parallel with the decreasing prevalence of PV infections in the population [96]. The reduced herd immunity would further have affected young children via the reduced transfer of protective maternal antibodies and also since children would have encountered PV later in life, when maternal antibodies had waned. Thus, the lack of protective maternally transferred antibodies combined with a later exposure to PV allowed the virus to spread more vigorously, resulting in severe tissue damage and poliomyelitis in some susceptible individuals.

In 2000, Viskari et al. applied the poliovirus hypothesis on EV infections and T1D development in an attempt to explain the high incidence of T1D in countries with a low number of observed EV infections [97]. Hence, the 'extended' poliovirus hypothesis states that a low frequency of EV infections in the background population can increase the risk for severe complications in infected genetically susceptible individuals, such as β-cell damage and T1D development [97]. The heightened risk for T1D development is in this model explained by the reduced level of maternally transferred antibodies and/or by children being exposed to diabetogenic EVs later in life, when the protective maternal antibody effect has waned [97-100]. We recently published direct evidence for the extended poliovirus hypothesis applied on EV infections and T1D in a mouse model for virus-induced diabetes ([101], *Paper III*). It has also been suggested that the immunity obtained if encountering a pathogen under the protection of maternal antibodies can persist up to adulthood [102]. Indirect evidence for the extended poliovirus hypothesis have also been shown in human studies, where high maternal titers of EV-specific antibodies in serum and breast milk correlated with reduced frequency of such EV infections in children [103].

3.4 VIRUSES AND T1D: DIRECT INFECTION

One way viruses may cause diabetes is through a direct infection of the β-cells. In this scenario, the immune system may attack and kill the infected β-cells, which will not only results in viral clearance but also T1D development. In this case T1D is not an autoimmune disease, but rather caused by the virus infection alone. This may be the case for fulminant diabetes, which has a very fast onset, or reflect a persistent infection, which may be reactivated until all β-cells are destroyed.

3.5 VIRUSES AND T1D: MOLECULAR MIMICRY

Another way through which viruses may cause T1D is via autoimmunity and molecular mimicry. This basically means that an immunogenic epitope of a virus (i.e. a part of a virus protein which the immune system can react to) has sequence similarities with a part of an endogenous host protein, and thereby a cross-activation of the immune system can occur [38, 104]. The result leads to a correct immune response towards the viral antigen by T- and B-cells, but unfortunately also to an autoimmune response towards the self-antigens, due to cross-reactivity, i.e. molecular mimicry [38, 104].

In the respect of autoimmune T1D and virus infections, it is interesting that the non-structural viral 2C protein of CVB4 has been suggested to have a shared epitope with the host proteins glutamate decarboxylase (GAD) -65 and -67 [105-107]. GAD autoantibodies have also been shown to be present before T1D onset in humans and proposed as an autoantigen in T1D development (e.g. [5, 49, 108, 109].

3.6 VIRUSES AND T1D: BYSTANDER ACTIVATION

A third way viruses may be involved in T1D development is through autoimmunity via bystander activation. This implies an unspecific activation of mainly autoreactive T-cells by activated APCs [38]. This can occur after infection, when highly activated APCs are presenting antigens to specifically activate antiviral T_C-cells through TCR-dependent interactions. However, surrounding autoreactive T-cells may potentially also be activated through TCR-independent pathways, due to released inflammatory cytokines. The activated antiviral T-cells killing infected cells may also further trigger bystander activation due to the release of inflammatory cytokines and autoantigens at the site of infection [38].

Bystander activation and autoimmunity is of interest for T1D and CVBs, since CVBs have been found to be pancreatropic [84]. Using a mouse model of autoimmune diabetes, the non-obese diabetes (NOD) mouse, several studies have shown that the exocrine cells in the pancreas are destroyed soon after CVB infections, but the islets are spared [110, 111], presented in Fig 2A and B ([112] and P. Larsson, V. Stone et al., unpublished results). Other studies have also showed that, depending on the time of infection, CVB infections can accelerate T1D development in NOD mice [113, 114]. This acceleration has been suggested to depend on bystander activation by the antiviral T_C-cells, which are killing the infected pancreatic exocrine tissue. A combination of secreted or cell-bound co-stimulatory molecules in the inflammatory milieu may activate surrounding islet-specific autoreactive T-cells to attack and kill the islets [38], as shown in Fig. 2C and D ([112] and P. Larsson, V. Stone et al., unpublished results). Further, persistent infections may also cause autoimmunity, through the constant presence of viral antigens, which may drive an immune response to keep a constant inflammatory process active at the site of infection [38].

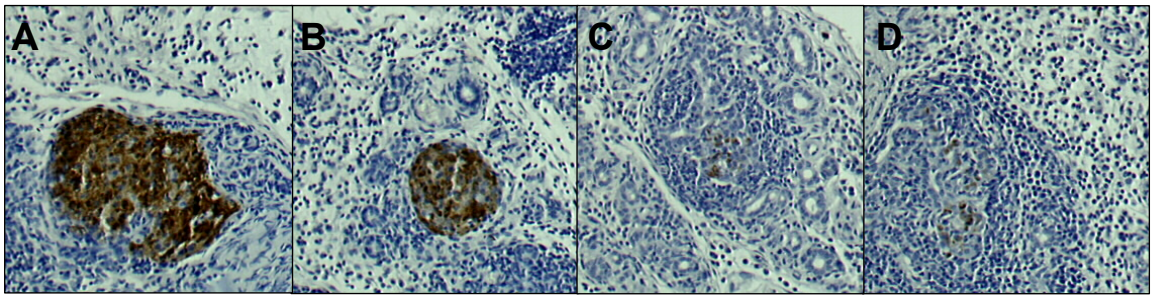

Figure 2. Insulin-stained islets after CVB4 infection of female Proins$^{-/-}$ NOD mice. A, B. Destroyed exocrine tissue but spared and healthy islets in a pancreas from a 6-week old mouse infected with CVB4, day 7 post infection. **C, D.** Completely destroyed exocrine tissue and destroyed islets in a pancreas from a 6-week old mouse infected with CVB4, day 7 post infection. IHC stainings for insulin with insulin-positive cells stained in brown ([112] and P. Larsson, V. Stone et al., unpublished results).

4 VACCINES

The only way to induce protective immunity against virus infections is through the use of vaccines [100]. Unlike bacterial infections, virus infections cannot be treated with antibiotics, a common misunderstanding among the general population. In fact, most virus infections cannot be treated at all but only prevented through vaccination and sanitation. Today, the use of vaccines have eradicated the deadly Variola virus that causes smallpox and poliovirus is very close to being eradicated. The use of child vaccination programs has also significantly reduced the number of pertussis and diphtheria cases in children.

4.1 HISTORY

The first modern vaccine was developed by Edward Jenner in 1796, who showed that the pus from cowpox blisters could confer protection against the deadly smallpox virus [115]. Despite earlier notions of the use of 'variolation' to induce immunity towards smallpox exist, Jenner is generally considered to be the first to have showed that the obtained immunity from cowpox and cowpox blister pus protected individuals against smallpox infection [115].

Later, vaccines against numerous viruses and bacteria have been developed which have lead to improved general health and a reduced infectious burden for the general population. In addition to Jenner, other front figures in vaccine development were: Louis Pasteur (developed vaccines against anthrax and rabies), Jonas Salk and Albert Sabin (developed the first inactivated and oral poliovirus vaccine, respectively) and Maurice Hilleman (responsible for developing vaccines against measles, mumps, hepatitis A& B and chickenpox). Recently, vaccination campaigns have also been introduced against human papillomavirus (HPV), a virus that can cause cervical cancer. Additionally, vaccination against the seasonal influenza virus is usually offered to individuals in risk groups every year.

4.2 HOW DOES A VACCINE WORK?

The idea of vaccination is to induce an immunogenic response and immunity towards a dangerous pathogen in a safe setting [100]. This is achieved through priming of the immune system using non-infectious antigens, like inactivated whole pathogens, viral or bacterial proteins or toxins. Attenuated (weakened and less virulent) viruses are also used, since they are cheap and provide a good immune response without the need for adjuvants or booster injections. However, there is a risk of attenuated viruses reverting to virulent forms through mutagenesis, as seen occasionally in some cases after oral poliovirus vaccination [116].

Upon vaccination, the host's immune response recognizes the foreign or immunogenic substance, which activates both the innate and adaptive immune system and their respective cellular components. This basically results in activated APCs, T- and B-cells, which leads to antibody production and generation of long-lived memory T-, and

B-cells, for a faster secondary response upon reactivation. The antibodies produced by the B-cells can thereafter neutralize the pathogen if the host would encounter it.

4.3 ADJUVANTS

For some antigens, the immune response needs to be boosted in order to be properly activated. Such a boosting effect is achieved through the use of adjuvants in the vaccine, which functions as unspecific activators of the immune system. Examples of adjuvants are bacterial toxins, aluminum hydroxide (alum), squalene, thimerosal, mineral oil and oil-water emulsions like AS03. Alum is commonly used in human vaccines today, for example in diphtheria-tetanus-pertussis, human papillomavirus and hepatitis vaccines.

4.4 SAFETY CONCERNS

The use of vaccines and adjuvants has however been questioned and debated from time to time. In the now infamous article published in 1998 by Wakefield et al., the authors reported an observed link between the vaccine used against measles, mumps and rubella and development of autism, in some children [117]. Despite the fact that this study was retracted, in part by ten out of twelve authors [118] and then completely retracted by the journal due to scientific irregularities and fraud [119], this article is still causing public disbelief in vaccines, resulting in parents denying vaccinations for their children.

Another debate about vaccine-related disease was spurred after vaccinations against the potential pandemic influenza A H1N1 virus in 2009-2010. In some European countries with high vaccine coverage, there was a marked increase in the number of narcolepsy cases developing after vaccination using the Pandemrix vaccine, an AS03-adjuvanted H1N1 vaccine [120-123]. Initially the vaccine was accused for causing the observed increase in narcolepy, and the use of AS03 as an adjuvant for influenza A vaccination was debated [124]. However, prior to the observed increase in narcolepsy after H1N1 vaccinations, a seasonal difference in the development of narcolepsy that followed the influenza A infection peaks was observed in China [123, 125]. Further, it was also noted that there was a clear peak of narcolepsy cases in H1N1 infected individuals in China [123, 125]. This suggested that the influenza A virus itself, especially the H1N1 strain, could cause narcolepsy since these patients were not vaccinated [123, 125]. All these findings were however recently explained in a study demonstrating that narcolepsy is indeed an autoimmune disease which can be caused by molecular mimicry, with an autoimmune T-cell epitope shared by the neuropeptide hypocretin and the H1N1 virus, specifically affecting genetically susceptible individuals [126]. Hence, in these susceptible individuals, both the H1N1 virus and the H1N1 vaccine combined with a potent adjuvant, appear to be able to trigger narcolepsy [126]. In the US, non-adjuvanted H1N1 vaccines were used and there was no increase in the number of narcolepsy cases, hence it remains to be determined if the use of a less potent adjuvant than the AS03 would have affected the development of narcolepsy observed in some European countries [126].

When developing a vaccine there are rigorous safety concerns involved, since a vaccine has to demonstrate a good immunogenic profile without causing side effects. As mentioned in the example above, there might be specific gene-virus/vaccine interactions causing autoimmunity that are very rare, which means that they might not be noticed in small studies. This is especially important if developing a vaccine against a virus associated with development of autoimmunity, since the vaccine itself must be shown to be safe in an autoimmune setting. Unfortunately, rare side effects are often impossible to predict, but the function and potential side effects must be meticulously tested prior to releasing novel vaccines to the public, in order to avoid issues of public disbelief in vaccines.

Over the years, vaccines have saved, and are continuously saving, countless of lives. The high level of herd immunity provided by thorough vaccination programs is key to reduce spread of pathogens and to protect genetically susceptible individuals from detrimental infections.

4.5 VACCINATION PROGRAMS

The current Swedish vaccination program for children born after 2009 is depicted in Table 1 (modified from [127]). As shown, boys receive a total of ten shots whereas girls receive thirteen, due to the three extra shots of HPV vaccine to reduce the risk for developing cervical cancer [127]. In addition to this broad vaccination program, children at risk are also offered hepatitis B vaccinations and the BCG vaccine against tuberculosis [127].

Table 1. The current Swedish vaccination program (2014), modified from [127].

The Swedish vaccination program for children born after 2009:

Age:	Vaccine:
3 months	Diphteria, tetanus, pertussis, polio & Haemophilus influenzae, type b
3 months	Pneumococci
5 months	Diphteria, tetanus, pertussis, polio & Haemophilus influenzae, type b
5 months	Pneumococci
12 months	Diphteria, tetanus, pertussis, polio & Haemophilus influenzae, type b
12 months	Pneumococci
18 months	Measels, mumps & rubella
5-6 years	Diphteria, tetanus, pertussis & polio
6-8 years	Measels, mumps & rubella
11-13 years	HPV (girls only, 3 injections)
14-16 years	Diphteria, tetanus & pertussis

4.6 DEVELOPMENT OF NEW VACCINES

Although numerous vaccines exist, there are still many communicable diseases for which we lack functional vaccines. However not fatal, a classic example for a disease caused by viruses is the common cold, which can be caused by numerous different viruses and combinations of these, for which we lack vaccines and functional

treatments except symptomatic treatments [128]. More severe diseases for which we lack vaccines include human immunodeficiency virus (HIV), hepatitis C, dengue virus and, among children, respiratory syncytial virus (RSV). Due to the high mutation rate of surface antigens on influenza A viruses, new vaccines have to be developed every year since one vaccine cannot cover all different combinations of influenza A surface antigens.

One should note that the above chapters regarding vaccines have focused on antiviral vaccines. There are several non-viral disease for which we also lack vaccines, many of them being of parasitic nature. Examples of non-viral disease for which we lack vaccines include malaria, hookworms and leishmania, which cause countless of infections and deaths every year.

4.7 MATERNAL ANTIBODIES

4.7.1 Transfer of protection

Due to the immature immune system of newborn babies, they do not respond to vaccinations [99, 129]. This is the reason why babies are generally not vaccinated before the age of three months. This may provide a window of opportunity for certain pathogens, to which children will be vaccinated against later on, to infect.
This risk of may be reduced by the passive antibody protection mediated from the mother [99, 103]. The two antibody isoforms capable of transferring maternal protection are IgGs, which are transferred over the placental barrier during pregnancy, and IgAs, which are transferred via the breast milk.

However not yet recommended in Sweden, the Centers for Disease Control and Prevention (CDC) in the USA now recommend pregnant women to receive a booster vaccination against tetanus, diphtheria and pertussis (a vaccine combination known as Tdap) during gestation week 27-36 [130]. This booster injection may maximize the maternal immune response towards these pathogens and also the transfer of maternal antibodies to the fetus [130]. The National Health Service (NHS) in the UK also mentions that a booster of pertussis vaccine in pregnant women could reduce the risk for pertussis in infants, however the NHS has not officially recommended vaccinations for pregnant women [131]. Since no single pertussis vaccine is used, the NHS suggestion is to receive a combination vaccine against tetanus, diphtheria, pertussis and polio [131]. Indeed, vaccination of pregnant women is an interesting vaccine strategy to provide children with protection already from birth.

In the context of CVBs infecting children, it has been shown that many children encounter a primary infection before the age of 3 months [132], which is within the time frame where maternal antibodies, if present, can protect the child from infections [99, 103]. Some studies, but not all, have also found a significant protection against β-cell autoimmunity and T1D development in children that are breast fed longer [59, 133].

4.7.2 Transfer of disease

Antibodies transferred from the mother to the fetus can also be pathogenic in some cases. A classic example of detrimental antibodies transferred from mother to fetus is the transfer of anti-Rh antibodies from an Rh^- mother to an Rh^+ fetus, if the mother has previously given birth to an Rh^+ sibling [27]. Anti-Rh antibodies that cross the placental barrier can cause a severe hemolytic disease called erythroblastosis fetalis, destroying the fetal red blood cells leading anemia, ranging from mild to fatal [27]. Destruction of the red blood cells causes conversion of hemoglobin to bilirubin which can lead to brain damage in the fetus, due to the accumulation of bilirubin in the brain [27].

Other examples of diseases transferred from mother to child depending on antibodies are Sjögrens syndrome, systemic lupus erythematosus (SLE) and Grave's disease. Pregnant women with rheumatic diseases like Sjögrens syndrome or SLE risk transferring antibodies to the fetus that can results in severe fetal heart block [134, 135].

5 AIMS OF THIS THESIS

The overall aim of this thesis was to expand our current knowledge on how and if CVBs may be involved in regulating T1D development. The four papers included here were all based on individual hypotheses, but they all resulted in new knowledge regarding the effects CVBs may have on T1D development and suggesting new strategies for how this may be counteracted.

Specific aims:

- To determine which cellular components are involved in regulating diabetes development in diabetes-prone mice after CVB4 infections. *(Paper I)*.
- To describe the mechanism for the protective effect by αGC against CVB4-accelerated diabetes development in diabetes-prone mice. *(Paper I)*.
- To test the efficacy and safety of a novel CVB1-specific vaccine in mice. *(Paper II)*.
- To study the effects on autoimmunity and diabetes development by CVB1 and a novel CVB1 vaccine in diabetes-prone NOD mice. *(Paper II)*.
- To test if maternal antibodies can protect genetically susceptible offspring against CVB3 infection and CVB3-induced diabetes in a mouse model for virus-induced diabetes. *(Paper III)*.
- To evaluate how human intestinal epithelial cells (IECs) respond to IFN-λs in respect to inducing an antiviral state. *(Paper IV)*.
- To determine if the two IEC lines CaCo-2 and HT-29 express the two main receptors used by CVBs for cellular entry, CAR and DAF *(Paper IV)*.

6 REFLECTIONS ON MATERIAL, METHODS AND RESEARCH DESIGN

In this chapter I will discuss the research design, material and methods that I have used in Paper I-IV. I will also reflect on technical difficulties I have encountered and how some methods had to be developed further in order to fully work in our experimental setup.

6.1 CELL LINES

Throughout the studies included in this thesis, several different cell lines have been used. For culturing details and medium compositions, please see the material and methods sections in *Paper I-IV*.

HeLa, GMK and Vero cells were used for virus propagations, titrations and titrations of neutralizing antibodies in *Paper I-IV*. The human colonic epithelial cell lines CaCo-2 and HT-29 were used to study the effect of IFN-λs on IECs in *Paper III*.

6.2 VIRUSES

In total four different strains of CVBs were used in the studies described in this thesis. The original stocks of CVB3 Nancy and CVB4 E2 were kindly provided by Dr Gun Frisk (Uppsala University, Sweden). A plasmid encoding the CVB3-eGFP virus was kindly provided by Professor Lindsay Whitton (The Scripps Research Institute, USA). The CVB1Nm strain was provided by Sanofi Pasteur (France).

CVB3 Nancy, CVB3-eGFP and CVB4 E2 were propagated and titrated using HeLa cells. Titrations were done a standard plaque assay in 6-well plates.

The CVB1Nm strain was propagated and titrated in Vero cells. Titrations were done using the $CCID_{50}$ end-point dilution method, and titers were calculated according to the Reed and Muench formula [136]. However, some titrations were also done by real-time PCR, as described in material and methods in *Paper II*.

Our initial idea for *Paper III* was to use an oral infection model to mimic the natural route of infection, but the low reproducibility in obtaining systemic viral spread and the need for high, and biologically non-relevant, concentrations of virus in our pilot experiments forced us to use i.p. infections throughout the study. This resulted in the use of i.p. infections for all infected mice included in *Paper I-III*.

6.3 VACCINE

The prototype CVB1 vaccine used in *Paper II* was developed by Vactech Oy (Finland) and Sanofi Pasteur (France). CVB1Nm stocks were propagated in Vero cells and then purified using a two-step centrifugation and the use of a 13% sucrose cushion. Purified virus stocks were then inactivated in PBS supplemented with 0.1% Tween80 and 0.025% formaldehyde for 72h at 37°C. Complete inactivation of the virus stocks were

monitored using Vero cells and $CCID_{50}$ end-point titrations, according to the Reed & Muench formula [136].

Due to the successful immunizations using the formalin-inactivated poliovirus vaccine without adjuvants [137], the prototype CVB1 vaccine used here also non-adjuvanted. Pilot studies in BALB/c mice showed good immunogenic properties by the vaccine (data not shown), hence all further vaccinations were done using a non-adjuvanted vaccine and a dose of 10^6 $CCID_{50}$ equivalents of CVB1, that was injected sub cutaneously intra scapular (i.s.).

6.4 NEUTRALIZATION ASSAY

There are several different methods to titrate the concentration of antibodies in serum: end-point dilution curves ($CCID_{50}$ titrations), plaque-neutralization assays or flow cytometry. Due to the lack of an established protocol in our lab, one initial experimental challenge we had was to set up a functional neutralization assay.

Initial attempts were done to set up a fast and reliable method of detecting neutralizing antibodies based on flow cytometry, with the hypothesis that this method would allow for a higher throughput than the traditional $CCID_{50}$ titrations and plaque-reduction assays. The method was based on a simple protocol where serial diluted serum was incubated with a pre-defined amount of CVB3 or the GFP-encoding CVB3-eGFP before the mixture was added to a specific number of HeLa cells. The blocking of virus replication would then be compared to freely infected cells using flow cytometry to measure GFP fluorescence or other fluorescent secondary antibodies used to detect cells stained intracellular for the viral VP1-protein. However, this technique proved to be harder to use than initially thought, and after several failed attempts to improve the method and detection, we decided to change to another method.

The $CCID_{50}$ end-point dilution method is used in many different laboratories. The sensitivity and reproducibility of the assay is indeed dependent on perfect cell growth, most commonly tested in 96-well plates. After failed attempts to use this high-throughput titration assay with HeLa cells, we decided to abandon even this model and resort to adopting a previously established protocol for a standard plaque-reduction assay in order to detect and titrate neutralizing antibodies in serum samples. Despite being a slower method, the reproducibility and sensitivity of this assay was deemed higher than both the other methods.

After initiating collaboration with the lab of Professor Heikki Hyöty (University of Tampere, Finland), we adopted their protocol for a plaque-neutralization assay and I set up this assay in our lab. Using their protocol as a basis, but with HeLa cells and human serum previously confirmed to contain neutralizing antibodies against CVB3, I developed a protocol for testing different viruses and antibodies, which is now widely used in our group for titration on neutralizing antibodies. The final protocol for the method is stated in the material and method section in *Paper III*.

Titrations of antibodies against CVB1 were also done using a plaque-neutralization assay, but here with GMK cells instead of HeLa cells. The slightly different protocol used for these titrations is presented in the material and method section in *Paper II*.

6.5 ANIMAL HUSBANDRY

The use of research animals is somewhat ethically controversial. However, it provides immunologists with an invaluable tool for studying immune responses in complex whole organisms. Hence, to study how the immune system responds to potentially diabetogenic virus infection, research animals are needed.

All animals included in *Papers I-IV* were housed in specific pathogen-free environments in approved facilities and all experiments were approved by local ethic committees and carried out according to Swedish and French law. For additional detail regarding individual experiments, please see the material and methods section in *Papers I-IV*.

6.5.1 Non-obese-diabetic (NOD) mice

The NOD mouse model dates back to the early 1980's, when Makino et al. published their first study on a mouse model for autoimmune diabetes [138]. NOD mice develop spontaneous autoimmune diabetes from around 10 weeks of age. Hallmarks of diabetes development in the NOD mice are insulitis, autoantibodies against β-cell antigens and a T- and B-cell dependent autoimmune etiology. Apart from the clearly displayed insulitis, all of these hallmarks are also observed in human T1D development. There is a clear sex-dependent difference in diabetes development among NOD mice, with approximately 60-80% of the females developing diabetes but only 20-30% of the males. Hence female NOD mice are used in most studies. The diabetes incidence in NOD mice is also dependent on animal housing standards, where a less clean animal house might provide a lower incidence of diabetes. The NOD mouse model is the most used model for studying autoimmune diabetes.

NOD mice were used in *Papers I-III* for evaluating diabetes development in different settings. For *Paper III*, NOD mice were also used to determine autoimmune safety of a prototype CVB1 vaccine and potential induction of autoimmunity after CVB1Nm infection and vaccination using formalin-inactivated CVB1Nm.

6.5.2 Proinsulin 2–deficient NOD mice

For the studies conducted in *Paper I*, we took advantage of the genetically modified Proins2$^{-/-}$ NOD mice, which lack the gene encoding for proinsulin-2. Due to the lack of proinsulin-2-presentation to T-cells in the thymus during T-cell development, these mice develop autoimmune diabetes with an accelerated onset compared to the normal NOD mice [139]. Generally, the Proins$^{-/-}$ NOD mice develop diabetes around 6-8 weeks of age [139]. Like in NOD mice, diabetes development is preceded by islet autoimmunity and insulitis [139]. The use of Proins$^{-/-}$ NOD mice significantly shortened the duration of the experiments conducted in *Paper I*.

6.5.3 Vα14 transgenic NOD mice

In *Paper I*, we also used transgenic NOD mice expressing the invariant T-cell receptor α-chain Vα14-Jα18 (Vα14-NOD). As previously shown, these mice have an increased frequency of iNKT cells that can regulate diabetes development in the NOD mouse [22].

Vα14-NOD and Proins2$^{-/-}$ NOD mice were also crossed in order to obtain Vα14 Proins2$^{-/-}$ NOD. These mice were used to study iNKT cells isolated from murine pancreatic islets and their response to CVB4 and αGalCer in *Paper I*.

6.5.4 BDC2.5 Cα$^{-/-}$ transgenic NOD mice

The CD4 T-cells from the TCR transgenic BDC2.5 Cα$^{-/-}$ NOD (BDC2.5 Cα$^{-/-}$ NOD) have been shown to be islet specific and have diabetogenic properties [140]. To study the effect of suppressive macrophages on diabetogenic T-cells in *Paper I*, we isolated T-cells from BDC2.5 Cα$^{-/-}$ NOD mice and co-cultured them with pancreatic macrophages from αGalCer and αGalCer+CVB4 treated Proins2$^{-/-}$ NOD mice in a T-cell proliferation assay.

6.5.5 Socs-1 transgenic NOD mice

In *Paper III* we used Socs-1 transgenic NOD (Socs-1 tg) mice as a model for CVB-induced diabetes [110, 111, 141, 142]. The Socs-1 tg mice express the Suppressor-of-Cytokine-Signaling (Socs) 1 protein under the control of a human insulin promotor, which specifically renders their β-cells unable to respond to IFN signaling. Upon a systemic CVB infection that spreads to the pancreas, Socs-1 tg mice develop diabetes within 5-11 days post infection, due to the failing interferon-induced antiviral defense in the β-cells [110, 111].

Heterozygous Socs-1-tg mice were bred with wild-type NOD mice, rendering ~50% of the offspring carrying the Socs-1 gene. DNA screening for the presence of Socs-1 was done by PCR on tail or ear biopsies from all mice. The detailed generation and breeding of Socs-1 tg mice have been described previously [110, 111, 141, 142].

I carried out the initial titrations of CVB3 Nancy, CVB4 E2 and CVB1Nm in order to obtain a systemic infection resulting in diabetes development in i.p. infected Socs-1 tg mice.

6.5.6 BALB/c mice

In *Paper II* we used BALB/c mice for the initial vaccinations and CVB1 infections. The BALB/c strain was used as a model of a non-diabetic and non-autoimmune ‘standard mouse’. BALB/c mice are also generally susceptible to infections, hence a good model to use for vaccine studies.

6.6 FLOW CYTOMETRY

In order to study the individual cellular components of the immune system in *Paper I*, we used flow cytometry and fluorescence-activated cell sorting (FACS). Using primary cells from mice, this allowed us to look for specific cells and their activity depending on different treatments. Single cells from murine pancreatic islets were prepared after CVB4 infection and stained with antibodies to distinguish between different immune cells. Cells were stained using the following surface antibodies: CD45, CD11b, CD11c, Ly-6G, Ly6C, F4/80, 120G8, CD115, CD62L, CD4 and CD8. αGalCer-loaded CD1d tetramers were also used to identify iNKT cells. Intracellular stainings were also done for Ki-67 and cytokines IL-4, IL-13 and IFN-γ. Non-specific Fc binding was blocked by an anti CD16/CD32 antibody. Staining details and protocol is presented in the material and methods of *Paper I*.

We also used flow cytometry in *Paper IV* in order to detect surface expression of the two main receptors used by CVBs for cellular entry, CAR and DAF, on the human IEC lines CaCo-2 and HT-29. Primary antibodies against CAR and DAF were after staining visualized using a secondary antibody for CAR and streptavidin-conjugated APC to detect the biotinylated DAF antibody.

All flow cytometry experiments were conducted using a BD Fortessa or BD Acurri C6 flow cytometer or sorted using a BD FACSAria II.

6.7 IN SITU HYBRIDIZATION (ISH)

In *Paper I* we performed in situ hybridization (ISH) in order to detect viral RNA in tissues harvested from mice after infection. The protocol used for the in situ hybridization has been published previously [88, 89, 143], and was here used with some modifications. Sections were counterstained using Kernechtrot, Light green or Hematoxylin and after dehydration in 100% EtOH, a dehydration step in xylene was skipped and cover glasses were mounted using 90% glycerol in PBS. Using ISH in *Paper I*, we tried to detect infected islets in the pancreas of CVB4 infected mice. However, we could not detect any infected islets in the pancreata from CVB4 infected mice at any time point.

We also used ISH in *Paper II* to detect infected pancreata after CVB1 infection (supplementary figure 1A-C in *Paper II*).

During the staining process using ISH, I made an attempt to further improve the protocol by changing the counterstaining used. Background stainings using Kernechtrot provides good contrast to the blue precipitate from NBT/BCIP, but the resolution of the pancreas in general was not ideal. A change to Light green improved the resolution and visibility of the pancreatic morphology, however, this was further improved by using hematoxylin for counterstaining. The resolution of the organ morphology was indeed much improved, but the blue color provided by hematoxylin counterstaining made detection of viral RNA using NBT/BCIP somewhat harder to visualize. We are currently still working on technical improvements of the ISH protocol in order to improve detection of viral RNA in pancreatic sections.

6.8 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

When analyzing organ morphology after infection, all pancreata were fixed in formalin prior to sectioning and hematoxylin and eosin staining (H&E). H&E stainings were used specifically to monitor pancreatic destruction and morphology after CVB infections.

Further, we have also performed immunohistochemistry (IHC) to stain pancreata using insulin and glucagon antibodies, to find islets (Fig. 2 in *Paper III* and Supplementary figure 1 in *Paper II*). The combination of insulin and glucagon staining in consecutive sections allowed us to find islets that did not produce insulin, which may have been affected by a virus infection.

The protocols for preparing tissue sections and stainings have been previously described [144]. The analysis of all stained tissue sections were conducted blinded by two independent observers, as previously described [110].

6.9 INSULIN AUTOANTIBODY (IAA) TITRATIONS

We titrated IAA concentrations in serum samples saved from mice before and after treatments in *Paper II*. IAA levels were quantified using a previously established radiolabel-binding assay [145]. After standardization, the cut-off limit for IAA positivity was set to 0.881 relative units. Using the cut-off value we could compare both the actual titers of IAAs and the frequency of seroconverting mice after each treatment (Fig. 4A-C in *Paper II*).

6.10 SERUM TRANSFERS

In *Paper III* we conducted adoptive serum transfers of heat-inactivated confirmed antibody-positive and negative sera from NOD mice to naïve Socs-1 tg mice. Serum (400µl/mouse) was injected i.p. to recipient mice and presence of neutralizing antibodies in serum was assessed 24h post transfer.

6.11 QUANTITATIVE REAL-TIME PCR

We have used quantitative real-time PCR (qRT-PCR) to detect expression levels of different genes in *Paper I and IV*, and we used qRT-PCR to detect virus in *Paper II*. Please see material and methods section of *Papers I, II and IV* for detailed protocols on genes tested, primers used and PCR machines used for analysis.

7 RESULTS AND DISCUSSION

7.1 *Paper I*

Protection Against Type 1 Diabetes upon Coxsackievirus B4 Infection and iNKT Cell Stimulation: Role of Suppressive Macrophages

T1D may result from an attack by autoreactive T-cells on the insulin-producing β-cells. Despite the fact that several genetic and environmental factors have been identified and suggested to regulate susceptibility to disease, T1D etiology remains elusive [5, 49]. Enterovirus infections, especially by CVBs, have been linked to T1D development [64, 79, 85, 146]. Depending on the timing of infection, CVB4 infection can accelerate diabetes development in NOD mice [91, 113]. However, protection from diabetes development has also been observed after virus infections in NOD mice. Previous studies suggested that protection from diabetes development in diabetes-prone mice infected with lymphocytic choriomeningitis virus (LCMV) was mediated by iNKT cells, which promoted antiviral CD8 T-cell responses but still protected the islets from autoreactive CD8 T-cells [147, 148]. iNKT cells can be activated and stimulated by the ligand αGC, and it has been shown that diabetes an be prevented in diabetes-prone mice by increased numbers of αGC-stimulated iNKT cells [22, 149, 150]. Despite their suppressive role diabetes development, iNKT cells also have potent immunmodulatory functions in enhancing the immune response towards different pathogens [151, 152]. We were therefore interested in evaluating iNKT cell responses to CVB4 and determine the role for iNKT cells in regulating diabetes development in CVB4-infected diabetes-prone mice.

We first confirmed the previous finding that CVB4 could accelerate diabetes onset [113] and compared diabetes development among untreated, αGC-treated, CVB4 infected and combined αGC-treated and CVB4 infected NOD and Proins$^{-/-}$ NOD mice (Fig. 1A-D in *Paper I*). Proins$^{-/-}$ NOD mice were used due to their accelerated diabetes onset compared to NOD mice [139]. As expected, CVB4 infected mice had an accelerated diabetes onset and αGC-treatment alone had no effect on diabetes development. However, mice treated with αGC and CVB4 had a significantly lower incidence of diabetes. There was no difference in pancreatic virus titers after CVB4 infection with our without αGC-treatment (Fig. 1F in *Paper I*). ISH also showed similar levels of virus in the exocrine tissue of the pancreas and no virus in the islets from CVB4 infected Proins$^{-/-}$ NOD mice with or without αGC-treatment. In situ hybridization showed no infection of islets at any time point, as seen in Fig. 3A-F ([112] and P. Larsson, V. Stone et al., unpublished results). This suggested that activated iNKT cells regulate diabetes development after CVB4 infection.

Next we analyzed the mRNA expression of inflammatory cytokines and suppressive enzymes in the islets from untreated, αGC-treated, CVB4 infected and combined αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice. We observed significantly reduced expression levels of the inflammatory cytokines IL-4, IL-13 and IFN-γ and increased expression levels of the suppressive enzymes iNOS, IDO1, IDO2, Arginase I and Ym1/Ym2, when comparing islets from the combination treated mice to those from

CVB4 infected mice (Fig. 2 in *Paper I*). These findings suggest that iNKT activated in the presence of CVB4 can establish a less inflammatory and more immunosuppressive milieu in the islets than what is observed after CVB4 infection alone.

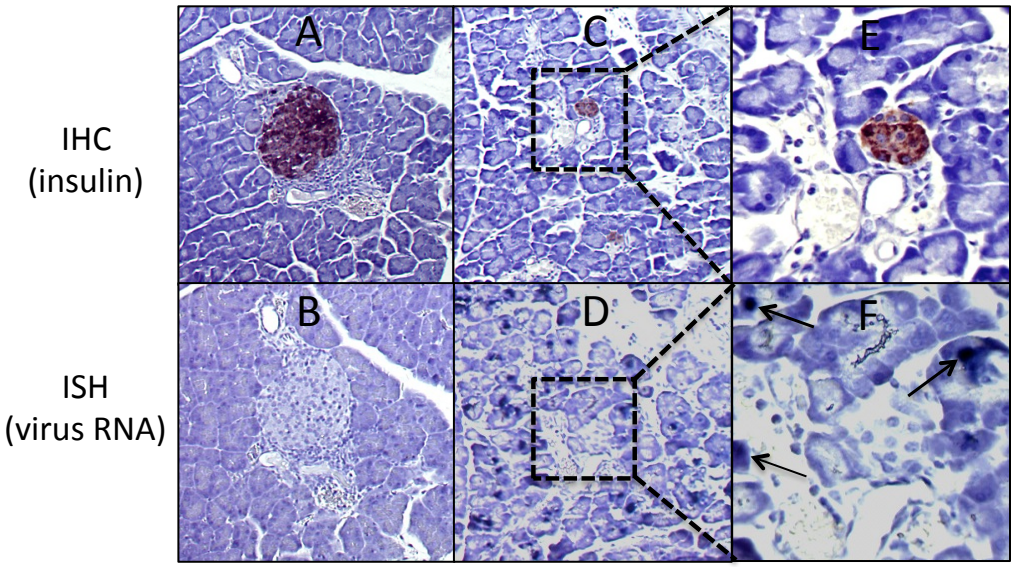

Fig. 3. Pancreatic sections from Proins$^{-/-}$ NOD stained for insulin and EV RNA. Insulin staining using IHC (A, C, E) and ISH staining for viral RNA (B, D, F) in pancreatic section from an uninfected mouse using 10x magnification (A, B), and from a CVB4 infected mouse using 10x (C, D) and 25x (E, F). Each staining is a representative staining for each condition. Arrows indicate cells stained positive for viral RNA ([112] and P. Larsson, V. Stone et al., unpublished results).

The upregulation of Ym1/Ym2 is interesting, since this enzyme is mainly expressed by the suppressive M2 macrophages [153]. We therefore isolated single cells from islets from untreated, αGC-treated, CVB4 infected and combined αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice and FACS sorted them using antibodies against CD45, CD11b, CD11c and 120G8. The cells were sorted as CD45^{-}, CD11c^{+}, CD11c^{low}/ 120G8^{+}, CD11b^{-}/CD11c^{-} and CD11b^{+}/CD11c^{-} and thereafter analyzed for mRNA expression of suppressive enzymes. The CD11b^{+}/CD11c^{-} myeloid cells had the highest expression of all analyzed suppressive enzymes (Fig. 3A and B in *Paper I*), and these cells were further characterized using F4/80, Ly-6G, Ly-6C and CD115 antibodies. Subsequent analysis showed that these cells represented CD45^{+}/CD11b^{+}/ F4/80^{+}/Ly-6C^{+}/CD115^{+} macrophages [154]. As previously shown, these macrophages expressed inflammatory cytokines if exposed to CVB4 alone [155-157], however, this expression was change to an expression of suppressive enzymes if exposed to a combination of αGC and CVB4 (Fig. 2 and Fig. 4B in *Paper I*). This suggested that macrophages infiltrating the islets after CVB4 infection expressed inflammatory cytokines, but upon CVB4 infection with αGC, iNKT cells could make these macrophages expressing suppressive enzymes instead.

We then further characterized the response to CVB4 in iNKT cells isolatd from the islets of Vα14/Proins$^{-/-}$ NOD mice. Vα14 transgenic mice were used since these mice express a 10-fold increase in frequency and numbers of iNKT cells. Isolated islets cells

from untreated, αGC-treated, CVB4 infected and combined αGC-treated and CVB4 infected Vα14/Proins$^{-/-}$ NOD mice, were stimulated with PMA/Ionomycin and Brefeldin A before they were stained using an APC-conjugated αGC-loaded CD1d tetramer and antibodies against CD69, Ki-67, IFN-γ, IL-4 and IL-13. CVB4 infection alone lead to induced activation of pancreatic iNKT cells that upregulated CD69 but not the proliferative marker Ki-67. αGC treatment alone increased proliferation and production of cytokines, but did not activate iNKT cells to upregulate CD69 (Fig. 5 in *Paper I*). The role of the cytokines IL-4, IFN-γ and IL-13 in suppressive macrophage function was then examined by PCR in islets from combined αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice also treated with antibodies blocking IL-4, IFN-γ or IL-13. Indeed, blocking of IFN-γ resulted in significantly decreased mRNA expression levels of iNOS, IDO1, IDO2 and Ym1/Ym2, whereas blocking of IL-13 reduced the expression levels of Arginase I (Fig. 6 in *Paper I*). Blocking of IL-4 did not have any effect. This suggested that cytokines expressed by activated iNKT cells are key mediators for the activation of suppressive macrophages in mice after CVB4 infection.

In order to investigate the role of the suppressive enzymes iNOS, IDO1, IDO2, and Arginase I, combined αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice were also treated with specific inhibitors for these enzymes or mock-treated. The inhibitors against iNOS (1400W) and Arginase I (nor-NOHA) had no effect in blocking the protective effect on diabetes development, but blocking IDO1/2 with inhibitor 1MT restored diabetes development in these mice (Fig. 7A in *Paper I*). The observation that 1MT alone did not have an effect on spontaneous diabetes development in Proins$^{-/-}$ NOD mice but the effect in αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice was the same as all three inhibitors together, suggesting that IDO1/2 alone played a significant role in the protection from diabetes development (Fig. 7A and B in *Paper I*).

Since the IDO1/2 expression in islets was induced by IFN-γ, we blocked IFN-γ in αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice and observed the same restoration of diabetes development as we did when blocking IDO1/2 with 1MT (Fig. 7D in *Paper I*). We also tested the suppressive macrophages in a T-cell proliferation assay, where macrophages isolated from islets from CVB4 infected and combined αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice were co-cultured with diabetogenic CD4 T-cells isolated from BDC2.5 mice. We observed an increased T-cell proliferation after co-culture with macrophages from CVB4 infected mice, but a significantly reduced proliferation of T-cells co-cultured with macrophages from combined αGC-treated and CVB4 infected mice (Fig. 7E in *Paper I*). The reduced proliferation was abrogated by addition of 1MT to the culture. We further performed adoptive transfers of macrophages isolated from islets from CVB4 infected and combined αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice and then transferred to CVB4 infected Proins$^{-/-}$ NOD mice. Macrophages transferred from CVB4 infected mice increased the observed incidence of diabetes but macrophages isolated from combined αGC-treated and CVB4 infected mice reduced the incidence of diabetes (Fig. 7F in *Paper I*). All this suggests that macrophages might have a dual role in diabetes development and protection from diabetes, all depending on how they are activated and stimulated after a CVB4 infection.

Finally, we examined T-cells isolated from islets from untreated, αGC-treated, CVB4 infected, combined αGC-treated and CVB4 infected and combined αGC-treated, 1MT treated and CVB4 infected Proins$^{-/-}$ NOD mice. We observed that all infected mice had significantly higher numbers of CD4 and CD8 T-cells in the islets than untreated and αGC-treated mice had (Fig. 8A and B in *Paper I*). When analyzing the frequency of IFN-γ^+ cells among the CD8 T-cells, we observed that mice that had developed diabetes had significantly higher frequencies of IFN-γ^+ cells, independent on previous treatments (Fig. 8C and D in *Paper I*). We also noted that IFN-γ^+ IGRP CD8 T-cells were only found among CVB4 infected diabetic mice (Fig. 8C in *Paper I*), further supporting a role for CD8 T-cells in CVB4-induced diabetes, as suggested previously [113, 158]. This suggested that the protective effect seen on diabetes development in combined αGC-treated and CVB4 infected Proins$^{-/-}$ NOD mice does not depend on reduced T-cell numbers, but rather suggests that the suppressive effects by the iNKT cell-induced macrophages can inhibit autoreactive T-cells locally in the pancreas.

In summary, our data indicates manipulations of iNKT cells can induce suppressive functions in macrophages. Upon αGC and CVB4 activation, islet iNKT cells produce IFN-γ which can activate macrophages to produce immunosuppressive enzymes, like IDO1/2, in the islets, which can prevent diabetes development through the local inhibition of autreactive T-cells. We have also showed a dual role for macrophages in diabetes development, where macrophages can be both causative and suppressive effector cells. We believe that our results can be used for novel therapeutic approaches in order to manipulate iNKT cells and suppressive macrophages.

7.2 *Paper II*

Preclinical Evaluation of Efficacy and Safety of a New Vaccine Against Coxsackievirus B1 – Implications for Type 1 Diabetes Development

EV infections have been implicated as environmental triggers in the etiology of T1D [67-69]. Several epidemiological studies have also suggested a causal linkage between EV infections and development of islet autoimmunity [64, 80-83]. However, most studies have neglected to sequence or serotype their viral findings further, hence we cannot say which types of EVs may be causative agents for T1D development. Historically the CVBs, especially CVB4, have been suggested to have diabetogenic properties, and CVB4 has indeed been isolated from diabetic patients. However, recently two multi-center studies appointed CVB1 a diabetogenic role, as it was demonstrated that CVB1 infections were associated with development of β-cell autoimmunity in some children [83] and that CVB1 infections were more common in children who have developed T1D [93]. The identification of a specific virus associated with development of T1D is interesting, since it suggests that only a few specific EV strains might be diabetogenic. Isolation of such viruses also suggests a possibility to vaccinate against these serotypes in order to test the hypothesis that CVB1 can trigger β-cell autoimmunity and T1D development.

Some attempts have been made to develop new vaccines against EVs, the most recent against EV71 [159, 160] and Coxsackievirus serotype A16 [161, 162]. EV71 has evolved as a novel epidemic in Asian countries, causing hand, foot and mouth disease

(HFMD) and severe encephalitis in patients [159]. There are however, to our knowledge, no vaccines that are targeting CVB1 infections. Hence, the aim of *Paper II* was to develop and pre-clinically evaluate function and autoimmune safety for a novel prototype CVB1 vaccine in mice.

We first titrated the levels of neutralizing antibodies in the circulation of vaccinated BALB/c mice (Fig. 1A in *Paper II*). The vaccination was deemed safe since no adverse side effects was observed in the vaccinated mice, and the cumulative weight increase after vaccination matched that of mock-treated controls (Fig. 1B in *Paper II*). Upon viral challenge with the live homologous CVB1 strain, vaccinated mice had significantly reduced virus titers in targeted tissues. The viremia levels at both day 2 and 7 post infection and the pancreatic virus titers day 7 post infection was significantly lower, or undetectable, in vaccinated mice compared to that observed in mock-treated mice (Fig. 1C in *Paper II*). We could also observe a weight loss in the mock-treated BALB/c mice after CVB1 challenge, a hallmark of a CVB infection in mice ([163] and P.G. Larsson and M. Flodström-Tullberg, unpublished observations). This was however not observed in the vaccinated mice after CVB1 challenge (Fig. 1D in *Paper II*). These results showed that the vaccine induced neutralizing antibodies that cold protect BALB/c mice from CVB1 infection and that the use of the vaccine was safe in BALB/c mice.

Thereafter, we investigated the safety profile of the vaccine using NOD mice, a model for autoimmune diabetes. Prediabetic NOD mice have also been shown to develop diabetes with an accelerated onset after various CVB infections [113, 114, 164], a finding that was recently confirmed in human T1D patients [90]. Also, like in humans [42], NOD mice develop autoantibodies towards β-cell antigens which precedes diabetes development [165]. Since it has not been tested before, we initially tested whether a CVB1infection would have the same accelerating effect on diabetes development as other CVBs [112-114, 164]. Hence, prediabetic female NOD mice were infected with CVB1 at 14 weeks of age, after which diabetes development was compared to that of untreated or mock-treated female NOD mice (Fig. 2B and C in *Paper II*). As expected, CVB1 infected NOD mice showed an accelerated onset of diabetes (Fig. 2B and C in *Paper II*). Knowing that CVB1 could accelerate diabetes onset in prediabetic female NOD mice, we next evaluated diabetes development in vaccinated, mock-treated or untreated female NOD mice. The vaccinations were done using three injections, just like in the BALB/c mice, and we observed high titers of neutralizing antibodies after vaccination (Fig. 3B in *Paper II*). Also in the NOD mice, vaccination was deemed safe as no adverse side effects was observed and the cumulative weight increase matched that of mock-treated and untreated mice (Fig. 3C in *Paper II*).

We could not observe any differences in incidence or acceleration of diabetes onset among the vaccinated NOD mice compared to mock-treated and untreated controls (Fig. 3D in *Paper II*). This suggested that, unlike infection with the live CVB1 virus, the vaccine does not affect the process leading to diabetes development in NOD mice. However, a previous study did observe a protective effect on diabetes development in NOD mice after immunizing them with formalin-inactivated CVB4 [166]. However, since we did not use any adjuvants in our study, these observations could be explained

by the differences in the experimental setup. The suggested dependency of T_{reg} cells was however not addressed in our study [166].

We further confirmed the notion that our vaccine had no affect on diabetes development by comparing induction of autoimmunity in the form of IAAs, in untreated, mock-treated, CVB1 infected and vaccinated NOD mice. We could not observe any significant increase in the IAA titers after neither CVB1 infection nor vaccination compared to respective mock-treated and untreated controls. We also compared the frequency of animals seroconverting to IAA positivity after treatments but there were no significant differences. However, it has been suggested previously that an immunization with formalin-inactivated CVB4 in combination with Freund's adjuvant could increase the titers of IAAs in mice [166]. Although our results do not support this finding, we did not use any adjuvants, which may explain the observed discrepancies. However, we did compare the effect on IAA levels after CVB1 vaccination to that seen after CVB1 infection, and we could not observe any differences, although CVB1 vaccination did accelerate diabetes development in NOD mice. This may suggests a negligible role for IAAs in diabetes development in NOD mice.

In respect of the observations that narcolepsy is an autoimmune disease, which may be triggered by both the H1N1 virus and the H1N1 vaccine Pandemrix through previously unidentified gene-virus/vaccine interactions [126], there is a possibility that such gene-virus/vaccine interactions may exist for T1D and the virus/vaccine tested here. Despite our safety studies in the NOD mouse model, we cannot rule out this possibility if the vaccine is further developed for human use.

In total, our results showed that vaccinated mice produced neutralizing antibodies, which provided protection against CVB1 infection *in vivo*. We also showed that the vaccine did not cause any adverse side effects in BALB/C and NOD mice, suggesting that the vaccine was well tolerated. Further, the vaccine did not trigger or accelerate β-cell autoimmunity, in the form of IAAs, or accelerate diabetes onset in pre-diabetic NOD mice. Lastly, we also showed that the live CVB1 could accelerate diabetes onset on prediabetic female NOD mice, but the effect on diabetes development did not correlate with induced β-cell autoimmunity in the form of IAA. In summary, our results indicate that this vaccine could be developed further and adapted for human use, in order to develop a vaccine that could be used to test the hypothesis that CVBs are involved in the development and etiology of human T1D.

7.3 *Paper III*

Previous Maternal Infection Protects Offspring from Enterovirus Infection and Prevents Experimental Diabetes Development in Mice

Despite numerous studies suggesting an association between EV infections and T1D development, some controversies exist. Based on the link between EV infections and T1D development, the natural expectation would be that the T1D incidence should be highest in countries with a high prevalence of observed EV infection. However, the opposite has been observed when comparing data from several different countries,

suggesting an inverse correlation between the incidence of T1D and the prevalence of observed EV infections. This counter-intuitive observation has been observed and explained before for poliovirus infections and development of severe paralytic poliomyelitis [96]. In 2000, Viskari et al. further suggested to apply the so called polivirus hypothesis [96] on enterovirus infections and T1D development, in order to explain the observed inverse correlation between the two [97]. Some indirect evidence have been presented in support of the extended poliovirus hypothesis suggested by Viskari et al., but there has been no direct experimental proof-of-concept for maternally transferred antibodies protecting offspring from potentially diabetogenic CVB infections [103, 132, 167]. Hence, we wanted to determine if maternally transferred antibodies could protect genetically susceptible offspring from virus-induced diabetes. In *Paper III* we therefore tested the extended poliovirus hypothesis applied on CVB infections and diabetes development in two different mouse models.

Firstly, we infected NOD mice with a low dose CVB3, in order to establish whether NOD mice develop neutralizing antibodies to CVB3. Virus infection was confirmed in all infected mice by blood samples in which viremia was assessed day 3 post infection (Supplementary figure 1A in *Paper III*). Infected mice then developed high titers of neutralizing antibodies at day 14 post infection (Supplementary figure 1B in *Paper III*). We also tested the specificity of the antibodies from the infected mice in a cross-neutralization test with CVB4, which showed that the induced antibodies were serospecific for CVB3 (data not shown).

Having established that NOD mice raise neutralizing antibodies to CVB3, we infected female NOD mice with a low dose of CVB3. After confirming antibody-positivity, immunized females were set up in heterozygous breeding with Socs-1 tg males, a mouse model for virus-induced diabetes, rendering 50% of the offspring Socs-1 tg. In parallel, naïve NOD females were also bred with Socs-1 tg males, see the experimental model presented in Fig. 4. All offspring were thereafter tested for the presence of neutralizing antibodies in serum at the day of weaning. We showed that offspring from immunized dams had high titers of neutralizing antibodies present in their circulation, whereas offspring from naïve mothers did not (Fig. 1A in *Paper III*). We also showed that these antibodies could protect offspring from infection with a high dose of CVB3, since the virus titers were significantly lower in both blood and pancreas in infected offspring from immunized mothers compared to offspring from naïve females (Fig. 1B and C in *Paper III*).

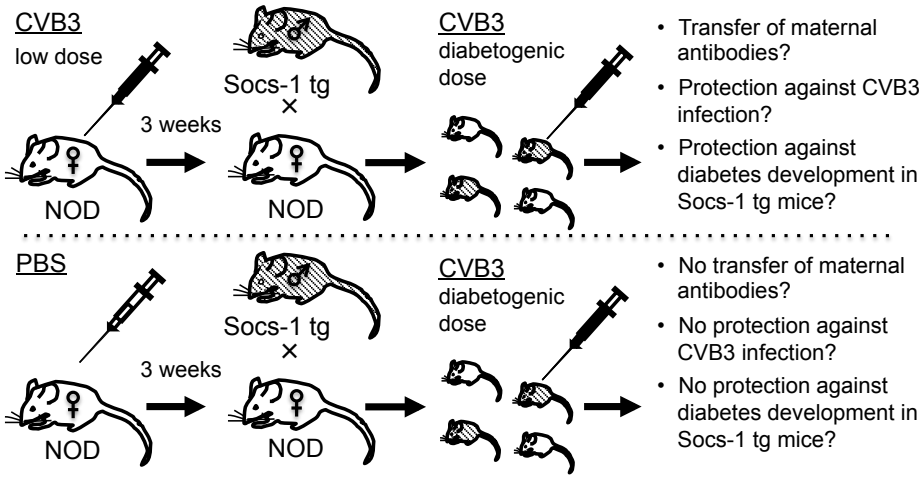

Figure 4. Experimental setup for maternal transfer of antibodies to offspring. Female NOD mice immunized with a low dose CVB3 or mock-treated were, after viral clearance, bred with Socs-1 tg NOD mice. Maternal antibody transfer was assayed in all offspring but protection from CVB3 infection and diabetes development after infection was only tested in Socs-1 tg offspring (adopted from *Paper III* [101], with permission from the publisher).

When analyzing the Socs-1 tg offspring from immunized and naïve females alone, we also observed that offspring from immunized females were protected from diabetes development after a diabetogenic CVB3 infection (Fig. 2A-C in *Paper III*). A clear difference was observed in diabetes development after challenging the Socs-1 tg offspring with a diabetogenic dose of CVB3 (Fig. 2B in *Paper III*), where none of the Socs-1 tg offspring from immunized females developed diabetes. Such a protection from infection and diabetes development in offspring from immunized females has indirectly been observed before by, when Tirabassi et al. showed that rats born from Kilham rat virus (KRV) immunized females were protected from infection and KRV-induced diabetes [167]. However, compared to our study, Tirabassi et al. neglected to show any data regarding the transfer of maternal antibodies or titers of neutralizing antibodies [167].

We further showed the maternal protection from infection by IHC stainings of pancreata from CVB3-infected Socs-1 tg offspring originating from immunized and naive mothers (Fig. 2C in *Paper III*), where we could clearly see that offspring from immunized females were protected from infection.

Despite the fact that we awaited viral clearance from the immunized females, even before we set them up in breeding, we cannot rule out the possibility that the maternal infection could have affected the permissiveness of the offspring by other means than the transferred antibodies. Therefore we conducted experimental adoptive serum transfers of confirmed antibody-positive and negative serum from immunized and naïve mice to naïve Socs-1-tg mice. All sera had been heat-inactivated prior to the transfers, in order to rule out any potential transfer of virus (data not shown). Antibody titers in the circulation of mice receiving serum transfers was analyzed 24h post transfer, and we observed high titers of neutralizing in mice receiving antibody-positive sera compared to no neutralizing activity in serum of mice receiving antibody-negative sera mothers (Fig. 3A in *Paper III*). Furthermore, recipients of antibody-positive sera

had almost non-detectable levels of viremia three days after challenge with a diabetogenic dose of CVB3, compared to mice receiving antibody-negative sera (Fig. 3B in *Paper III*). Finally, we also showed that Socs-1 tg mice receiving antibody-positive sera were completely protected from developing diabetes after CVB3 infection, whereas 50% of the Socs-1 tg mice receiving antibody-negative sera developed diabetes rapidly after infection.

A potential limitation with this study is the use of the Socs-1 tg mouse model for virus-induced diabetes, since diabetes development in this model is solely dependent on a virus infection in the β-cells. Using for example the NOD or the LCMV/RIP-GP/NP mouse models, future studies could test whether maternal antibodies can protect offspring from virus-induced T-cell mediated diabetes development. Also, our results do not supply any additional information to whether EVs can trigger diabetes or not. Such data may only come from vaccination studies showing an effect on human T1D development.

Taken together, we have here shown data that suggests proof-of-concept for the poliovirus hypothesis applied on CVB infections and development of diabetes in genetically susceptible mice. This implies that the extended poliovirus hypothesis suggested by Viskari et al., may be used to explain the observed inverse correlation between T1D incidence and the number of EV infections in different populations. Furthermore, our results suggest that maternal antibodies indeed can protect offspring from potentially diabetogenic virus infections, which if extrapolated, may suggest novel vaccination strategies for pregnant women. This idea is supported by the recent recommendations by the CDC in the United States, to give all pregnant women a booster vaccination against tetanus, diphtheria and pertussis (a vaccine combination known as Tdap) during gestation week 27-36, in order to maximize the transferred protection to the children [130].

7.4 *Paper IV*

Human Intestinal Epithelial Cells Enter an Antiviral State upon Stimulation with Interferon Lambda

Type I IFNs (mainly IFN-α and β) are important for antiviral responses in most cells [29, 30]. The effect of the IFN-λs (type III IFN) may however only have local effects, due to the relatively restricted expression of the IFN-λ receptor. It has been suggested that mainly epithelial cells express the IFN-λ receptor [30, 168-170], but this has been expanded by other studies showing that for example primary hepatocytes [171] and human pancreatic islets [172] also expressed the IFN-λ receptor. Our study showing that human islets produced IFN-λ after CVB3 infection and that IFN-λ could reduce islet permissiveness to CVB infection, also suggests an important role for IFN-λs in in the antiviral response to CVBs [172]. Other studies have shown a potent role for IFN-λs in the antiviral defense against rotavirus infections in the intestine [170]. This is interesting, since the primary site of replication for CVBs is in the IECs.

In *Paper IV* we were interested in examining how human IECs respond to IFN-λ stimulation, in respect of induction of antiviral defense genes previously suggested to

be involved in the establishment of an antiviral state. Previous studies have shown that the expression of the antiviral genes PKR, MxA, ISG15, OAS-2 and iNOS are of importance in the antiviral response to CVB infections [144, 173, 174]. Hence, in *Paper IV* we tested if two human IEC lines CaCo-2 and HT-29 could upregulate these antiviral defense genes after IFN-λ stimulation.how the two human IEC lines after IFN-λ stimulation. We also determined the expression of the two main receptors used by CVBs for cellular entry, CAR and DAF, in CaCo-2 and HT-29 cells, in order to determine if these IECs could be used to test if IFN-λs can regulate IEC permissiveness to CVB infections.

We first confirmed the previous finding that CaCo-2 and HT-29 cells expressed mRNA for the IFN-λ receptor subunits IFNλR1 and IL-10R2 [169]. Both CaCo-2 and HT-29 cells had detectable mRNA expression levels of the receptor subunits, comparable to what has been observed before for human islets [172], which was used as positive controls (Fig. 1 in *Paper IV*). Successful confirmation of mRNA expression of both receptor subunits in both cell lines was indicative that these cells would be able to respond to IFN-λ stimulation *in vitro*. Of note here is that mRNA expression for the receptor subunits does not automatically suggest functional protein translation. However, since we did observe a response after IFN-λ stimulation of both IEC lines, we concluded that the receptor mRNA expression indeed indicated expression of a functional IFN-λ receptor complex on CaCo-2 and HT-29 cells (Fig. 1 and 2 in *Paper IV*).

Having established this, we continued to study the activation of antiviral defense genes in CaCo-2 and HT-29 cells after IFN-λ stimulation. In part, previous studies have shown that these cells could upregulate transcription factors and ISGs after IFN-λ stimulation [169]. Our results confirmed the previous findings of MxA and OAS-2 upregulation after IFN-λ stimulation [169] (Fig. 2 in *Paper IV*). Our study further expanded this with the findings that CaCo-2 and HT-29 cells also upregulated the expression of PKR and ISG15 after IFN-λ stimulation. However, we could not observe induction of iNOS after IFN-λ stimulation of either cell line. IFN-α was used as a positive control for induction of the respective mRNA.

As previously mentioned, mRNA expression levels are not enough to claim that a protein is upregulated. Hence, using Western blotting, we determined expression of MxA protein levels in both CaCo-2 and HT-29 cells, before and after IFN-λ stimulation (Fig. 3 in *Paper IV*). MxA was upregulated in both cell lines compared to control cells by both IFN-λ1 and IFN-λ2. However only done for one of the studied genes, this indicates that the induced mRNA expression of genes involved in an antiviral state is also translated into proteins. IFN-α stimulation was used as a positive control.

During this study, we observed a marked difference when comparing the induced mRNA expression levels and MxA protein expression after IFN-α and stimulation to that after IFN-λ1 and IFN-λ2 stimulation (Fig. 2 and 3 in *Paper IV*). With the cytokine concentrations and time points we used, the effect on both gene expression and protein translation was consistently more prominent after IFN-α stimulation than after IFN-λ1 and IFN-λ2 stimulation. Such differences have been observed previously [171, 175], and it is also in line with observations from our recent study on IFN-λ and IFN-α

stimulated pancreatic islets [172]. However, conflicting data has also been published, showing a more prominent antiviral effect induced by IFN-λ stimulation [176]. These differences could depend on varying receptor expression on the different cells and tissues studied. Another explanation is that the differences reflect a dose-dependent effect, suggesting a non-comparable effect after stimulating cells with either 1000U/ml IFN-α or 100ng/ml of the respective IFN-λs. Indeed, in *Paper IV*, we did not perform a dose-response curve with higher doses than 100ng/ml of each IFN-λs, as seen in Fig. 5 (P. Larsson, V. Stone et al., unpublished data), hence it is possible that the observed effect would have been more prominent if using higher doses of IFN-λs. However, throughout the studies in *Paper IV* we used 1000U/ml of IFN-α and 100ng/ml IFN-λs, since these concentrations have been used before with a clearly induced effect and minimized cytotoxicity [169, 171, 172, 177, 178].

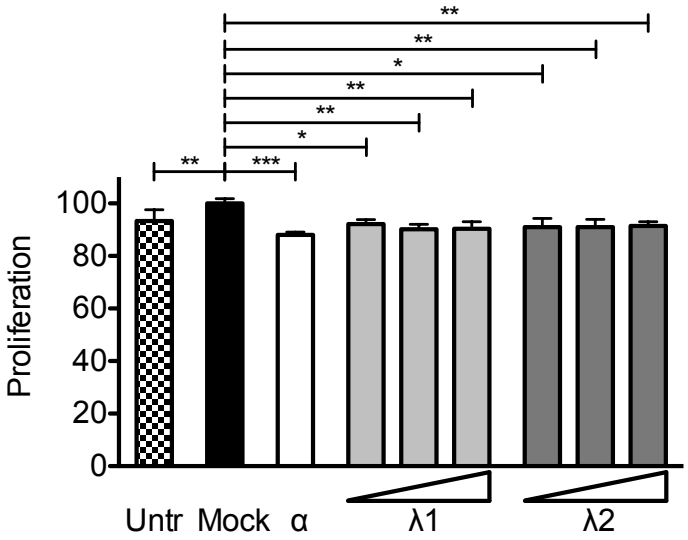

Figure 5. Proliferation in IFN-stimulated HT-29 cells. HT-29 cells were untreated or treated with IFN-buffer, 1000U/ml IFN-α, or 1, 10 or 100ng/ml IFN-λ1 or IFN-λ2 for 24h. Cell proliferation was normalized to that of buffer-treated cells. Data was presented as means ± SD, n=4 for each sample (P. Larsson, V. Stone et al., unpublished data).

Finally, we also tested whether CaCo-2 cells and HT-29 cells express the two main receptors used by CVBs for cellular entry, CAR and DAF, using flow cytometry with HeLa cells as positive controls [71, 72]. Both cell lines expressed CAR and DAF, but CAR was not expressed to the same extent as on HeLa cells (Fig. 2 in *Paper IV*). HeLa cell expression of CAR and DAF was high and stable, 97% ± 3% (n=3) and 98% ± 2% (n=3), respectively. Of the HT-29 cells, 67% ± 2% (n=3) expressed CAR and 99% ± 1% (n=3) expressed DAF. Expression of CAR on CaCo-2 cells was less stable and 57% ± 17% (n=3) were positive for CAR, whereas 98% ± 2% (n=3) expressed DAF. All data was presented as means ± SD. The fact that both tested cell lines were positive for CAR and DAF, suggests that these cells could indeed get infected with CVBs.

However, a potential limitation with our results describing CAR and DAF expression on the cellular surface is that this was only shown by flow cytometry. We cannot say how or where the cells may express CAR and DAF, if stained in confluent cell layers.

It has been suggested previously that CAR is not accessible from the apical side of polarized epithelial cells [76]. This may suggest that CVBs that depend solely on CAR may not be able to infect these cells *in vitro* in polarized cells.

In summary, the fact that CaCo-2 and HT-29 cells respond to IFN-λ signaling by entering an antiviral state and the finding that both cell lines express the receptors used by CVBs, suggests that these cell lines could be used in future experiments to evaluate whether IFN-λ can regulate permissiveness to CVBs in IECs. Furthermore, our findings are also interesting in respect to the suggestion that EVs might cause a persistent infection in the intestinal mucosa of T1D patients [88, 89], since our model suggests a novel way of studying these infections to determine what can regulate IEC permissiveness to CVBs.

8 CONCLUDING REMARKS

Paper I:

- iNKT cells can be activated and manipulated in order to induce suppressive macrophages, which in turn can inhibit diabetes development locally in the pancreas of CVB4-infected diabetes-prone mice.

- Islet-resident macrophages may have a dual role in diabetes development in CVB4-infected diabetes-prone mice, since they can be of an inflammatory phenotype (expressing IL-1β, IL-6 and TNF-α) or suppressive phenotype (expressing IDO1/2).

Paper II:

- The novel prototype CVB1 vaccine induced high titers of neutralizing antibodies in two different mouse strains, even without the use of adjuvants. The vaccine protected mice against CVB1 infection and was safe for use in two different mouse strains, with no adverse side effects observed.

- CVB1-vaccination of diabetes-prone NOD mice did not affect diabetes development, whereas live infection with CVB1 did. Vaccination did neither trigger autoimmunity in the form of IAAs in NOD mice.

- If further developed for human use, this vaccine could be used to test the hypothesis that CVB1 can trigger autoimmunity and T1D development in genetically susceptible individuals.

Paper III:

- Our results showed experimental proof-of-concept for how maternal antibodies can protect genetically susceptible offspring from virus infection and diabetes development in a mouse model for virus-induced diabetes. This may further lend support for using the extended poliovirus hypothesis in order to explain the inverse correlation between T1D incidence and the prevalence of observed EV infections.

- Our results further shows that maternal antibodies indeed can protect offspring from diabetogenic virus infections, which if extrapolated, may suggest beneficial effects of adding an EV vaccine in future vaccination programs for pregnant women.

Paper IV:

- We have here shown that the two human IEC lines CaCo-2 and HT-29 can enter an antiviral state upon IFN-α and IFN-λ treatments.

- CaCo-2 and HT-29 cells express the two main receptors CVBs use to infect cells, CAR and DAF, which suggests that these cell lines can be used to study if IFN-λ can regulate permissiveness to CVB infections in IECs.

9 FUTURE PERSPECTIVES ON DIABETES RESEARCH

T1D is a truly complex disease with both genetic factors and environmental factors involved in regulating susceptibility to disease development. Genetic differences may be studied and found quite easily, by comparing genes and gene expression in T1D cases versus controls. However, studying the environmental factors involved in regulating disease susceptibility is more complicated, since once diabetes is presented in patients, it is very hard to track backwards which environmental factors might have been involved in triggering the disease. Combining both genetic an environmental factors makes it even harder to understand the etiology of T1D; which factors are actually regulating each other, what is the hen and what is the egg?

To come around this problem, several national and international long-term prospective cohort studies have been initiated, for example the DIPP, TEDDY, ENDIA and TRIGR studies [179-182]. These studies have enrolled children from birth depending on different inclusion and exclusion criteria, but the common denominator is that all studies are continuously saving blood, serum and stool samples over time. Some studies also provide questionnaires for the participants in order to save matched information regarding for example infection history and symptoms from the participants. By saving all this, scientists can then lock backwards in a diabetic patient's history of samples, in order to find specific events that may have triggered onset of autoimmunity or T1D. This is a great tool for diabetes researchers and these cohort studies are indeed extraordinary initiatives. Upon final analysis, these studies will be able to provide invaluable sets of data, which hopefully can lead to new discoveries on which environmental factors are involved in T1D development.

I think that the most important thing here is to read the final results from these studies with an open mind. If possible, we should all take one step back from what we are currently working on, away from our old habits and potentially narrow-minded views, and instead try to use these studies to see the whole picture of T1D. It might be that we will find that T1D is a very heterogeneous disease, with several different complex mechanisms that all can regulate diabetes development independently of each other. In any case, providing that we can understand the etiology of T1D and the different pathways involved in regulating development of disease, we may be able to tailor specific preventative therapies for those at risk, based on different genetic and environmental factors for different patients.

I believe that the recent findings of CVB1 to be specifically diabetogenic are important observations, and this may be one factor that can regulate T1D development in certain genetically susceptible individuals. However, since most people who are infected with CVB1 do not develop T1D, we need to further address what other factors that, in combination with CVB1, may regulate susceptibility to T1D. Hopefully, this can be answered by the aforementioned prospective studies.

When reflecting on these paragraphs and ideas, I find it truly strange that many scientists in the diabetes field are so blinded by their own hypotheses that they stubbornly refuse to listen and learn from new findings in other research fields than their own. Examples of this scenario can unfortunately be observed on any diabetes conference you go to. However, if not listening to new findings with an open mind, how can we obtain new knowledge to implement in our own studies to understand T1D etiology?

Another thing that is hampering the scientific community is actually the funding system for science, since people won't share their unique ideas before these are fully published, due to the fact that we are all competing for the same research grants. I do not have any constructive suggestions on how to solve this, since of course grants should go to the scientist who presents the best application with the most promising ideas, but I would like to emphasize that the funding system for science may not be ideal.

Finally, for me having T1D, it is a bit depressing to realize that whatever we do, we will not likely be able to "cure" diabetes. We will hopefully be able to prevent it in the future, but unless we can finally map the etiology of T1D, there is actually very little point in trying to develop transplantation strategies or even growing new β-cells from stem cells. Unless we understand why the β-cells were killed in the first place, they will likely be killed again if we just replace them.

Hence, the near future for T1D patients and T1D treatment most likely lies in technical developments and new smart insulin pumps with integrated continuous glucose sensors, which upon individual programming, resembles an artificial pancreas.

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11 REFERENCES

1. World Health Organization T. The World Health Organization: Diabetes. 2013.
2. American Diabetes A. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2009;32 Suppl 1:S62-67.
3. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. Canadian Medical Association journal. 1922;12:141-146.
4. Daneman D. Type 1 diabetes. Lancet. 2006;367:847-858.
5. Atkinson MA. The pathogenesis and natural history of type 1 diabetes. Cold Spring Harbor perspectives in medicine. 2012;2.
6. Palmer JP, Hampe CS, Chiu H, Goel A, Brooks-Worrell BM. Is latent autoimmune diabetes in adults distinct from type 1 diabetes or just type 1 diabetes at an older age? Diabetes. 2005;54 Suppl 2:S62-67.
7. Leslie RD, Williams R, Pozzilli P. Clinical review: Type 1 diabetes and latent autoimmune diabetes in adults: one end of the rainbow. The Journal of clinical endocrinology and metabolism. 2006;91:1654-1659.
8. Canadien Diabetes Association T. The Canadien Diabetes Association: The history of diabetes. 2012.
9. Sakula A. Paul Langerhans (1847-1888): a centenary tribute. Journal of the Royal Society of Medicine. 1988;81:414-415.
10. von Mering J, Minkowski O. Diabetes mellitus nach Pankreasextirpation. Centralblatt für klinische Medicin (Leipzig). 1889;23:393-394.
11. Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care. 2000;23:1516-1526.
12. Gale EA. The rise of childhood type 1 diabetes in the 20th century. Diabetes. 2002;51:3353-3361.
13. The DIAMOND Project Group. Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999. Diabet Med. 2006;23:857-866.
14. Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. Lancet. 2009;373:2027-2033.
15. Harjutsalo V, Sjoberg L, Tuomilehto J. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. Lancet. 2008;371:1777-1782.
16. Berhan Y, Waernbaum I, Lind T, Mollsten A, Dahlquist G, Swedish Childhood Diabetes Study G. Thirty years of prospective nationwide incidence of childhood type 1 diabetes: the accelerating increase by time tends to level off in Sweden. Diabetes. 2011;60:577-581.
17. Rahier J, Goebbels RM, Henquin JC. Cellular composition of the human diabetic pancreas. Diabetologia. 1983;24:366-371.
18. Suckale J, Solimena M. Pancreas islets in metabolic signaling--focus on the beta-cell. Frontiers in bioscience : a journal and virtual library. 2008;13:7156-7171.
19. Parkin J, Cohen B. An overview of the immune system. Lancet. 2001;357:1777-1789.
20. Turvey SE, Broide DH. Innate immunity. The Journal of allergy and clinical immunology. 2010;125:S24-32.
21. Van Kaer L, Joyce S. Innate immunity: NKT cells in the spotlight. Curr Biol. 2005;15:R429-431.

22. Lehuen A, Lantz O, Beaudoin L, Laloux V, Carnaud C, Bendelac A, Bach JF, Monteiro RC. Overexpression of natural killer T cells protects Valpha14-Jalpha281 transgenic nonobese diabetic mice against diabetes. The Journal of experimental medicine. 1998;188:1831-1839.
23. Novak J, Lehuen A. Mechanism of regulation of autoimmunity by iNKT cells. Cytokine. 2011;53:263-270.
24. Cerundolo V, Silk JD, Masri SH, Salio M. Harnessing invariant NKT cells in vaccination strategies. Nature reviews Immunology. 2009;9:28-38.
25. Diana J, Lehuen A. NKT cells: friend or foe during viral infections? Eur J Immunol. 2009;39:3283-3291.
26. Lehuen A, Diana J, Zaccone P, Cooke A. Immune cell crosstalk in type 1 diabetes. Nature reviews Immunology. 2010;10:501-513.
27. Kindt T, Goldsby R, Osborne B. Kuby Immunology. 6th ed. New York: W.H. Freeman and Company; 2006.
28. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nature reviews Immunology. 2008;8:958-969.
29. Durbin RK, Kotenko SV, Durbin JE. Interferon induction and function at the mucosal surface. Immunological reviews. 2013;255:25-39.
30. Donnelly RP, Kotenko SV. Interferon-lambda: a new addition to an old family. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2010;30:555-564.
31. Stetson DB, Medzhitov R. Type I interferons in host defense. Immunity. 2006;25:373-381.
32. Sadler AJ, Williams BR. Interferon-inducible antiviral effectors. Nature reviews Immunology. 2008;8:559-568.
33. Bonilla FA, Oettgen HC. Adaptive immunity. The Journal of allergy and clinical immunology. 2010;125:S33-40.
34. Dighiero G, Lymberi P, Guilbert B, Ternynck T, Avrameas S. Natural autoantibodies constitute a substantial part of normal circulating immunoglobulins. Ann N Y Acad Sci. 1986;475:135-145.
35. von Herrath M, Sanda S, Herold K. Type 1 diabetes as a relapsing-remitting disease? Nature reviews Immunology. 2007;7:988-994.
36. Badami E, Sorini C, Coccia M, Usuelli V, Molteni L, Bolla AM, Scavini M, Mariani A, King C, Bosi E, Falcone M. Defective differentiation of regulatory FoxP3+ T cells by small-intestinal dendritic cells in patients with type 1 diabetes. Diabetes. 2011;60:2120-2124.
37. Beaudoin L, Laloux V, Novak J, Lucas B, Lehuen A. NKT cells inhibit the onset of diabetes by impairing the development of pathogenic T cells specific for pancreatic beta cells. Immunity. 2002;17:725-736.
38. Fujinami RS, von Herrath MG, Christen U, Whitton JL. Molecular mimicry, bystander activation, or viral persistence: Infections and autoimmune disease. Clin Microbiol Rev. 2006;19:80-+.
39. Selin LK, Wlodarczyk MF, Kraft AR, Nie S, Kenney LL, Puzone R, Celada F. Heterologous immunity: immunopathology, autoimmunity and protection during viral infections. Autoimmunity. 2011;44:328-347.
40. Strachan DP. Hay fever, hygiene, and household size. BMJ. 1989;299:1259-1260.
41. Eisenbarth GS. Type I diabetes mellitus. A chronic autoimmune disease. N Engl J Med. 1986;314:1360-1368.
42. Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, Winkler C, Ilonen J, Veijola R, Knip M, Bonifacio E, Eisenbarth GS. Seroconversion to

multiple islet autoantibodies and risk of progression to diabetes in children. JAMA. 2013;309:2473-2479.
43. Coppieters KT, Dotta F, Amirian N, Campbell PD, Kay TW, Atkinson MA, Roep BO, von Herrath MG. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. The Journal of experimental medicine. 2012;209:51-60.
44. Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE. Fields Virology, 5th ed. In: Straus SE, editor. Philadelphia, PA, USA: Lippincott Williams & Wilkins; 2007.
45. Koshland DE. The seven pillars of life. Science. 2002;295:2215-2216.
46. Breitbart M, Rohwer F. Here a virus, there a virus, everywhere the same virus? Trends in microbiology. 2005;13:278-284.
47. Imagawa A, Hanafusa T, Miyagawa J, Matsuzawa Y. A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. Osaka IDDM Study Group. N Engl J Med. 2000;342:301-307.
48. Imagawa A, Hanafusa T. Pathogenesis of fulminant type 1 diabetes. The review of diabetic studies : RDS. 2006;3:169-177.
49. Todd JA. Etiology of type 1 diabetes. Immunity. 2010;32:457-467.
50. Noble JA, Erlich HA. Genetics of type 1 diabetes. Cold Spring Harbor perspectives in medicine. 2012;2:a007732.
51. Smyth DJ, Cooper JD, Bailey R, Field S, Burren O, Smink LJ, Guja C, Ionescu-Tirgoviste C, Widmer B, Dunger DB, Savage DA, Walker NM, Clayton DG, Todd JA. A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. Nat Genet. 2006;38:617-619.
52. Barnett AH, Eff C, Leslie RD, Pyke DA. Diabetes in identical twins. A study of 200 pairs. Diabetologia. 1981;20:87-93.
53. Redondo MJ, Rewers M, Yu L, Garg S, Pilcher CC, Elliott RB, Eisenbarth GS. Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. BMJ. 1999;318:698-702.
54. Redondo MJ, Jeffrey J, Fain PR, Eisenbarth GS, Orban T. Concordance for islet autoimmunity among monozygotic twins. N Engl J Med. 2008;359:2849-2850.
55. Hermann R, Knip M, Veijola R, Simell O, Laine AP, Akerblom HK, Groop PH, Forsblom C, Pettersson-Fernholm K, Ilonen J, FinnDiane Study G. Temporal changes in the frequencies of HLA genotypes in patients with Type 1 diabetes--indication of an increased environmental pressure? Diabetologia. 2003;46:420-425.
56. Gillespie KM, Bain SC, Barnett AH, Bingley PJ, Christie MR, Gill GV, Gale EA. The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. Lancet. 2004;364:1699-1700.
57. Vaarala O, Paronen J, Otonkoski T, Akerblom HK. Cow milk feeding induces antibodies to insulin in children--a link between cow milk and insulin-dependent diabetes mellitus? Scandinavian journal of immunology. 1998;47:131-135.
58. Vaarala O, Knip M, Paronen J, Hamalainen AM, Muona P, Vaatainen M, Ilonen J, Simell O, Akerblom HK. Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes. Diabetes. 1999;48:1389-1394.

59. Ziegler AG, Schmid S, Huber D, Hummel M, Bonifacio E. Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. JAMA. 2003;290:1721-1728.
60. Norris JM, Barriga K, Klingensmith G, Hoffman M, Eisenbarth GS, Erlich HA, Rewers M. Timing of initial cereal exposure in infancy and risk of islet autoimmunity. JAMA. 2003;290:1713-1720.
61. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, Hu C, Wong FS, Szot GL, Bluestone JA, Gordon JI, Chervonsky AV. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature. 2008;455:1109-1113.
62. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Drew JC, Ilonen J, Knip M, Hyoty H, Veijola R, Simell T, Simell O, Neu J, Wasserfall CH, Schatz D, Atkinson MA, Triplett EW. Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal. 2011;5:82-91.
63. Mejia-Leon ME, Petrosino JF, Ajami NJ, Dominguez-Bello MG, de la Barca AM. Fecal microbiota imbalance in Mexican children with type 1 diabetes. Scientific reports. 2014;4:3814.
64. Gamble DR, Kinsley ML, FitzGerald MG, Bolton R, Taylor KW. Viral antibodies in diabetes mellitus. British medical journal. 1969;3:627-630.
65. Honeyman MC, Coulson BS, Stone NL, Gellert SA, Goldwater PN, Steele CE, Couper JJ, Tait BD, Colman PG, Harrison LC. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. Diabetes. 2000;49:1319-1324.
66. Hyoty H, Taylor KW. The role of viruses in human diabetes. Diabetologia. 2002;45:1353-1361.
67. Hober D, Sauter P. Pathogenesis of type 1 diabetes mellitus: interplay between enterovirus and host. Nat Rev Endocrinol. 2010;6:279-289.
68. Yeung WC, Rawlinson WD, Craig ME. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. BMJ. 2011;342:d35.
69. Ghazarian L, Diana J, Simoni Y, Beaudoin L, Lehuen A. Prevention or acceleration of type 1 diabetes by viruses. Cellular and molecular life sciences : CMLS. 2013;70:239-255.
70. Gale EA. Congenital rubella: citation virus or viral cause of type 1 diabetes? Diabetologia. 2008;51:1559-1566.
71. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science. 1997;275:1320-1323.
72. Bergelson JM, Chan M, Solomon KR, St John NF, Lin H, Finberg RW. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc Natl Acad Sci U S A. 1994;91:6245-6248.
73. Shaw CA, Holland PC, Sinnreich M, Allen C, Sollerbrant K, Karpati G, Nalbantoglu J. Isoform-specific expression of the Coxsackie and adenovirus receptor (CAR) in neuromuscular junction and cardiac intercalated discs. Bmc Cell Biol. 2004;5.
74. Cohen CJ, Shieh JTC, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. Proceedings of the National Academy of Sciences of the United States of America. 2001;98:15191-15196.
75. Coyne CB, Bergelson JM. CAR: a virus receptor within the tight junction. Advanced drug delivery reviews. 2005;57:869-882.

76. Bozym RA, Morosky SA, Kim KS, Cherry S, Coyne CB. Release of intracellular calcium stores facilitates coxsackievirus entry into polarized endothelial cells. PLoS pathogens. 2010;6:e1001135.
77. Lind K, Huhn MH, Flodstrom-Tullberg M. Immunology in the clinic review series; focus on type 1 diabetes and viruses: the innate immune response to enteroviruses and its possible role in regulating type 1 diabetes. Clin Exp Immunol. 2012;168:30-38.
78. Dotzauer A, Kraemer L. Innate and adaptive immune responses against picornaviruses and their counteractions: An overview. World journal of virology. 2012;1:91-107.
79. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med. 1979;300:1173-1179.
80. Hyoty H, Hiltunen M, Knip M, Laakkonen M, Vahasalo P, Karjalainen J, Koskela P, Roivainen M, Leinikki P, Hovi T, et al. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. Diabetes. 1995;44:652-657.
81. Lonnrot M, Korpela K, Knip M, Ilonen J, Simell O, Korhonen S, Savola K, Muona P, Simell T, Koskela P, Hyoty H. Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study. Diabetes. 2000;49:1314-1318.
82. Sarmiento L, Galvan JA, Cabrera-Rode E, Aira L, Correa C, Sariego S, Fonseca M, Cubas-Duenas I, Hung LH, Resik S, Cilio CM. Type 1 diabetes associated and tissue transglutaminase autoantibodies in patients without type 1 diabetes and coeliac disease with confirmed viral infections. J Med Virol. 2012;84:1049-1053.
83. Laitinen OH, Honkanen H, Pakkanen O, Oikarinen S, Hankaniemi MM, Huhtala H, Ruokoranta T, Lecouturier V, Andre P, Harju R, Virtanen SM, Lehtonen J, Almond JW, Simell T, Simell O, Ilonen J, Veijola R, Knip M, Hyoty H. Coxsackievirus B1 Is Associated With Induction of beta-Cell Autoimmunity That Portends Type 1 Diabetes. Diabetes. 2014;63:446-455.
84. Ylipaasto P, Klingel K, Lindberg AM, Otonkoski T, Kandolf R, Hovi T, Roivainen M. Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells. Diabetologia. 2004;47:225-239.
85. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, Mosca F, Boggi U, Muda AO, Prato SD, Elliott JF, Covacci A, Rappuoli R, Roep BO, Marchetti P. 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;104:5115-5120.
86. Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. Diabetologia. 2009;52:1143-1151.
87. Krogvold L, Frisk G, Hyoty H, Oikarinen S, Oikarinen M, Edwin B, Buanes T, Hanssen KF, Dahl-Jørgensen K. Enterovirus in live pancreatic islets at the diagnosis of human type 1 diabetes (T1D). ADA poster. 2013;1689-P.
88. Oikarinen M, Tauriainen S, Honkanen T, Oikarinen S, Vuori K, Kaukinen K, Rantala I, Maki M, Hyoty H. Detection of enteroviruses in the intestine of type 1 diabetic patients. Clin Exp Immunol. 2008;151:71-75.

89. Oikarinen M, Tauriainen S, Oikarinen S, Honkanen T, Collin P, Rantala I, Maki M, Kaukinen K, Hyoty H. Type 1 diabetes is associated with enterovirus infection in gut mucosa. Diabetes. 2012;61:687-691.
90. Stene LC, Oikarinen S, Hyoty H, Barriga KJ, Norris JM, Klingensmith G, Hutton JC, Erlich HA, Eisenbarth GS, Rewers M. Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). Diabetes. 2010;59:3174-3180.
91. Coleman TJ, Gamble DR, Taylor KW. Diabetes in mice after Coxsackie B 4 virus infection. British medical journal. 1973;3:25-27.
92. Hultcrantz M, Huhn MH, Wolf M, Olsson A, Jacobson S, Williams BR, Korsgren O, Flodstrom-Tullberg M. Interferons induce an antiviral state in human pancreatic islet cells. Virology. 2007;367:92-101.
93. Oikarinen S, Tauriainen S, Hober D, Lucas B, Vazeou A, Sioofy Khojine A, Bozas E, Muir P, Honkanen H, Ilonen J, Knip M, Keskinen P, Saha M-T, Huhtala H, Stanway G, Bartsocas CS, Ludvigsson J, Taylor KW, Hyoty H, group TVs. Virus antibody survey in different European populations indicates risk association between Coxsackievirus B1 and type 1 diabetes. Diabetes. 2014;63:655-662.
94. Viskari H, Ludvigsson J, Uibo R, Salur L, Marciulionyte D, Hermann R, Soltesz G, Fuchtenbusch M, Ziegler AG, Kondrashova A, Romanov A, Knip M, Hyoty H. Relationship between the incidence of type 1 diabetes and enterovirus infections in different European populations: results from the EPIVIR project. J Med Virol. 2004;72:610-617.
95. Viskari H, Ludvigsson J, Uibo R, Salur L, Marciulionyte D, Hermann R, Soltesz G, Fuchtenbusch M, Ziegler AG, Kondrashova A, Romanov A, Kaplan B, Laron Z, Koskela P, Vesikari T, Huhtala H, Knip M, Hyoty H. Relationship between the incidence of type 1 diabetes and maternal enterovirus antibodies: time trends and geographical variation. Diabetologia. 2005;48:1280-1287.
96. Nathanson N, Martin JR. The epidemiology of poliomyelitis: enigmas surrounding its appearance, epidemicity, and disappearance. Am J Epidemiol. 1979;110:672-692.
97. Viskari HR, Koskela P, Lonnrot M, Luonuansuu S, Reunanen A, Baer M, Hyoty H. Can enterovirus infections explain the increasing incidence of type 1 diabetes? Diabetes Care. 2000;23:414-416.
98. Gamble DR. The epidemiology of insulin dependent diabetes with particular reference to the relationship of virus infection to its etiology. Epidemiologic reviews. 1980;2:49-70.
99. Zinkernagel RM. Maternal antibodies, childhood infections, and autoimmune diseases. N Engl J Med. 2001;345:1331-1335.
100. Zinkernagel RM. On natural and artificial vaccinations. Annu Rev Immunol. 2003;21:515-546.
101. Larsson PG, Lakshmikanth T, Svedin E, King C, Flodström-Tullberg M. Previous maternal infection protects offspring from enterovirus infection and prevents experimental diabetes development in mice. Diabetologia. 2013;56:867-874.
102. Navarini AA, Krzyzowska M, Lang KS, Horvath E, Hengartner H, Niemialtowski MG, Zinkernagel RM. Long-lasting immunity by early infection of maternal-antibody-protected infants. Eur J Immunol. 2010;40:113-116.
103. Sadeharju K, Knip M, Virtanen SM, Savilahti E, Tauriainen S, Koskela P, Akerblom HK, Hyoty H. Maternal antibodies in breast milk protect the child from enterovirus infections. Pediatrics. 2007;119:941-946.

104. Oldstone MB. Virus-induced autoimmunity: molecular mimicry as a route to autoimmune disease. Journal of autoimmunity. 1989;2 Suppl:187-194.
105. Kaufman DL, Erlander MG, Clare-Salzler M, Atkinson MA, Maclaren NK, Tobin AJ. Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. The Journal of clinical investigation. 1992;89:283-292.
106. Tian J, Lehmann PV, Kaufman DL. T cell cross-reactivity between coxsackievirus and glutamate decarboxylase is associated with a murine diabetes susceptibility allele. The Journal of experimental medicine. 1994;180:1979-1984.
107. Vreugdenhil GR, Geluk A, Ottenhoff THM, Melchers WJG, Roep BO, Galama JMD. Molecular mimicry in diabetes mellitus: the homologous domain in coxsackie B virus protein 2C and islet autoantigen GAD(65) is highly conserved in the coxsackie B-like enteroviruses and binds to the diabetes associated HLA-DR3 molecule. Diabetologia. 1998;41:40-46.
108. Hou J, Said C, Franchi D, Dockstader P, Chatterjee NK. Antibodies to glutamic acid decarboxylase and P2-C peptides in sera from coxsackie virus B4-infected mice and IDDM patients. Diabetes. 1994;43:1260-1266.
109. Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK. Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. The Journal of clinical investigation. 1994;94:2125-2129.
110. Flodstrom M, Maday A, Balakrishna D, Cleary MM, Yoshimura A, Sarvetnick N. Target cell defense prevents the development of diabetes after viral infection. Nat Immunol. 2002;3:373-382.
111. Flodstrom M, Tsai D, Fine C, Maday A, Sarvetnick N. Diabetogenic potential of human pathogens uncovered in experimentally permissive beta-cells. Diabetes. 2003;52:2025-2034.
112. Ghazarian L, Diana J, Beaudoin L, Larsson PG, Puri RK, Van Rooijen N, Flodstrom-Tullberg M, Lehuen A. Protection against type 1 diabetes upon Coxsackievirus B4 infection and iNKT cell stimulation: role of suppressive macrophages. Diabetes. 2013.
113. Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CJ, Atkinson MA. Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. Diabetes. 2000;49:708-711.
114. Tracy S, Drescher KM, Chapman NM, Kim KS, Carson SD, Pirruccello S, Lane PH, Romero JR, Leser JS. Toward testing the hypothesis that group B coxsackieviruses (CVB) trigger insulin-dependent diabetes: inoculating nonobese diabetic mice with CVB markedly lowers diabetes incidence. J Virol. 2002;76:12097-12111.
115. Riedel S. Edward Jenner and the history of smallpox and vaccination. Proceedings. 2005;18:21-25.
116. Cann AJ, Stanway G, Hughes PJ, Minor PD, Evans DM, Schild GC, Almond JW. Reversion to neurovirulence of the live-attenuated Sabin type 3 oral poliovirus vaccine. Nucleic acids research. 1984;12:7787-7792.
117. Wakefield AJ, Murch SH, Anthony A, Linnell J, Casson DM, Malik M, Berelowitz M, Dhillon AP, Thomson MA, Harvey P, Valentine A, Davies SE, Walker-Smith JA. Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children. Lancet. 1998;351:637-641.
118. Murch SH, Anthony A, Casson DH, Malik M, Berelowitz M, Dhillon AP, Thomson MA, Valentine A, Davies SE, Walker-Smith JA. Retraction of an interpretation. Lancet. 2004;363:750.

119. Lancet TEoT. Retraction--Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children. Lancet. 2010;375:445.
120. Partinen M, Saarenpaa-Heikkila O, Ilveskoski I, Hublin C, Linna M, Olsen P, Nokelainen P, Alen R, Wallden T, Espo M, Rusanen H, Olme J, Satila H, Arikka H, Kaipainen P, Julkunen I, Kirjavainen T. Increased incidence and clinical picture of childhood narcolepsy following the 2009 H1N1 pandemic vaccination campaign in Finland. PloS one. 2012;7:e33723.
121. Poli F, Overeem S, Lammers GJ, Plazzi G, Lecendreux M, Bassetti CL, Dauvilliers Y, Keene D, Khatami R, Li Y, Mayer G, Nohynek H, Pahud B, Paiva T, Partinen M, Scammell TE, Shimabukuro T, Sturkenboom M, van Dinther K, Wiznitzer M, Bonhoeffer J. Narcolepsy as an adverse event following immunization: case definition and guidelines for data collection, analysis and presentation. Vaccine. 2013;31:994-1007.
122. Läkemedelsverket. Registerstudie med fokus på neurologiska och immunrelaterade sjukdomar efter vaccination med Pandemrix. Läkemedelsverket2013. p. 1-15.
123. Mahlios J, De la Herran-Arita AK, Mignot E. The autoimmune basis of narcolepsy. Current opinion in neurobiology. 2013;23:767-773.
124. Melen K, Partinen M, Tynell J, Sillanpaa M, Himanen SL, Saarenpaa-Heikkila O, Hublin C, Olsen P, Ilonen J, Nohynek H, Syrjanen R, Kilpi T, Vuorela A, Kirjavainen T, Vaarala O, Julkunen I. No serological evidence of influenza A H1N1pdm09 virus infection as a contributing factor in childhood narcolepsy after Pandemrix vaccination campaign in Finland. PloS one. 2013;8:e68402.
125. Han F, Lin L, Warby SC, Faraco J, Li J, Dong SX, An P, Zhao L, Wang LH, Li QY, Yan H, Gao ZC, Yuan Y, Strohl KP, Mignot E. Narcolepsy onset is seasonal and increased following the 2009 H1N1 pandemic in China. Annals of neurology. 2011;70:410-417.
126. De la Herran-Arita AK, Kornum BR, Mahlios J, Jiang W, Lin L, Hou T, Macaubas C, Einen M, Plazzi G, Crowe C, Newell EW, Davis MM, Mellins ED, Mignot E. CD4+ T Cell Autoimmunity to Hypocretin/Orexin and Cross-Reactivity to a 2009 H1N1 Influenza A Epitope in Narcolepsy. Science translational medicine. 2013;5:216ra176.
127. Vårdguiden. Vaccinationsprogram för barn. 2013.
128. Eccles R. Understanding the symptoms of the common cold and influenza. The Lancet infectious diseases. 2005;5:718-725.
129. PrabhuDas M, Adkins B, Gans H, King C, Levy O, Ramilo O, Siegrist CA. Challenges in infant immunity: implications for responses to infection and vaccines. Nat Immunol. 2011;12:189-194.
130. CDC. Vaccines and Preventable Diseases: Tdap for Pregnant Women: Information for Providers. 2013.
131. (NHS) NHS. Whooping cough vaccination in pregnancy. 2012.
132. Salur L, Oikarinen S, Tauriainen S, Mandel M, Hyoty H, Uibo R. Enterovirus infections in young infants: are children still protected by maternal antibodies? Human vaccines. 2011;7:966-971.
133. Sadauskaite-Kuehne V, Ludvigsson J, Padaiga Z, Jasinskiene E, Samuelsson U. Longer breastfeeding is an independent protective factor against development of type 1 diabetes mellitus in childhood. Diabetes Metab Res Rev. 2004;20:150-157.
134. Sonesson SE, Salomonsson S, Jacobsson LA, Bremme K, Wahren-Herlenius M. Signs of first-degree heart block occur in one-third of fetuses of pregnant

women with anti-SSA/Ro 52-kd antibodies. Arthritis and rheumatism. 2004;50:1253-1261.
135. Salomonsson S, Sonesson SE, Ottosson L, Muhallab S, Olsson T, Sunnerhagen M, Kuchroo VK, Thoren P, Herlenius E, Wahren-Herlenius M. Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis, and mediate congenital heart block. The Journal of experimental medicine. 2005;201:11-17.
136. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. The American Journal of Hygiene. 1938;27:493-497.
137. Furesz J. Developments in the production and quality control of poliovirus vaccines - historical perspectives. Biologicals : journal of the International Association of Biological Standardization. 2006;34:87-90.
138. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese, diabetic strain of mice. Jikken dobutsu Experimental animals. 1980;29:1-13.
139. Thebault-Baumont K, Dubois-Laforgue D, Krief P, Briand JP, Halbout P, Vallon-Geoffroy K, Morin J, Laloux V, Lehuen A, Carel JC, Jami J, Muller S, Boitard C. Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice. The Journal of clinical investigation. 2003;111:851-857.
140. Katz JD, Wang B, Haskins K, Benoist C, Mathis D. Following a diabetogenic T cell from genesis through pathogenesis. Cell. 1993;74:1089-1100.
141. Flodstrom-Tullberg M, Yadav D, Hagerkvist R, Tsai D, Secrest P, Stotland A, Sarvetnick N. Target cell expression of suppressor of cytokine signaling-1 prevents diabetes in the NOD mouse. Diabetes. 2003;52:2696-2700.
142. Hultcrantz M, Jacobson S, Hill NJ, Santamaria P, Flodstrom-Tullberg M. The target cell response to cytokines governs the autoreactive T cell repertoire in the pancreas of NOD mice. Diabetologia. 2009;52:299-305.
143. Hohenadl C, Klingel K, Mertsching J, Hofschneider PH, Kandolf R. Strand-specific detection of enteroviral RNA in myocardial tissue by in situ hybridization. Molecular and cellular probes. 1991;5:11-20.
144. Flodstrom-Tullberg M, Hultcrantz M, Stotland A, Maday A, Tsai D, Fine C, Williams B, Silverman R, Sarvetnick N. RNase L and double-stranded RNA-dependent protein kinase exert complementary roles in islet cell defense during coxsackievirus infection. J Immunol. 2005;174:1171-1177.
145. Knip M, Virtanen SM, Seppa K, Ilonen J, Savilahti E, Vaarala O, Reunanen A, Teramo K, Hamalainen AM, Paronen J, Dosch HM, Hakulinen T, Akerblom HK. Dietary intervention in infancy and later signs of beta-cell autoimmunity. N Engl J Med. 2010;363:1900-1908.
146. Gamble DR, Taylor KW, Cumming H. Coxsackie viruses and diabetes mellitus. British medical journal. 1973;4:260-262.
147. Diana J, Griseri T, Lagaye S, Beaudoin L, Autrusseau E, Gautron AS, Tomkiewicz C, Herbelin A, Barouki R, von Herrath M, Dalod M, Lehuen A. NKT cell-plasmacytoid dendritic cell cooperation via OX40 controls viral infection in a tissue-specific manner. Immunity. 2009;30:289-299.
148. Diana J, Brezar V, Beaudoin L, Dalod M, Mellor A, Tafuri A, von Herrath M, Boitard C, Mallone R, Lehuen A. Viral infection prevents diabetes by inducing regulatory T cells through NKT cell-plasmacytoid dendritic cell interplay. The Journal of experimental medicine. 2011;208:729-745.
149. Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, Kronenberg M, Koezuka Y, Delovitch TL, Gombert JM, Leite-De-Moraes M, Gouarin C, Zhu R, Hameg A, Nakayama T, Taniguchi M, Lepault F, Lehuen A, Bach JF, Herbelin A. Activation of natural killer T cells by alpha-galactosylceramide

treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nature medicine. 2001;7:1057-1062.
150. Simoni Y, Diana J, Ghazarian L, Beaudoin L, Lehuen A. Therapeutic manipulation of natural killer (NK) T cells in autoimmunity: are we close to reality? Clin Exp Immunol. 2013;171:8-19.
151. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, 3rd, Zhou D, Saint-Mezard P, Wang V, Gao Y, Yin N, Hoebe K, Schneewind O, Walker D, Beutler B, Teyton L, Savage PB, Bendelac A. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature. 2005;434:525-529.
152. Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. Nature reviews Microbiology. 2007;5:405-417.
153. Parsa R, Andresen P, Gillett A, Mia S, Zhang XM, Mayans S, Holmberg D, Harris RA. Adoptive transfer of immunomodulatory M2 macrophages prevents type 1 diabetes in NOD mice. Diabetes. 2012;61:2881-2892.
154. Geissmann F, Auffray C, Palframan R, Wirrig C, Ciocca A, Campisi L, Narni-Mancinelli E, Lauvau G. Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunology and cell biology. 2008;86:398-408.
155. Eizirik DL, Mandrup-Poulsen T. A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis. Diabetologia. 2001;44:2115-2133.
156. Uno S, Imagawa A, Okita K, Sayama K, Moriwaki M, Iwahashi H, Yamagata K, Tamura S, Matsuzawa Y, Hanafusa T, Miyagawa J, Shimomura I. Macrophages and dendritic cells infiltrating islets with or without beta cells produce tumour necrosis factor-alpha in patients with recent-onset type 1 diabetes. Diabetologia. 2007;50:596-601.
157. Cantor J, Haskins K. Recruitment and activation of macrophages by pathogenic CD4 T cells in type 1 diabetes: evidence for involvement of CCR8 and CCL1. J Immunol. 2007;179:5760-5767.
158. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nature medicine. 1998;4:781-785.
159. Bek EJ, Hussain KM, Phuektes P, Kok CC, Gao Q, Cai F, Gao Z, McMinn PC. Formalin-inactivated vaccine provokes cross-protective immunity in a mouse model of human enterovirus 71 infection. Vaccine. 2011;29:4829-4838.
160. Kolpe AB, Kiener TK, Grotenbreg GM, Kwang J. Display of enterovirus 71 VP1 on baculovirus as a type II transmembrane protein elicits protective B and T cell responses in immunized mice. Virus research. 2012;168:64-72.
161. Liu Q, Yan K, Feng Y, Huang X, Ku Z, Cai Y, Liu F, Shi J, Huang Z. A virus-like particle vaccine for coxsackievirus A16 potently elicits neutralizing antibodies that protect mice against lethal challenge. Vaccine. 2012;30:6642-6648.
162. Cai Y, Liu Q, Huang X, Li D, Ku Z, Zhang Y, Huang Z. Active immunization with a Coxsackievirus A16 experimental inactivated vaccine induces neutralizing antibodies and protects mice against lethal infection. Vaccine. 2013;31:2215-2221.
163. Huhn MH, McCartney SA, Lind K, Svedin E, Colonna M, Flodstrom-Tullberg M. Melanoma differentiation-associated protein-5 (MDA-5) limits early viral replication but is not essential for the induction of type 1 interferons after Coxsackievirus infection. Virology. 2010;401:42-48.

164. Drescher KM, Kono K, Bopegamage S, Carson SD, Tracy S. Coxsackievirus B3 infection and type 1 diabetes development in NOD mice: insulitis determines susceptibility of pancreatic islets to virus infection. Virology. 2004;329:381-394.
165. Zhang L, Nakayama M, Eisenbarth GS. Insulin as an autoantigen in NOD/human diabetes. Current opinion in immunology. 2008;20:111-118.
166. Davydova B, Harkonen T, Kaialainen S, Hovi T, Vaarala O, Roivainen M. Coxsackievirus immunization delays onset of diabetes in non-obese diabetic mice. J Med Virol. 2003;69:510-520.
167. Tirabassi RS, Guberski DL, Blankenhorn EP, Leif JH, Woda BA, Liu Z, Winans D, Greiner DL, Mordes JP. Infection with viruses from several families triggers autoimmune diabetes in LEW*1WR1 rats: prevention of diabetes by maternal immunization. Diabetes. 2010;59:110-118.
168. Sommereyns C, Paul S, Staeheli P, Michiels T. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS pathogens. 2008;4:e1000017.
169. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diebold J, Diepolder H, Adler B, Auernhammer CJ, Goke B, Dambacher J. IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression. American journal of physiology Gastrointestinal and liver physiology. 2005;289:G960-968.
170. Pott J, Mahlakoiv T, Mordstein M, Duerr CU, Michiels T, Stockinger S, Staeheli P, Hornef MW. IFN-lambda determines the intestinal epithelial antiviral host defense. Proc Natl Acad Sci U S A. 2011;108:7944-7949.
171. Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P, Chan C, Birks C, Foster D, Clegg CH, Wietzke-Braun P, Mihm S, Klucher KM. Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology. 2006;44:896-906.
172. Lind K, Richardson SJ, Leete P, Morgan NG, Korsgren O, Flodstrom-Tullberg M. Induction of an antiviral state and attenuated coxsackievirus replication in type III interferon-treated primary human pancreatic islets. J Virol. 2013;87:7646-7654.
173. Chieux V, Chehadeh W, Harvey J, Haller O, Wattre P, Hober D. Inhibition of coxsackievirus B4 replication in stably transfected cells expressing human MxA protein. Virology. 2001;283:84-92.
174. Flodstrom M, Horwitz MS, Maday A, Balakrishna D, Rodriguez E, Sarvetnick N. A critical role for inducible nitric oxide synthase in host survival following coxsackievirus B4 infection. Virology. 2001;281:205-215.
175. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol. 2003;4:69-77.
176. Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, Paludan SR. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol. 2006;80:4501-4509.
177. Hou W, Wang X, Ye L, Zhou L, Yang ZQ, Riedel E, Ho WZ. Lambda interferon inhibits human immunodeficiency virus type 1 infection of macrophages. J Virol. 2009;83:3834-3842.

178. Tian RR, Guo HX, Wei JF, Yang CK, He SH, Wang JH. IFN-lambda inhibits HIV-1 integration and post-transcriptional events in vitro, but there is only limited in vivo repression of viral production. Antiviral research. 2012;95:57-65.
179. Knip M, Simell O. Environmental triggers of type 1 diabetes. Cold Spring Harbor perspectives in medicine. 2012;2:a007690.
180. Hagopian WA, Erlich H, Lernmark A, Rewers M, Ziegler AG, Simell O, Akolkar B, Vogt R, Jr., Blair A, Ilonen J, Krischer J, She J, Group TS. The Environmental Determinants of Diabetes in the Young (TEDDY): genetic criteria and international diabetes risk screening of 421 000 infants. Pediatric diabetes. 2011;12:733-743.
181. Penno MA, Couper JJ, Craig ME, Colman PG, Rawlinson WD, Cotterill AM, Jones TW, Harrison LC. Environmental determinants of islet autoimmunity (ENDIA): a pregnancy to early life cohort study in children at-risk of type 1 diabetes. BMC pediatrics. 2013;13:124.
182. Group TS, Akerblom HK, Krischer J, Virtanen SM, Berseth C, Becker D, Dupre J, Ilonen J, Trucco M, Savilahti E, Koski K, Pajakkala E, Fransiscus M, Lough G, Bradley B, Koski M, Knip M. The Trial to Reduce IDDM in the Genetically at Risk (TRIGR) study: recruitment, intervention and follow-up. Diabetologia. 2011;54:627-633.