Inflammatory Mediators and Enterovirus Infections in Human Islets of Langerhans

ANNIKA MOËLL
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoiet, Dag Hammarskjöldsväg 20, Uppsala, Saturday, April 5, 2008 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Type 1 diabetes (T1D) is due to a selective loss of the insulin producing β-cells. However, the process responsible for this loss is still unknown. There is accumulating evidence that enteroviruses (EVs) are involved in T1D. In addition to direct virus-induced cytolysis, EVs could facilitate β-cell destruction by inducing inflammatory cytokines. Induction of such genes has previously been shown in EV-infected islets in vitro. Modulation of inflammatory mediators expressed in the islets could be a possible strategy to reduce β-cell destruction.

In the first paper we screened uninfected isolated human islets for genes with the potential to induce or modulate an immune response. We found that several of the genes expressed in the islets encode proteins with a powerful biological activity, such as IL-1β, IL-8, MIP-2α, MCP-1 and MIF. This indicates that the islets themselves can express several triggers of inflammation, and if expressed in vivo these mediators would probably contribute to β-cell destruction.

The vitamin B3 derivate, nicotinamide (NA), has been shown to modulate expression of factors important for coagulation and inflammatory responses. Addition of NA into isolated islet cultures resulted in a reduced expression of the pro-inflammatory chemokine MCP-1 and the coagulation activator tissue factor, suggesting that NA may have implications for both inflammatory responses and the pro-coagulant activity of islets.

We successfully isolated EVs from three newly diagnosed T1D patients. All isolates showed tropism for human islets and β-cells in vitro and clearly affected islet function. We also found that EV infection induced islet secretion of the chemokines IP-10 and MCP-1 and that this induction could be blocked or reduced by addition of NA to the culture medium. Interestingly, NA also reduced viral replication and virus-induced islet destruction.

To conclude, this thesis provides new information about expression and modulation of inflammatory mediators in infected and uninfected human islets that could trigger inflammatory reactions leading to β-cell destruction. Moreover, it further strengthens the causal relationship between EV and T1D.

Keywords: diabetes, islets of Langerhans, enterovirus, inflammatory mediators, cytokines, nicotinamide

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ISSN 1651-6206
urn:nbn:se:uu:diva-8501 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-8501)
To my family ♥
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### Abbreviations

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<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackievirus and adenovirus receptor</td>
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<td>CBV</td>
<td>Coxsackie B virus</td>
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<td>CPE</td>
<td>Cytopathic effect</td>
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<td>DAF</td>
<td>Decay-accelerating factor</td>
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<td>ds RNA</td>
<td>Double stranded RNA</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>EV</td>
<td>Enterovirus</td>
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<td>GAD65</td>
<td>Glutamic acid decarboxylase 65</td>
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<td>GMK</td>
<td>Green monkey kidney</td>
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<tr>
<td>HeLa</td>
<td>Henrietta Lacks cells</td>
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<td>IA-2</td>
<td>Islet cell antigen 2/tyrosine phosphatase</td>
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<tr>
<td>IBMIR</td>
<td>Instant blood-mediated inflammatory reaction</td>
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<td>ICA</td>
<td>Islet cell antigens</td>
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<td>ICAM-1</td>
<td>Intracellular adhesion molecule 1</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IFN</td>
<td>Interferons</td>
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<td>IL</td>
<td>Interleukins</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>IP-10</td>
<td>Interferon inducible peptide-10</td>
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<td>IRF</td>
<td>Interferon regulatory factors</td>
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<td>MCP-1</td>
<td>Monocyte chemotactic factor-1</td>
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<td>MHC</td>
<td>Major histocompatibility factor</td>
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<td>MIF</td>
<td>Migration inhibitory factor</td>
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<td>MIP-2α</td>
<td>Macrophage inflammatory protein 2α</td>
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<td>NA</td>
<td>Nicotinamide</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor KB</td>
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<td>NO</td>
<td>Nitric oxide</td>
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<td>NOD</td>
<td>Non obese diabetic</td>
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<tr>
<td>2´5´OAS</td>
<td>2´5´oligoadenylate synthetase</td>
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<tr>
<td>PARP-1</td>
<td>Poly ADP-ribose polymerase 1</td>
</tr>
<tr>
<td>PKR</td>
<td>dsRNA-dependent protein kinase</td>
</tr>
<tr>
<td>RD</td>
<td>Rhabdo myosarcoma</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TAT</td>
<td>Thrombin-antithrombin complex</td>
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<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Tissue culture infectious dose 50</td>
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<td>TF</td>
<td>Tissue factor</td>
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<td>T1D</td>
<td>Type 1 diabetes</td>
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<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Introduction

The insulin producing β-cells are located in the islets of Langerhans, which are small clusters of endocrine cells embedded throughout the exocrine tissue in the pancreas. Insulin is crucial for the regulation of the glucose metabolism and must be present for glucose, the main “fuel” of the body, to enter the cells.

In type 1 diabetes (T1D) the body has lost its ability to produce insulin as a result of β-cell destruction. With an increasing incidence rate worldwide, T1D is a major health care problem. Still, the trigger of this disease remains unknown and insights into the etiology of the disease are mandatory if we are to develop strategies to prevent the deleterious outcomes of this disease.

This thesis summarizes studies of inflammatory mediators and EV infections in the human islets of Langerhans - possible triggers of β-cell destruction.
Background

Type 1 diabetes

Diabetes has been recognized since antiquity but the pathogenesis of diabetes has only been understood experimentally since the beginning of the 1900 [1]. Today it is known that the lack of insulin in individuals with T1D is due to a selective loss of the insulin producing β-cells. However, the process responsible for this loss is still unknown.

Clinical features and epidemiology

T1D, also known as insulin-dependent diabetes mellitus or juvenile diabetes, usually appears in childhood or adolescence although disease onset can appear at any age [2]. The classical clinical symptoms such as thirst, weight loss, and polyuria (secondary to hyperglycemia) develop acutely and it has been commonly stated that 60-80% of the β-cell mass has been lost by the time the symptoms appear [2]. However, a recent study found that the percentage reduction in β-cell mass was highly correlated with the age at onset, with the greatest reduction in β-cell mass in the youngest patients. On average, a 40% reduction in β-cell mass was sufficient to precipitate clinical symptoms at 20 years of age [3]. More than 70% of newly diagnosed T1D patients have one or more autoantibodies directed against islet cell antigens (ICA), insulin, glutamic acid decarboxylase (GAD65) or tyrosine phosphatase (IA-2) [2]. Autoantibodies are not believed to be directly involved in the pathogenesis but are considered to be markers of ongoing β-cell destruction and are therefore used to identify subjects at high risk of T1D. The autoantibodies can often be detected months or years before diagnosis, suggesting that β-cell destruction takes place over a longer period of time. In contrast to this slow type of T1D, accumulating evidence has indicated the presence of very rapid form of T1D, reviewed in [4]. The duration of onset of this type is considered to be only a few days.

The incidence of T1D has increased worldwide over the last few decades [5, 6] and is predicted to affect 6 millions by the year 2010 [7]. There is a great variation in the prevalence and incidence of T1D between countries and different ethnic groups. Finland, followed by Sardinia and the other
Nordic countries, has the highest reported incidence with ~50/100 000/year. The lowest incidence rate is seen in Asia with 0.5-1.3/100 000/year (reviewed in [8]. A seasonal incidence has been noted in all parts of the world, with an increased disease onset during the colder seasons suggesting an infectious etiology [9].

Pathology
The characteristic histological findings of the pancreas from T1D patients are the near total lack of insulin producing β-cells while the other endocrine cells (α, δ and PP cells) are preserved [10]. Inflammatory infiltrates of different immune cells (insulitis) are sometimes seen in the early stages of the disease [11]. The distribution of islets with insulitis in the human pancreas can be very uneven, with some pancreatic lobules showing normal histology and others being totally infiltrated [12].

Immune-mediated destruction of β-cells
Due to the presence of immune cells in the islets and islet cell specific autoantibodies in the blood of T1D patients, T1D is believed to be an autoimmune disease.

Lymphocytes and macrophages
CD8+ lymphocytes are the predominant type of lymphocytes in the islets of newly diagnosed T1D patients, but also CD4+, B-cells, NK cells and macrophages can be found [9].

The role of B-cells and autoantibodies in T1D is not fully understood. Although B-cells could play a part in T1D as antigen presenting cells, T1D has been reported in a patient suffering from B-cell deficiency suggesting that neither autoantibodies nor B-cells are critically involved in the pathogenesis of the disease [13].

NK cells can lyze islets in vitro [14] but the function of NK cells in human T1D is poorly studied. Infiltration of NK cells together with T-cells, macrophages, dendritic cells have been demonstrated in pancreas biopsies from enterovirus-positive T1D patients and recent onset T1D patients[15, 16].

Infiltrating macrophages and dendritic cells have also been found in islets from newly diagnosed T1D patients [16, 17]. Macrophages are believed to participate in the process of β-cell destruction as antigen presenting cells, by producing cytokines or by phagocytosis of damaged β-cells [17].

Cytokines and chemokines
Pro-inflammatory cytokines and chemokines secreted from infiltrating immune cells might also trigger β-cell destruction. The cytokines interleukin 1-
β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) can be produced by activated T-cells and macrophages and have been shown to induce rodent and human β-cell death mostly by apoptosis (reviewed in [18]). Secretion of cytokines by the infiltrating cells may induce expression of nitric oxide (NO) in the islets, which itself is destructive to the islets (reviewed in [19]). In addition to directly inducing β-cell death, exposure to cytokines may induce induction of Fas expression, rendering the β-cells susceptible to lysis by T-cells [18].

Chemokines are small cytokines involved in the inflammation and migration of leukocytes. They are predominantly expressed by macrophages or endothelial cells but also by CD8+ cells, and are induced by cytokines such as TNF-α and IFN-γ [20]. The secretion of chemokines can modulate the composition of the infiltrating immune cells [21]. For example, the chemokine receptor CXCR3 is predominantly found on activated Th1 type T-cells and secretion of the ligand, interferon inducible protein 10 (IP-10/CXCL10) [22], may direct the immune response towards a more aggressive Th1 type T-cell dominated response [20].

Direct damage to β-cells

10-20% of newly diagnosed T1D patients do not carry autoantibodies to any of the major autoantigens (reviewed in [2]) and insulitis is rarely found after the age of 17 years [11]. Despite numerous of studies of T1D it is not clear whether autoimmunity is the cause or the result of the disease process. Direct damage of β-cells by exogenous factors is an alternative mechanism to immune-mediated β-cell destruction. Toxins or lytic viruses with tropism for β-cells are possible candidates that could cause direct β-cell damage. This damage could also be the first insult to the islets leading to a leakage of antigens that could trigger an autoimmune response. A single large dose of the toxin streptozotocin can induce β-cell death in rodents probably as a result of direct toxic effect, but given in small multiple doses it induces an immune-mediated destruction of the β-cells [23].

Genetic factors

The HLA gene region has the strongest influence on disease susceptibility [24]. There are both protective HLA alleles (DR2) as well as HLA alleles associated with increased risk (DR3 and DR4) [24] and about 95% of T1D patients carry HLA-DR3, HLA-DR4, or both [8]. It is possible that individuals carrying risk alleles might be less efficient at eliminating self-reactive immune cells and therefore be more prone to developing autoreactivity.
Environmental factors

T1D is thought to be a multifactorial disease with both genetic and environmental factors being of importance. The high discordance rate between monozygotic twins [25, 26], the changes in diabetes incidence of migrating populations as well as the rapidity of the increase of diabetes world wide [27] clearly indicate that environmental factors play an important role. Dietary factors, chemicals and virus have been discussed as potential environmental factors that might trigger T1D [28]. These factors may act alone or in combination and may initiate or enhance β-cell destruction in genetically susceptible individuals.

Among the dietary factors, cow’s milk proteins have received the most attention and early exposure to cow’s milk proteins in infancy has been linked with a risk of T1D in epidemiological studies [29, 30]. The seasonal variation observed in the presentation of clinical diabetes [5, 31] and in the appearance of the first autoantibodies in young children with increased susceptibility to T1D has suggested an infectious agent as a trigger of β-cell autoimmunity [32]. Several viruses such as EV, congenital rubella, rotavirus, cytomegalovirus, Epstein-Barr, varicella zoster virus, retrovirus and mumps virus have been associated with T1D (reviewed in [33]). Prenatal rubella infection is well established as an initiator of T1D and 20% of the patients develop disease [34]. However, since the introduction of vaccination programs in the Western countries rubella is now rare and probably not a common cause of T1D in these countries. Enteroviruses (EVs) have tropism for islets in vivo [15, 35, 36] and the evidence of EV involvement in T1D is growing stronger. It is not likely that all EV infections will lead to β-cell destruction since these infections are quite common. Most likely, the virus strain and time of infection together with the genetic susceptibility of the host determines the outcome of the infection.

Treatment and cure of type 1 diabetes

Today, there are no treatments available to stop the destruction of the β-cells but the acute complications of T1D can be successfully treated with life-long daily insulin injections. However, most patients develop secondary complications and despite improved diabetes care in recent decades, T1D is still one of the leading causes of end-stage renal disease, blindness and amputation.

The only current cure is transplantation of whole pancreas or isolated pancreatic islets. The former procedure is well established but requires major surgery and is associated with high morbidity and mortality [37]. In contrast, islet transplantation is a less invasive procedure, in which the islets are injected into the liver via the portal vein, with a smaller risk and lower levels of morbidity [38]. However, the transplanted patients have to undergo life-long treatment with immunosuppressive drugs something that has to be bal-
anced against the advantages of restoring physiological blood glucose metabolism. For this reason, transplant candidates are currently selected among patients with unstable T1D or those who have already received a donated kidney (and are on immunosuppressive treatment anyway).

The functional capacity of the transplanted islets corresponds to only 20% of that in a non-diabetic [39], indicating low graft survival rates. In most cases, islets isolated from several donors are needed to produce insulin independence in the patient [40].

The IBMIR
The infusion of islets into the portal vein involves interaction with all the various components of blood. Previous findings have demonstrated that the islets induce an acute inflammatory reaction when they come into contact with whole blood [41]. This reaction, termed the instant blood-mediated inflammatory reaction (IBMIR) is characterized by the activation of the coagulation and complement systems and a rapid binding of activated platelets to the islet surface leading to clot formation and infiltration of leukocytes [42]. Tissue factor (TF) is the main trigger of blood coagulation in vivo and islet-produced TF can initiate the IBMIR during clinical islet transplantation [43]. The IBMIR provides an explanation for the loss of islets during islet transplantation and offer new strategies to improve the outcome of clinical islet transplantation.

Human enterovirus
Classification of enterovirus
EVs belong to the Picornaviridae family, and are classified into five species; Poliovirus and Human Enterovirus A-D [44]. The different species of EV are further divided into serotypes, which by definition are all the viruses which are neutralized by treatment with a type specific serum. For example the serotype coxsackie B virus-4 (CBV-4) is included in species B. As many as 97 different EV serotypes are recognized and the number is increasing [45]. Due to the rapid mutation rate of RNA-virus many strains of virus with different characteristics can exist within a single serotype [46].

Clinical manifestation of enteroviral infections
EV infections occur frequently in the population, particularly among young children [47, 48] and the virus is transmitted primarily by fecal-oral route. The infections are most common in the late summer and autumn and less frequent during the rest of the year [48, 49]. As the name implies the normal site of replication is in gastrointestinal mucosa but it can also replicate in the
upper respiratory tract [50]. After replication in the mucosa the virus can spread through the lymphatics to the circulation to secondary replication sites which determine the symptoms of the infection. Although the infections are usually subclinical or mild, on rare occasions, some EVs can cause severe complications such as myocarditis, paralysis, meningitis and neonatal systemic diseases [51-53]. Chronic inflammatory diseases such as chronic myocarditis, Sjögrens disease and T1D have also been associated with EV infections, outlined in several reviews [33, 51, 54].

Enterovirus lifecycle

The virion
The EV virion is ~30 nm in diameter and consists of a positively sensed single-stranded RNA genome surrounded by a protein shell arranged with icosahedral symmetry. The genome consists of ~ 7400 nucleotides and the coding region is flanked by non-coding regions at the 5´ and 3´ ends.

Attachment and entry
The virus enters the host cell through attachment to specific cell-surface receptors. To date, several receptors for EVs have been identified, including the poliovirus receptor [55], intracellular adhesion molecule 1 (ICAM-1) [56], decay-accelerating factor (DAF) [57, 58], human coxsackievirus and adenovirus receptor (CAR)[59] and some integrin receptors [36, 60, 61]. The mechanism of entry differs depending on the virus and the receptor used in a particular cell type. Binding to some receptors initiates conformational changes in the viral capsid and a release of the genome [62], a process called uncoating, leaving the empty capsid outside the cell. In other cases binding to a receptor leads to an uptake of the receptor and the virus through caveolae-mediated endocytosis [63] before the uncoating occurs.

Translation and replication
Once taken up, the viral genome is made available for translation. The EV genome acts directly as mRNA and is translated into a single polypeptide by host cell ribosomes. In contrast to the host, EVs use a cap-independent mechanism and the translation is initiated via an internal ribosomal entry site located in the 5´ non-coding region. The translated polyprotein includes several proteases and cleaves itself into the precursor proteins: P1, P2 and P3. P1 is further cleaved into three capsid proteins VP0, VP3 and VP1. P2 and P3 are also further cleaved into seven non-structural proteins which interact with viral RNA (including the RNA-dependent RNA-polymerase) and various host-cell components [64]. After translation the system switches to RNA-replication and the viral RNA must free itself from the ribosomes so that it can function as a template during the RNA synthesis. The replication takes place in the cytoplasm on membranes of virus-induced vesicles formed from
the endoplasmatic reticulum (ER) [65, 66]. The virus-encoded RNA-dependent RNA-polymerase first makes a minus strand RNA copy of the genome which is used as a template to make multiple copies of the plus strand genome. Therefore double stranded RNA (dsRNA) is present as an intermediate form during replication.

**Virion assembly and release**

The capsid proteins VP0, VP3 and VP1 form protomers and 60 protomers assemble into a capsid. The primary function of the capsid is to protect and to carry the viral genome in and out of host cells. When the RNA genome is packed, the virions mature into infectious virus particles by the cleavage of VP0 into VP2 and VP4. The virus particles are then released from the cell by cell lysis or by other non-lytic mechanisms [67].

**Effect of viral replication on the host cell**

The virus is dependent on the host cell for its own replication, and has developed efficient ways to affect the cell to promote viral replication. Since EV replication takes place on intracellular membranes, one of the most prominent virus-induced changes of the host cell is the accumulation of membranous vesicles in the cytoplasm [68].

EV causes a specific inhibition of the host cell translation, preceded by the cleavage of eIF4GI and eIF4GII, components of the eIF4F complex involved in cap-dependent host protein synthesis [69]. This cleavage results in an inhibition of cap-dependent translation and the mediator for this process is the viral protease 2A [70, 71]. Shut-off of host cell translation is not required for EV replication [72] but probably increases the translation rate of viral proteins and reduces the synthesis of host defense proteins. EVs can also reduce protein transport in the host cell by inhibiting ER-to-Golgi traffic [73]. This may block antigen presentation by MHC class 1, reduce the expression of cell surface receptors and the secretion of cytokines from infected cells [74]. Moreover, EV can inactivate different transcription factors such as the TATA-box binding protein [75], cyclic AMP-responsive element binding protein [76] and NF-κB [77] and thereby downregulate the expression of cellular genes.

The biochemical and structural effects of the virus-induced metabolic and morphologic changes in the host cell are known as the cytopathic effect (CPE). In common cell lines used for propagation and isolation of EV, such as green monkey kidney cells (GMK) and Henrietta Lacks cells (HeLa), EV-induced CPE is characterized by rounding of the cells which make them refractory to light, followed by cell death and detachment of the cells from the surface. Not all productive infections result in CPE. The virus may not induce CPE if for example the replication is very slow [78] or if the RNA persists as a double stranded complex for some time without replication [79].
Antiviral response of the host cell

Despite inhibition of host cell protein synthesis and transport during virus infection, several host defense mechanisms can be induced. The membrane bound toll-like receptors (TLRs) can detect dsRNA and expression of TLR-3 has been detected in mouse and human islets cells [80]. The signaling pathway of these receptors results in activation of different transcription factors including NF-κB [81] and induction of genes that encode proteins important for the antiviral response such as inducible nitric oxide synthase (iNOS) [82], interferons (IFNs) and inflammatory cytokines [81]. IFNs inhibit the replication of many viruses potentially via induction of proteins such as dsRNA-dependent protein kinase (PKR), 2′5′oligoadenylate synthetase (2′5′ OAS) and RNase L [83]. Induction of these proteins results in a block of mRNA translation and degradation of RNA in the cell in an attempt to limit virus production [83].

It is not clear which of these antiviral response pathways, if any, are activated during EV infection of human islets. Some picornaviruses are able to counteract some of the antiviral signaling pathways triggered by dsRNA [84]. However, expression of IFN-α [85], IFN-β and several inflammatory cytokines [86, 87] has been reported in human islets after EV infection.

Enterovirus infections in isolated human islets

Primate or human cell lines are frequently used for EV propagation and the cellular effects of EV infections in these cells have been widely studied. The possible involvement of EV in T1D has gained interest the last decades. Due to the low availability of human islets for research most studies of EV and T1D have been performed on mouse or rat islets and little is known about the effects of EV infection in human islets or β-cells. The studies that have been performed have demonstrated the capacity of different EV serotypes to infect, damage and finally kill human islets in vitro. Several EVs have been found to have tropism for human islets or β-cells [88-90] and even though most EVs are lytic in human islets some EVs have been shown to cause persistent infections in human islet cells [85, 89, 91]. EV infection has also been shown to impair the insulin release after stimulation with high glucose [88, 90]. The impairment differed between strains within the same serotype, suggesting any serotype could be potentially diabetogenic. Induction of several inflammatory genes has been demonstrated in EV infected islets [86, 87] which might contribute to the homing and activation of immune cells in the islets during infection and/or an autoimmune response in vivo.

Enterovirus and type 1 diabetes

There are several observations that support the involvement of EV in T1D. EVs are common pathogens found to accompany or precede T1D develop-
ment and EV-RNA and IgM antibodies against EVs have been found in higher frequencies in newly diagnosed patients than in controls [92-96]. EVs can infect human islets in vitro [88-90] and a recent finding has confirmed that these viruses also can infect human islets in vivo [15, 36]. Furthermore, EV infections are more frequent in siblings developing T1D than in non-diabetic siblings [97]. Moreover, in children who develop autoantibodies, EV infections are more frequent than in control children [98, 99] and clusters of infections have been shown to precede the appearance of autoantibodies [100, 101]. EV has been isolated from newly diagnosed T1D patients in a few cases [102-105] and some were capable of causing diabetes in mice [102, 103].

It is possible that the high rates of EV infections among newly diagnosed diabetic patients are due to an increased susceptibility for infections. However, this tendency has not been seen with other viruses. Neither is infection related to high blood glucose level or type 2 diabetes mellitus [54]. In patient studies many different serotypes have been associated with T1D and EV infections have also been implicated in a variety of other diseases. This reflects the existence of multiple strains with various degrees of virulence, also within a single serotype. It has been shown that individual nucleotide substitutions in the non-coding and coding regions of the viral genome determine virulence [106, 107]. Also, recent findings show that EVs can naturally delete sequence information in the genomic 5’ terminus [108]. Because of the importance of the deleted region for virus replication these strains replicated very slowly without causing CPE but were able to transmit replicating virus into other cell cultures [78]. In vivo, the adaptive immune response which clears the cytolytic strains may confer a selective advantage to these more slowly replication strains. A defective replication may be beneficial for prolonged viral genetic survival under specific circumstances. Therefore, different strains with different characteristics should be used when studying the effects of EV infections on human islets and the potential mechanisms that could trigger T1D.

Possible mechanisms of enterovirus-induced β-cell destruction

Although EV infections have long been considered an exogenous factor of major importance for the development of T1D, there is still a considerable lack of knowledge as to the mechanisms involved. However, several possible mechanisms have been suggested. The mechanisms might be different for different EVs and might also be able to coexist.

1. Direct effects: Infection with a lytic virus that is highly tropic for the β-cells could destroy the β-cells by virus-induced cytolysis. A series of lytic infections might lead to a gradual loss of β-cells that finally results in T1D. A
lytic infection might also result in an exposure of previously hidden β-cell antigens [9] necessary for the activation of an autoimmune response.

2. **Immune-mediated destruction**: β-cell destruction might also be caused by an immune response directed against virus antigens [33]. If the virus is able to cause a persistent infection, this could lead to a chronic inflammation. Alternatively, as mentioned above, several lytic infections might induce a gradual immune-mediated destruction.

3. **Molecular mimicry**: This model is based on the sequence homology between EV proteins and host cellular proteins (e.g., GAD65 and the CBV viral protein p2C) [109] and does not require virus infection of the islets. During infection the viral peptides are presented by antigen-presenting cells resulting in activation of specific T-cells that can cross-react with host protein expressed on β-cells [33]. It has been difficult to prove this theory since healthy control groups also show reactivity to islet cell antigens [110].

4. **Bystander mechanism**: A non-lytic infection or a local infection in some islets or in the exocrine tissue could result in a virus-induced inflammatory reaction with an increased secretion of cytokines and other inflammatory mediators [33] which could cause initial β-cell damage. This inflammatory milieu together with a release of islets antigens could lead to the initiation of a β-cell targeted autoimmune process.

**Figure 1: Possible mechanism of EV induced β-cell destruction**

Figure modified from Roivainen M, IJBCB, 2006
Antiviral strategy to prevent type 1 diabetes

If EV infections prove to be a trigger of T1D and if T1D is caused by a particular diabetogenic strain or if a common epitope is discovered then vaccination would be an efficient way to prevent T1D, although identifying risk subjects among the population will be challenging. If a wide variety of EV can cause the disease, an alternative strategy could be to use antiviral drugs with a broad specificity. Antiviral treatment given at the time of appearance of autoantibodies or at diagnosis of T1D might halt the progression of β-cell damage by inhibiting virus replication in the patients and save the remaining β-cells. Several anti-picornavirus substances are available for in vitro studies, but to our knowledge there are no anti-picornavirus substances at the stage of clinical trials today, and there is a need for potent and nontoxic compounds for the treatment of picornavirus infections.
Aims

The overall aim of this thesis was to study expression and modulation of inflammatory mediators in infected and uninfected islets that could trigger inflammatory reactions leading to β-cell destruction.

Paper I

In this study we aimed to screen isolated human islets for mRNA expression of genes with the potential to induce or modulate an immune response.

Paper II

Islet secretion of TF and MCP-1 could trigger coagulation and inflammatory responses and also plays a relevant role in the clinical outcome of islet transplantation. We therefore wanted to investigate if the presence of different compounds, previously known to affect TF expression in other cells, would affect the expression of these factors in isolated human islets.

Paper III

The aims of this paper were to study virus replication, viability and insulin release of human islets infected with EVs isolated from patients at onset of T1D.

Paper IV

EV has previously been shown to induce expression of pro-inflammatory cytokines and chemokines in human islets in vitro. The modulating effect of nicotinamide (NA) on MCP-1 expression found in paper II made us interested in studying the effect of NA on EV infected human islets. Virus replication, virus-induced islet destruction/CPE and secretion of the chemokines MCP-1 and IP-10 were studied with or without the presence of NA.
Material and Methods

A brief description of the methods used is presented here. More detailed information is given in Papers I-IV.

Cell culture

Human islet (papers I-IV)
Using a protocol approved by the local ethics committee, human islets of Langerhans were isolated as previously described [111] from brain-dead donors free of known pancreatic disease. The islet preparation was available for research because of too low islet yield after isolation. The quality of the islets was not different from that of islets used for clinical transplantation. Islets were cultured at 37°C (in 5% carbon dioxide) in supplemented CMRL-1066 medium with 10% heat-inactivated human serum. The medium was changed every other day. The purity of the islets was determined by microscopical characterization after staining with diphenylthiocarbazone. Islet viability/quality was assessed in a dynamic perfusion system examining the response to high glucose stimulation.

Culture of human islets in the presence of various substances (paper II)
Islets were cultured in supplemented CMRL medium without NA for 24 h. Islet culture then continued for 48 h with various test substances added individually to the medium: NA (0, 10, 25 or 50 mM) and L-arginine (1 mM), enalapril (40 μg/ml) or cyclosporin A (1 μM).

GMK cells (paper III and IV)
GMK cells were used for virus propagation and virus titrations. When confluent, they were cultured in EMEM supplemented with 2% newborn bovine serum.
Microarray analysis (paper I)
We used Atlas Human Cytokine/Receptor and Hematology/Immunology nylon membranes representing 268 and 406 genes respectively to investigate the gene expression in isolated human islets from five different donors cultured for 2-11 days after isolation. Total RNA was prepared from isolated human islets and radioactively labeled probes were produced and hybridized to the array membrane overnight at 68°C. After 1-3 days of exposure Fuji Bas 1800 II Imager was used to visualize the gene expression and the files were scanned. After subtracting the background values for each membrane, the expression of each different gene was calculated and related to the mean intensity of five housekeeping genes on the same array. The threshold was set so that an adjusted intensity (intensity minus the background) of at least three times the background value was considered a genuine signal.

The tubing loop system, blood and plasma analysis (paper II)
To study the interaction between islets and blood we used a previously described model [41, 112, 113]. This system consists of loops made of PVC tubing with an inner surface coated with heparin, designed to mimic a blood vessel. Heparinized connectors were used to hold the tubing loops together.
Islets cultured in CMRL medium supplemented with 0, 10, 25 and 50 mM of NA were harvested and washed. 5 µl of islets re-suspended in 100 µl CMRL medium was added to the loops containing fresh human blood. A control loop without islets containing blood with 100 µl CMRL medium was included. The test tubes with or without islets were incubated on a rocking device at 37°C to generate a blood flow of ~45 ml/min (corresponding to the portal flow). At 5, 15, 30 and 60 min 1 ml of blood was collected from each loop into EDTA-containing tubes for further hematologic analysis. Samples of blood at 0 min, before the blood was added to the tubing were also included.
Plasma levels of thrombin-antithrombin (TAT) were quantified using a commercial EIA kit.

Viruses

Virus strains (paper III and IV)
The following virus strains were used in this study: T1 and T2 (paper III) were isolated from a mother and her son who were both diagnosed with T1D
on the same day [105]; Adrian (A) (paper III and IV) and Eric (E) (paper III), were isolated from two identical twins of whom one was diagnosed with T1D (A); the CBV-4 E2 strain (paper III) was isolated from a diabetic boy who died of ketoacidosis [102]; the CBV-4 strain VD2921 (paper IV) was plaque-purified after isolation from a patient suffering from aseptic meningitis and has been shown to cause a non-lytic infection of islet cells [88, 91, 114]. All strains used belong to the EV genus

Virus isolation (paper III)
Stool samples from the patients after diagnosis of T1D were re-suspended with glass beads in a phosphate buffer and the clarified suspensions were used to inoculate GMK, Rhabdo myosarcoma (RD) and HeLa cells. The inoculated cells were examined every day for the appearance of CPE. The isolates from the twin boys revealed typical EV CPE on GMK and HeLa cells and none of the isolates caused CPE on RD cells, known to lack the CBV receptor CAR. Neutralization with antiserum pools suggested that the isolates from the twin boys were Echovirus 21.

Virus inoculations (paper III and IV)
Human islets were inoculated by addition of $10^3$ tissue culture infectious dose-50 (TCID$_{50}$) of the virus to the cultures. The inoculated cells were left for 30-60 minutes to allow the virus to attach, after which fresh culture medium was added to the cells. Islet culture was continued for 5-7 days and aliquots of culture medium were withdrawn for TCID$_{50}$ titrations on GMK cells. Mock-infected islets from the same donor were used as controls.

Detection of virus replication (paper III and IV)
Virus replication was determined by TCID$_{50}$ titrations on GMK cells. Samples of culture medium were collected from the infected human islet cultures on day 0 and then at regular intervals until days 5-7 post infection (pi). To titrate the virus content in the medium on the respective days, the samples were serially diluted and each dilution was added in duplicate to GMK cells in 96-well plates. The virus titre was determined as the reciprocal of the highest dilution able to induce CPE in 50% of the inoculated GMK cell culture.

Immunostaining for EV (paper III)
HeLa cells cultured on culture slides infected with the four isolates or with CBV-4 E2 strain (positive control) were fixed in acetone. Antibodies against
the EV capsid protein VP1 were added. The binding of the primary antibody was visualized by addition of PicTure-Plus kit containing a polymer conjugate of horseradish peroxidase and Fab fragments. The same staining protocol was performed on PFA-fixed paraffin-embedded human islets infected with the isolates (day 3 and day 6 pi).

**Studies of cell morphology and viability of infected islets**

**Assessment of CPE (papers III and IV)**
Infected cells were examined for CPE every day using a light microscope. The degree of CPE was ranked from 0 to 4 +, with 0 indicating no CPE; 1+, weak changes, 5-25% of the cells being disrupted or showing changes in morphology; 2+ and 3+, 25-50% and 50-90%, respectively, of the cells being affected as described above; 4+, ≥ 90% of the cells being completely disrupted. The same ranking was used to assess islet destruction.

**Electron microscopy (paper III)**
The morphological changes induced by the isolates were also studied using electron microscopy. Infected and uninfected islets (day 3 and day 7 pi) were fixed in 2% glutaraldehyde and 1% formaldehyde, followed by 1% osmium tetroxide, dehydrated in graded ethanol and embedded in TAAB-812-resin. The 500 Å slides were counterstained with uranyl acetate and lead citrate before examination under the electron microscope.

**Viability (paper III)**
The viability of islets infected with the isolates and uninfected islets was assessed by initial trypsinization followed by staining with trypan-blue on day 3 and day 7 pi. The number of dead cells was counted in a blinded manner.

**Glucose stimulation of infected islets (paper III)**
Static glucose stimulation tests were performed on human islets infected with the four isolates as well as on uninfected control islets. On day 3 pi culture medium was changed to RPMI containing either 5.5 or 16.5 mM glucose. Day 4 pi the media was changed back to 5.5 mM glucose. Samples of culture
medium were collected before and after the medium changes for subsequent measurements of insulin. The insulin secretion in response to high glucose was calculated by subtracting the insulin content of the culture medium on day 4 from the content on day 3 pi. A high range rat insulin ELISA, which cross-reacts with human insulin, was used for the measurements of insulin to avoid dilution of the samples. The insulin response to high glucose was calculated per islet.

Cytokine and chemokine measurement

TF and MCP-1 measurement (paper II)
Islets were collected after the 24 h baseline period and after 48 h of culture with the various substances. All harvested islets were homogenized and analyzed for TF and MCP-1 using commercial ELISAs.

IP-10 and MCP-1 measurement (paper IV)
Samples of culture medium from infected islets cultures were analyzed for IP-10 and MCP-1 using commercial ELISAs. Culture samples from uninfected human islets cultured with and without NA were used as controls.

Statistical analysis (paper II, III and IV)
In paper II, mean values were compared using the ratio t-test. The correlation between MCP-1 and TF expressed by islets and their TF content in relation to coagulation activation (measured by TAT) was calculated by linear regression. Because of the differences in individual blood characteristics and variation in pro-inflammatory mediators, we expressed the data as percentage of the value for a control tubing loop included in each experiment instead of absolute numbers. This tubing loop consisted of whole blood, medium in which islets were re-suspended (but with no islets) and any drugs being tested.

In paper III, Wilcoxon signed ranks test and Mann-Whitney test were used to calculate differences in insulin release. Differences in the degree of islet destruction/cytolysis were tested with the independent samples t-test for comparing paired samples. This test was also used to calculate differences in the frequencies of dead cells between infected and uninfected cells.

In paper IV, differences in virus titre and chemokine secretion were analyzed with Wilcoxon signed ranks test

A p value of < 0.05 was considered statistically significant in all papers.
Consideration regarding the research design and methods

Experiment using human islets (papers I, II, III and IV)

Because of ethical considerations and shortage of human islets most of our current information of T1D comes from animal studies. Although animal models are valuable tools for studying certain aspects, it is important to bear in mind that results from animal studies can not be related to the situation in humans without caution. For instance, immune responses, expression of receptors and cytokines may differ between species.

Isolated human islets from brain dead donors are a unique material in which to study T1D. However, factors such as the cause of death of the donor, warm and cold ischemia time, stress-related hormones released prior to death and the isolation process could all affect the quality of the islets. Also, the culture conditions utilized are not perfect and hypoxia and nutrient deprivation could influence expression of different stress factors, receptors, etc.

Microarray analysis (paper I)

Although we used purified islets (50-80% pure), an mRNA contribution from non-endocrine cells within the islets, such as macrophages, endothelial cells and exocrine tissue, that are present in the islet preparations cannot be excluded. However, we presume that in the in vivo situation all the cells within an islet can potentially contribute to the immune response induced.

It should also be emphasized that the variation in absolute transcript numbers between the different cytokines studied does not necessarily reflect the importance of a specific cytokine. Also, since we only studied the expression on mRNA level the expression of these cytokines as proteins is unknown.

The tubing loop system (paper II)

For this tubing loop system the differences between experimental groups are more important than the absolute values. There are several reasons for this: The same aliquot of blood re-circulates in the tubing during the whole ex-
experiment, a situation that contributes to the accumulation of coagulation activation products. Also, the heparin-coated inner surface is not a perfect model of the vascular endothelium which displays other anticoagulant activities related to expression of thrombomodulin and the activation of fibrinolysis. Finally, the air enclosed in the tubing contributes to the activation of the blood.

Virus infections of human islets (paper III and IV)

Working with both human islets and EV strains isolated from patients at onset of T1D is a great opportunity to obtain new information leading to a better understanding of the mechanism(s) behind EV-induced β-cell destruction.

Successful isolation of virus at onset of T1D is not very common. We managed to isolate three strains from T1D patients (A, T1 and T2) and one strain from a non-diabetic family member (E). Three of the isolates have not yet been genotyped but results from neutralization tests suggest that A and E were Echovirus 21. The isolate T1 has been genotyped and after sub-cultivation also serotyped as CBV-5. The T2 strain could not be serotyped with conventional methods suggesting that this isolate might contain two different viruses or two different serotypes.

When human islets were infected with the isolates (paper III) the increase in viral titre was later (day 3-6 pi) than has been shown in previous islet studies [88, 91]. This late increase in viral titre could be due to the change of media on day 3 and day 4 pi or that these isolates had a slower replication cycle. Also, the CBV-4 strains used in other publications had been passaged several times and the isolates only twice. Differences in replication could also be due to donor differences.
Results and Discussion

Paper I

Expression of immune modulating proteins in the islets in vivo could have an important role in the process of β-cell destruction seen in T1D. In addition, such expression could markedly influence the outcome of intraportal islet transplantation. Using the technique of cDNA array, mRNA expression of hundreds to thousands of genes can be analyzed simultaneously. Because of the finite access of human islets we chose to use this technique to screen isolated human islets for inflammatory and cytokine genes.

Atlas Human Cytokine/Receptor array representing 269 genes was used to examine the gene expression. 51 genes excluding the housekeeping genes were expressed with only small variations between the five different islet preparations (Table 1, paper I). Gene expression was also examined using Atlas Human Hematology/Immunology array, which confirmed the results from the first array with no major additional gene products identified. Of the 51 genes identified, MCP-1, migration inhibitory factor (MIF), vascular endothelial growth factor (VEGF) and thymosin β-10 were detected in islets from all donors. Several of the genes expressed in the islets encode proteins with powerful biological activity such as IL-1β, IL-8, macrophage inflammatory protein 2α (MIP-2α), MCP-1 and MIF.

IL-1β is a pro-inflammatory mediator that also has direct cytotoxic effects on rodent β-cells in vitro [115, 116]. IL-8 and MIP-2α are involved in the recruitment of neutrophils. MCP-1 attracts monocytes, T-cells and NK cells [21] and is produced by various cell types, including β-cells, in response to pro-inflammatory stimuli [117-120]. The secretion of MCP-1 from islets has also been shown to be important for the outcome of islet transplantation, where low MCP-1 secretion was the most relevant factor for long-lasting insulin independence [121]. MIF was expressed in all islet preparations. This protein has been found to be expressed at high levels in rat β-cells where it is secreted together with insulin to act as an autocrine factor to stimulate insulin release [122]. Circulating MIF levels are elevated during stress or systemic inflammatory processes and this protein may play a central role in the control of insulin secretion during various disease states [123]. There seemed to be a correlation between MIF and the expression of MCP-1, and MIF has previously been shown to inhibit MCP-1-induced migration of monocytes [124].
Angiogenesis is crucial for the revascularization of the islets after transplantation and VEGF is the dominant stimulus for this process [125]. VEGF is expressed in islets both in vitro and in vivo [126] and hypoxia has been shown to increase the expression of this factor [127, 128]. The observed high expression of VEGF in all islet preparations in this study is most likely due to hypoxia during transport, islet isolation and culture. Thymosin β-10 expression, which was also was present in all islet preparations, seems to be modulated by VEGF [129]. Thymosin β-10 is associated with cell growth in proliferating tissue and is over-expressed in different cancer cells [130]. The reason for the expression of thymosin β-10 in the islets is unknown but it may be speculated that also this expression is correlated to hypoxia.

A constitutive expression of all these inflammatory genes in the islets seems unlikely and the expression is probably induced by several factors including the process of brain-death and organ procurement. Moreover, the culture condition of human islets is not optimal, for practical reasons. The finding that more genes were expressed early after isolation (day 2) compared to after long-term culture (day 7-11) suggests that it could be of advantage to culture the islets before transplantation to reduce the expression of inflammatory mediators.

In summary, many genes with a potent inflammatory capacity were expressed in isolated human islets. This indicates that during stress the islets themselves can express several triggers of inflammation, and if expressed in vivo these mediators would probably contribute to β-cell destruction. In addition, modulation of the gene expression during culture could be a promising strategy to reduce or enhance expression of selected genes of importance for islet transplantation and islet engraftment.

Paper II

The pro-inflammatory cytokine MCP-1 and the coagulation activator TF can trigger inflammatory responses and therefore play a relevant role in the clinical outcome of islet transplantation.

In this study we cultured isolated human islets with different compounds (NA, L-arginine, enalapril and Cyclosporin A) known to affect TF in other cells [131-134]. The intracellular content of TF and MCP-1 was measured after 48 h of culture in medium supplemented with the various compounds. NA reduced the expression of both TF and MCP-1 in a dose-dependent manner (Figure 1, paper II). Optimal inhibition was achieved with concentrations of 10-25 mM. The decrease of TF by NA, L-Arginine and enalapril correlated well with MCP-1 levels (Figure 2, paper I). Cyclosporin A, suppressed MCP-1 levels to 50% compared to controls but had limited effect on TF, indicating that TF and MCP-1 are only partially regulated by the same mechanism(s).
NA is thought to interfere indirectly with NF-κB, a nuclear transcription factor for pro-inflammatory cytokines such as TF and MCP-1 [135, 136]. This inhibition involves interference of NA with poly ADP-ribose polymerase 1 (PARP-1), a nuclear chromatin-associated protein activated by DNA strand breaks [137]. In addition to NF-κB, several other transcription factors are known to regulate hypoxia-induced TF in monocytes, including hypoxia-inducible factor-1, activator protein 1 and early-growth-response gene product [138-140].

To test if the down-regulation of TF had any effect on the islets procoagulant activity, islets cultured with NA were exposed to fresh ABO-identical blood in the tubing loop system. The islets were cultured for 48 h in 0, 10, 25 or 50 mM of NA before the experiment and the TF content was then measured. The TF content of the islets correlated strongly to the potency to initiate the IBMIR, as reflected by the formation of the coagulation activation marker, TAT (Figure 3, paper II). Islets cultured with the different concentrations of NA were also evaluated in a dynamic perfusion system by stimulation with glucose and assessment of insulin secretion. NA did not affect islet function (Figure 4, paper II). In addition, insulin content was measured and no adverse effect of NA was observed.

In summary, the expression of the inflammatory mediators TF and MCP-1 in isolated human islets can be modulated by NA and islets cultured with NA displayed a reduced capacity to trigger the coagulation system in vitro. This indicates that NA may have implications for both inflammatory responses and the procoagulant activity of islets. Moreover, pretreatment of the islets before transplantation, to reduce the synthesis of TF in islets in culture, may be an efficient way of reducing the adverse effect of IBMIR during clinical islet transplantation. This would also be a valuable complement to systemic anticoagulant treatments, which have limitations due to potential adverse effects on hemostasis in the patients.

**Paper III**

Our group has previously published a case report were EVs (termed T1 and T2) were isolated from a mother and a son diagnosed with T1D on the same day [105]. In the present study we successfully isolated virus from two twin boys Eric (E) and Adrian (A), one of whom was diagnosed with T1D (A). An EV-RT-PCR was performed and the positive results indicate that these isolates belonged to the EV group. Isolated human islets were infected with the isolates and all strains replicated in human islet cells and were able to induce islet destruction/CPE to different degree, with Adrian being the most aggressive on day 7 pi (Table 2, paper III). Islets infected with the four isolates were also stained with a specific EV-antibody and only infected islets showed positive staining, confirming the results from the EV-PCR.
Trypan blue staining revealed that culture-wells with infected islets contained more dead cells than the wells with uninfected control islets on day 7 pi. Islets infected with the isolate from the non-diabetic twin contained fewer dead cells than islets infected with any of the T1D strains.

Electron microscopy of islets infected with three of the isolates (A, E and T1) revealed reorganization of ER and clusters of vesicles in the cytoplasm which are classical hallmarks for EV infection (Figure 4B, paper III). Also, virus particles could be seen inside some of these vesicles in cells infected with the A strain (Figure 4C, paper III).

The function of infected islets was also examined by measuring the insulin secretion in response to high glucose. All infected islets displayed a reduced ability to secrete insulin in response to high glucose on day 3 pi compared to controls (Table 3, paper III).

The onset of T1D within a family suggests that genetic predisposition is a risk factor for the disease. However, 90% of diabetic children do not have a first degree relative with the disease [141] and the concordance rate between monozygotic twins is less than 50% [25, 26] suggesting that the genetic susceptibility can only partly explain disease development. This indicates that environmental factors play an important role. The close time of onset of T1D within the families and the successful isolation of EVs from these patients together with the observed tropism of the isolates for human islets and β-cells and the effect on islet function, further strengthen a causal relationship between some EV and T1D.

Paper IV

EV infection might promote β-cell damage by inducing expression and secretion of inflammatory cytokines and chemokines and thereby stimulate immune cell infiltration into the pancreatic islets. Induction of such genes, including IP-10 and MCP-1, has been shown in EV-infected islets [86, 87, 142].

The modulating effects of NA on cytokine expression, discussed in paper II, made us interested in evaluating the effect of NA on EV-infected human islets. Isolated human islets were cultured with or without the presence of NA and then infected with Adrian (defined in paper III) or VD2921 (an EV strain which causes a non-lytic infection in islet cells[88, 91]). Virus replication and virus-induced islet destruction/CPE was studied. Uninfected islets cultured with or without the presence of NA were used as controls.

Both EV strains replicated well in the cultured human islets and addition of NA to the islet culture 24 hours before infection led to a lower virus titre compared to untreated islets (Figure 1, paper IV). A protective effect of NA on cell viability was seen when virus-induced islet destruction/CPE was studied, especially with the Adrian strain on the later days of infection (days
Pretreatment of GMK cells with NA also revealed that NA protected these cells from CPE. In addition, the dilutions of the EV strains that caused CPE on GMK cells was 1.7 and 2 log lower in the presence of NA. This indicates that the protective effect of NA is not islet specific.

IP-10 and MCP-1 are both potent attractants of different immune cells such as T-cells, NK cells and monocytes [21, 121, 143, 144] and infiltration of these cell types has been found in islets from EV-positive T1D patients and recent onset T1D patients [15, 16]. Elevated concentrations of IP-10 have also been found in serum from T1D patients [145, 146]. Therefore the secretion of IP-10 and MCP-1 from EV-infected and uninfected human islets cultured with or without NA was examined.

Secretion of IP-10 was induced by both VD2921 and Adrian on days 2-3 pi and days 5-7 pi (Figure 3, paper IV). The secretion from EV-infected islets seems to be dependent on virus replication, since uninfected islets from the same donor did not secrete this chemokine and inactivated virus has previously been shown to be unable to induce IP-10 expression in human islets [142]. Increased secretion of MCP-1 was detected from infected islets when compared to uninfected controls on days 5-7 pi (Figure 4, paper IV). As earlier shown (in paper I and paper II), uninfected islets also secreted MCP-1.

When islets were cultured with NA-supplemented medium for 24 hours before infection, the secretion of IP-10 from islets infected with Adrian was almost completely blocked (Figure 3, paper IV). Also, the secretion of IP-10 from islets infected with VD2921 was strongly reduced. In concordance with the results from paper II, NA reduced the islet secretion of MCP-1, both from infected and uninfected islets (Figure 4, paper IV).

NA is believed to affect cell death pathways and gene expression in islet cells leading to β-cell survival and an altered immune-regulatory balance probably via interaction with PARP-1 [137]. PARP-1 exerts a wide array of biological activities including DNA repair, replication and differentiation and regulation of transcription of inflammatory mediators [137]. The protective effects of PARP-1 inhibition likely result from improvement of cellular energetic status by prevention of NAD+ and energy metabolite depletion resulting in an increased cell survival and from inhibition of common pathway(s) of tissue injury such as NF-κB activation or oxidative stress-induced cytotoxicity [137, 147, 148]. NF-κB is involved in the transcription of pro-inflammatory cytokines, such as MCP-1 and was recently shown to be important also for the induction of IP-10 [149].

NA has been shown to delay diabetes in NOD-mice [150], but in the large European Nicotinamide Intervention trial, NA failed to prevent T1D in human subjects at high risk of developing the disease. In this trial 70% of the subjects had more than one autoantibody, suggesting that treatment was started too late when the destructive process of the β-cells might already be
ongoing. The failure could also be due to the low concentration achieved locally in the islets. If a virus is involved in T1D as an initiator of the immune process, infection would probably precede the appearance of autoantibodies.

The mechanism(s) behind the antiviral effect of NA found in our study further needs to be elucidated. However, it can be speculated that NA (or other PARP-1 inhibitors) could be of interest as a complement to antiviral treatment. Inhibition of viral replication and modulation of the cellular immune response might reduce the β-cell damage and save the remaining β-cells in the patients.
Conclusions

Paper I
1. Isolated human islets expressed several pro-inflammatory genes.

Paper II
1. NA reduced the expression of both TF and MCP-1 in isolated human islets - in a dose-dependent manner.

2. By decreasing the expression of TF, NA reduced the capacity of isolated human islets to trigger the coagulation system \textit{in vitro}.

Paper III
1. The increase in virus titres and the presence of virus particles in the β-cells showed that these EV strains isolated from T1D patients had tropism for islets and β-cells \textit{in vitro}.

2. All strains induced islet destruction/CPE and infected islets contained more dead cells compared to uninfected control islets.

3. All four isolates affected the islets ability to secrete insulin in response to high glucose.

Paper IV
1. NA inhibited viral replication and reduced EV-induced islet destruction/CPE in EV-infected human islets.

2. NA inhibited or reduced EV-induced secretion of IP-10 and MCP-1 from isolated human islets.
Acknowledgments

Det finns många personer som på ett eller annat sätt bidragit med inspiration, stöd och uppmuntran under arbetet med denna avhandling, tack till er alla!

Speciellt skulle jag vilja tacka:

Gun Frisk, min handledare, utan dina uppmuntrande ord och din smittande entusiasm hade jag inte klarat detta. Tack för ditt stöd och för att du alltid tar dig tid när jag kommer med frågor och funderingar.

Olle Korsgren, min handledare och ”the great mind” inom ö-forskningen. Tack för att du tog emot mig som exjobbare och uppmuntrade mig att fortsätta som doktorand. Tack också för dina värdefulla kommentarer, idéer och ditt kritiska öga. Hur du lyckas hålla så många ”bollar i luften” är för mig ett mysterium.

Min biträdande handledare Bo Nilsson, för dina råd och ditt stöd!

Tack till mina medförfattare för ett bra samarbete!

Min forskning hade inte varit möjlig utan det Nordiska nätverket.

Margareta ”Bumsan” Engkvist, vad skulle Klinimm vara utan dig? Du ställer alltid upp trots ditt fullbokade schema. Tusen tack för all hjälp och goda råd!

Graciela Elgue, som lärt mig allt om ELISOR och som är en klippa när det gäller korrekturläsning. Muchas gracias!

Ulrika Johansson, en av mina medförfattare som lärt mig massor om diverse labbmetoder och tekniker. Tack för att du tog hand om mig när jag kom till Klinimm som vilsen exjobbare.

Mona Persson, Ann-Sophie Lindberg och Elin Blom Ekberg, en eloge till er som hinner med allt pappersjobb fast ni ständigt blir avbrutna, stort tack!
Sanja Cabric och Lisa Moberg, jag kan inte ens tänka mig hur doktorandtiden skulle ha varit utan er! Tack för roliga stunder på och utanför labbet, massor av skratt, några tårar, och ett oändligt antal koppar ”java”.

AnnaKarin Lidehäll, kul att vi kunde göra den här resan tillsammans, snart är vi på andra sidan ”berget ” då får vi fira ordentligt!

Peter Schmidt, din underfundiga humor är svårslagen och jag kommer aldrig att glömma första gången jag såg ”pundardansen”. Ringer dig om någon ”småfläckig sumphöna” skulle dyka upp på tomten.

Nuvarande och tidigare medlemmar i Ö-gruppen, Andrew Friberg (for nice company in the Ph.D room), Masafumi and Megumi Goto (we all miss you so much), Henrik Krook (som uppmuntrade mig att doktorera), Helena Johansson och Torsten Eich (för sällskap i skidbacken i Igls), Anette von Malmborg och Magnus Stähle (för t Trevligt sällskap i Dublin), Nina Mikkola och Anna Svensson (som gör ett fantastiskt jobb på ö-labb), samt Christina Andersson, Daniel och Heide Brandhorst, José Caballero, Hideyuki Yamaya, Linda Holm, Christian Molnar, Viola Selling, Anna Mattsson och Karin Andersson för roliga stunder på ö-labbet och i fikahörnan!

Nuvarande och tidigare medlemmar i virusgruppen på Institutionen för kvinnors och barns hälsa, Oskar Skog och Monika Hudik, kul att ni börjat virusgruppen och lycka till med egna avhandlingar! Anna-Karin Berg, tack för ett gott samarbete, för hjälp med metoder och språkgranskning. Asma Elshebani, thank you for your hard work on our paper, for helping me with methods and for being a good friend in the lab. Maria Hindersson, tack för trevligt sällskap i Holland. All övrig personal på KBH, tack för trevliga fikastunder!


Tikomed-gänget och GIN-lab, Caroline Magnusson, Jonas Andersson, Naomi Forslund, Maria Wilén, Peetra Magnusson, Ida Rasmusson och Fredrik Carlsson.

Nuvarande och tidigare medlemmar av GIG gruppen, med Thomas Tötterman och Magnus Essand i spetsen. Valeria Giandomenico, Ole Forsberg, Arian Sadeghi, Angelika Danielsson, Moa Fransson, Sara Mangsbo, Camilla Lindqvist, Justyna Leja, Sofia Wikman, Helena Dzojic, Björn Carlsson,
Wing-Shing Cheng, Angelica Loskog, Gabriella Paul-Wetterberg och Berit Nilsson.

Sussane Gabrielsson, Malin Flodström-Tullberg och Monica Hultcrantz på KI, tack för ett gott samarbete!

Projektarbetare och rutinpersonal på Klinisk Immunologi.

Alla vänner och släktingar speciellt Ebba, Helena och Els-Marie, samt Olle, Christina.

Barbro, snällare moster får man leta efter. Min mormor, som inte finns med oss idag men som nog har koll på mig ändå.

Världen bästa lillebror, Peter och hans familj.

Mamma och Pappa, för att ni alltid stöttar mig och ställer upp i vått och torrt. Ni är världens bästa föräldrar, älskar er oändligt!

Linus, för att du tror på mig och får mig att kämpa och inte ge upp. Jag älskar dig! ”Har någon sagt att du är underbar”…..

Agnes, mitt hjärta, när du ler försvinner alla bekymmer! ♥
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)