Experimental Studies Aiming to Prevent Type 1 Diabetes Mellitus

TOBIAS RYDGREN
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Abstract

Type 1 diabetes mellitus (T1DM) is an autoimmune disease in which T-cells and macrophages invade the islets of Langerhans and selectively destroy the insulin producing β-cells, either directly or through the secretion of e.g. cytokines and nitric oxide (NO). This thesis has studied possible strategies to prevent T1DM. In β-cells and macrophages, NO is produced by inducible nitric oxide synthase (iNOS).

In the first study, we found that 1400W, a highly selective inhibitor of iNOS could prevent interleukin (IL)-1β induced suppression of rat islet function in vitro, but not diabetes induced by multiple low dose streptozotocin (MLDS), a well established animal model for autoimmune diabetes, in vivo.

Next, we wanted to test a new type of high affinity blocker of IL-1 action, called IL-1 trap, in vitro. Here we found that an IL-1 trap could prevent the suppressive effects by IL-1β on rat pancreatic islet function. Also, it was sufficient to block the action of IL-1β to prevent islet cell death induced by a combination of IL-1β, tumor necrosis factor-α and interferon-γ.

In study III, a murine IL-1 trap was found to prolong islet graft survival in the recurrence of disease (ROD) model, a T1DM model that involves syngeneic transplantation of healthy pancreatic islets to diabetic nonobese diabetic mice. Mice treated with IL-1 trap displayed an increased mRNA level of the cytokine IL-4 in isolated spleen cells. This suggests a shift towards Th2-cytokine production, which in part could explain the results.

Finally, simvastatin an anti-hypercholesterolemic drug that possesses anti-inflammatory properties e.g. by interfering with transendothelial migration of leukocytes to sites of inflammation was studied. We found that the administration of simvastatin could delay, and in some mice prevent, the onset of MLDS-diabetes, and prolong islet graft survival in the ROD model.

Keywords: Diabetes, Interleukin-1 trap, Simvastatin, Nitric oxide synthase, Cytokine, Pancreatic islet, Streptozotocin, Transplantation

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“Every now and then go away, even briefly, have a little relaxation, for when you come back to your work your judgment will be surer, since to remain constantly at work will cause you to lose power.”

–Leonardo da Vinci

“If you can’t explain it to your grandmother, you don’t really understand it yourself.”

–Albert Einstein
List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


III Rydgren T, Börjesson A, Sandler S. Administration of a murine interleukin-1 cytokine trap counteracts the recurrence of disease following syngeneic islet transplantation to diabetic NOD mice. (Manuscript)

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumine</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphatase dehydrogenase</td>
</tr>
<tr>
<td>GAD65</td>
<td>Glutamic acid decarboxylate 65</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A</td>
</tr>
<tr>
<td>IA-2</td>
<td>Islet antigen-2</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IgG1</td>
<td>Immunoglobulin G1</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1RI</td>
<td>Interleukin-1 receptor type I</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-1RAcP</td>
<td>Interleukin-1 receptor accessory protein</td>
</tr>
<tr>
<td>sIL-1RII</td>
<td>Soluble interleukin-1 receptor type II</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun terminal kinase</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function associated antigen-1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLDS</td>
<td>Multiple low doses of streptozotocin</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nonobese diabetic</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ROD</td>
<td>Recurrence of disease</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetyl-d,L-penicillamine</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
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</table>
Type 1 diabetes mellitus (T1DM) is considered to be one of the most common chronic diseases in childhood and adolescents. The incidence of T1DM is increasing globally by ~3 % per year and is predicted to exceed 30 per 100000 in many different populations by the year 2010 (1). T1DM is regarded as a complex autoimmune disorder which results in life-long requirement of insulin therapy. It is believed that an immune reaction selectively destroys the insulin producing β-cells in the pancreatic islets.

In the general white population, the risk for a person to sometime in life develop T1DM is ~0.4 % (2), while for monozygotic twins, where one has T1DM, the risk for the other one to later in life develop the disease is 30–40 % (3, 4). The fact that the risk is higher in monozygotic twins compared to the general population, but at the same time not 100 %, suggests that the cause of this autoimmune destruction of β-cells is a combination of genetic susceptibility and different environmental factors.

The human leukocyte antigen (HLA) gene region, and especially the HLA class II molecule, has been shown to be a major genetic determinant of T1DM. Since HLA class II molecules participate in antigen presentation, the mechanism of HLA-influenced susceptibility in T1DM is believed to involve antigen presentation to CD4⁺ T cells, thymic selection and immune responsiveness. For example, normal, or even protective, forms of the HLA molecule may form stable complexes with self antigens in the thymus, leading to efficient deletion of potentially autoreactive T cells, while in contrast, HLA molecules that are predisposing for T1DM may form less stable complexes which may result in inefficient T cell removal and the release of autoreactive T cells to the periphery (5, 6).

The importance of environmental factors to the disease risk is further supported by the seasonal and geographical variation of the incidence of T1DM. Most new cases of the disease occur in autumn and winter (7) when viral infections are most common, and the incidence of T1DM among French and Jewish children living in Canada is higher than their counterparts living in France and Israel (8). Suggested environmental factors include congenital rubella infection (9), infections by enteroviruses (10, 11), bovine milk ingestion (12) or even the early ingestion of cereals (prior to 3 months of age) (13, 14). The mechanism by which these environmental factors might induce autoimmune β-cell destruction is not fully understood. Some of the suggested hypotheses, however, include bystander autoimmunity (viruses)
and molecular mimicry (viruses and dietary products). The bystander hypothesis, posits that virus infects the β-cells without destroying them, but, when these virus infected cells are discovered and destroyed by the immune system, and the person in question has a genetic predisposition for T1DM, this could result in subsequent activation of resting autoreactive T cells. The molecular mimicry hypothesis suggests that sequence similarities between viral protein and/or dietary products and β-cell autoantigens could cause a β-cell specific immune response by generation of cross-reactive cytotoxic T cells or autoantibodies. Such sequence similarity exists between the β-cell antigen glutamic acid decarboxylase (GAD65) and a coxsackie B4 virus protein as well as between two certain epitopes of the β-cell antigen tyrosine phosphatase IA-2 and proteins of milk and wheat.

The hygiene hypothesis has also been much discussed as a cause of the increased T1DM incidence seen in western countries in later years. This concept suggests a correlation between the increase in autoimmune diseases, the decline in infectious diseases and the progress in hygiene in these countries. The mechanisms of the protective effect of infections on autoimmune diseases, mostly derived from animal models, include e.g. antigenic competition and immunoregulation (15). Concerning antigen competition, it has been known for a long time that immune responses to single antigens are usually stronger than the response to the antigen administered concomitantly with other antigens. It has also been postulated that the strong immune responses elicited by infectious agents, compete with immune responses against weaker antigens such as autoantigens and allergens, for homeostatic signals (15). Regarding the mechanism of immunoregulation, it has been suggested that regulatory cells stimulated by infectious agents may extend to dampen autoimmune responses as well as the infection. Also, in a study by Alyanakian et al. it was shown that transforming growth factor (TGF)-β was involved, and natural killer cells could be involved, in protecting mice against diabetes after the administration of a Gram-positive bacterial extract (16).

The resulting destruction of β-cells in T1DM is thought to be initiated by the presentation of β-cell specific autoantigens, by antigen presenting cells (APC), to CD4+ T helper (Th) cells. These APC release interleukin (IL)-12 which stimulates CD4+ Th cells to secrete interferon (IFN)-γ and IL-2. The secreted IFN-γ and IL-2 subsequently stimulate invading macrophages and β-cell autoantigen specific CD8+ cytotoxic T cells to kill the β-cells. Macrophages through the release of e.g. cytokines, such as IL-1β and tumor necrosis factor (TNF)-α, nitric oxide (NO) and free oxygen radicals and CD8+ cytotoxic T cells by the release of perforin and granzyme and by Fas-mediated apoptosis (17–19).
Streptozotocin

Streptozotocin (STZ) is a naturally occurring antibiotic that was first isolated from the bacteria *Streptomyces achromogenes* (20). STZ is composed of the cytotoxic moiety, 1-methyl-1-nitrosourea, attached to the C-2 position of D-glucose. During preclinical screening for the use of STZ in cancer chemotherapy, this since it had previously been demonstrated that other nitrosoureas had potent anticancer properties, it was found to be diabetogenic in rats and dogs (21) and later also in a variety of laboratory animals including mice, guinea pigs and hamsters (22, 23). The glucose moiety seems to be the essential component that specifically directs the STZ toxicity in pancreatic islets to the β-cells, sparing α- and δ-cells (24), since analogs of streptozotocin whose sugar moieties have been replaced by mannose, galactose, or α-O-methylglucose have been reported to be nondiabetogenic (25, 26). Evidence suggests that the uptake of STZ into rodent β-cells is mediated by the glucose transporter 2 (GLUT2) (27, 28). In contrast, humans predominately express the GLUT1 isoform on their β-cells (29), with very low or no affinity for STZ as a transport substrate (27). This could explain why streptozotocin is not toxic to human fetal pancreatic β-cells (30) and why patients with endocrine tumors that are treated with STZ do not develop diabetes.

Once inside the cell, STZ spontaneously decompose to form an isocyanate compound and a methyldiazoxyhydroxide (31). The latter decomposes further to form a highly reactive carbonium ion which is able to alkylate various cellular components such as DNA and proteins. Okamoto et al. has shown that this DNA alkylation by STZ trigger DNA repair mechanisms that involve the NAD degrading enzyme poly(ADP-ribose) synthetase, and hypothesize that, if STZ is given at cytotoxic doses, the subsequent depletion of the cellular NAD pool depresses cellular function and destroys the β-cells (32–36). Wilson et al. (37) hypothesize that concomitant with activation of poly(ADP-ribose) synthetase is a drop in ATP formation, most likely due to alkylation of enzymes vital for the generation of ATP (e.g. glycolytic or mitochondrial enzymes). This fall in ATP would impair the resynthesis of NAD, resulting in levels below critical.

The Multiple Low Dose Streptozotocin Model

In 1976, Like and Rossini published the first description of a new mouse model of T1DM, later called the multiple low dose streptozotocin (MLDS) induced diabetes model, in male CD-1 mice (38). Outbred CD-1 male mice, of 8 weeks of age, were given a daily dose of 40 mg/kg bodyweight of STZ for 5 consecutive days. In this model, that was later discovered to function in C57BL/KsJ mice as well (39), mice start to develop hyperglycemia and insulitis approximately 7 days after the first injection. MLDS induced diabetes
has been reported to be T lymphocyte dependent (40–43) and it is suggested that the pathogenesis of this model requires a cell mediated immune response, elicited by direct STZ-mediated β-cell injury.

In contrast, treatment with a single high dose of STZ (100 mg/kg body-weight) to CD-1 mice has been reported to cause hyperglycemia within 72 hours with only 2 out of 20 mice showing signs of mononuclear cell infiltration in the islets of Langerhans (insulitis), 1 week after administration of the drug (44).

Nitric oxide (NO) production

NO can be produced from L-arginine, by the enzyme nitric oxide synthase (NOS), in many different mammalian cells. The enzyme exists in 3 different isoforms: neuronal NOS (nNOS), first discovered in neuronal tissue, endothelial NOS (eNOS), first found in vascular endothelial cells and inducible NOS (iNOS), which is inducible by different agents in a variety of cells and tissues and produce a large amount of NO that in this case often function as a cytostatic and cytotoxic molecule (45). These isoforms are usually differentiated either on the basis of their Ca\(^{2+}\)-dependence (eNOS and nNOS) or Ca\(^{2+}\)-independence (iNOS) or on the basis of their constitutive (eNOS and nNOS) versus inducible (iNOS) expression.

Nitric oxide in type 1 diabetes (T1DM)

The iNOS isoform has been suggested to be involved in β-cell destruction in T1DM and it has been shown that iNOS is expressed both in rodent and human β-cells and that NO is produced in these cells in response to cytokines (46, 47). Moreover, in the NOD mouse, it has been shown that both β-cells and activated macrophages up-regulate the expression of iNOS during the pre-diabetic insulitis in the pancreatic islets (48, 49). Also, iNOS knockout mice were shown to be resistant to diabetes in the multiple low doses of streptozotocin (MLDS) T1DM model (50).

However, studies on human pancreatic islets have shown them to be less sensitive to cytokine induced NO production than murine pancreatic islets (47). This raises questions as to the pathophysiological relevance of NO in immune mediated β-cell death in humans. A recent study by Størting et al (51) showed that NO, from an NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP), may aggravate cytokine signaling via pro-apoptotic JNK in human β-cells. This suggest that NO may represent one of several factors contributing to the life-death balance in human β-cells.
Garvey et al were the first to identify a “highly selective” iNOS inhibitor called 1400W (N-(3-(aminomethyl)benzyl)acetamidine; Figure 1) (52). Until then all iNOS inhibitors tested on different disease models were at best “partially selective” or “highly selective” but only for one of the two constitutive isoforms (45). This selectivity for iNOS is necessary in order to isolate the effects mediated by iNOS in a disease model. It is also important to have as good selectivity as possible, in case it ever becomes a therapeutic drug, in order to prevent side effects that otherwise might arise by the inhibition of other isoforms.

Figure 1. Chemical structure of 1400W.

The nonobese diabetic (NOD) mouse

The NOD mouse originated from a cataract mouse observed among outbred ICR/Jcl mice in Japan 1966, which gave rise to the cataract Shionogi (CTS) strain (53, 54). The suspicion of a pathogenetic relationship to diabetes arose after noting, at an early generation of inbreeding, that mice without cataracts displayed elevated levels of fasting blood glucose (55). These mice were selectively bred in the hope of creating a mouse model for spontaneous diabetes development. At the same time a co-selected control line, exhibiting normal fasting blood glucose levels, was also selectively bred. Surprisingly, it was in this normoglycemic control line that the first case of spontaneous autoimmune type 1 diabetes occurred. Fortunately, the female mouse that first showed overt diabetes had given birth to two litters prior to manifestation of diabetes. From these and some animals from the high fasting blood glucose substrain the NOD mouse strain became established.

The spontaneous autoimmune diabetes that this strain develops shares many similarities to human T1DM, e.g. the presence of pancreas-specific autoantibodies, autoreactive CD4⁺ and CD8⁺ T cells (recognized antigens include e.g. GAD65 and IA-2) and the fact that the major histocompatibility complex (MHC) class II, corresponding to HLA class II in humans, contributed the main component of disease susceptibility (56).
The incidence of spontaneous diabetes in the NOD model is approximately 60–80% in females and much lower in males (57, 58). The onset of diabetes normally occurs at 12–14 weeks of age in female mice (and slightly later in male mice), but can occur as late as 25 weeks of age, or later (59). Infiltration of mononuclear cells into the periphery of the islets (perinsulitis) starts as early as 4 weeks of age in both genders (56). These infiltrations further progress to invade the whole islet during the subsequent few weeks, which results in marked insulitis in most animals by 10 weeks of age.

The recurrence of disease (ROD) model in NOD mice

In this model recently diabetic NOD female mice receive syngeneic transplants of a curative number of isolated pancreatic islets, under the left kidney capsule, from normoglycemic young male NOD mice. If these transplants are successful, the mice will become normoglycemic within 1–2 days and without any treatment, recurrence of disease (i.e. hyperglycemia as a result of the destruction of insulin producing islet β-cells) will occur within approximately 1 week (60). Evidence suggests that this destruction is caused by an autoimmune reaction and not by graft rejection (60–62). The fact that this model represents a way to control the autoimmune onset of hyperglycemia in NOD mice, makes it a valuable tool in searching for new treatment protocols for human T1DM and ROD in humans transplanted with pancreatic islets.

Cytokines and antagonism of cytokines

Cytokines are considered to be the coordinators of the immune system and as such play important roles in host defense against various pathogens. However, pro-inflammatory cytokines, such as IL-1, TNF-α and IFN-γ, has also been associated with tissue destruction in autoimmune disease and consequently represents important targets for immunomodulation.

These cytokines have also been suggested to be involved in the pathogenesis of T1DM, based on studies in the NOD mice, the BB rat, and in the pancreata of T1DM patients. It has also been well documented, in studies on pancreatic islets in vitro, that they are cytotoxic to β-cells, in pM-nM concentrations and can cause β-cell death in both rodent (alone or in combination), and human pancreatic islets (in combination) (63, 64).

In studies on cytokine gene expression in mononuclear cells infiltrated in the pancreatic islets of NOD mice, IFN-γ mRNA levels were found to correlate with β-cell destructive insulitis (65, 66). Additional evidence of a role for IFN-γ in T1DM comes from the finding that transgenic expression of IFN-γ by β-cells in normal mice leads to an immune-mediated insulitis, β-
cell destruction and diabetes (67). Furthermore, monoclonal antibodies to IFN-γ have been found to protect against diabetes development in NOD mice (68, 69) and BB rats (70).

In a study by Yang et al. the administration of an anti-TNF-α monoclonal antibody to NOD mice before age 3 weeks, decreased insulitis and completely prevented diabetes, whereas treatment started later (age >4 weeks) was less effective (71). In the same study, systemic administration of TNF-α to NOD mice before age 3 weeks accelerated diabetes development, while TNFα treatment after age 4 weeks decreased diabetes incidence. This suggest that TNF-α may promote the early development of autoimmunity but suppress an already established diabetogenic process. The role of TNF-α in the development of T1DM was further supported by the fact that transgenic expression of a soluble TNF receptor p55-human FcIgG3-fusion protein resulted in decreased insulitis and were completely protected from spontaneous diabetes as well as accelerated forms of the disease caused by the transfer of NOD spleen cells or cyclophosphamide injections (72).

Treatment with a soluble IL-1 receptor (73) as well as treatment with a polyclonal anti-IL-1β antibody (74) reduced the incidence of cyclophosphamide induced diabetes in NOD mice. In these two studies the insulitis was not reduced in treated mice, which is consistent with the view that IL-1 contributes to the final effector mechanisms of β-cell destruction and therefore IL-1 neutralization would not be expected to affect mononuclear infiltration (75). It has also been reported that administration of an IL-1 receptor antagonist (IL-1Ra) could protect transplanted syngeneic pancreatic islets against ROD in NOD mice (for as long as continuous administration was upheld) as well as, delay diabetes onset in BB rat (60, 76).

Today, the only agent approved for human treatment, against rheumatoid arthritis, that targets IL-1 is the IL-1Ra (anakinra, Kineret®). One of the disadvantages with this drug is its poor pharmacokinetic properties, with a relatively short half life in humans, resulting in the need for daily injections or intravenous infusions.

In 2003, Economides et al. described the creation of very high affinity cytokine blockers, called cytokine traps, for cytokines that use two distinct receptor components for cell signaling, e.g. IL-1, IL-4 and IL-6 (77). The IL-1 trap described consists of the extracellular domain of IL-1 receptor type I (IL-1RI) and IL-1 receptor accessory protein (IL-1RAcP) arranged inline and fused to the Fc-portion of IgG1. These Fc-portions then form disulphide-linked homodimers so that every IL-1 trap consists of two sets of the protein construct (Figure 2). An IL-1 trap consisting entirely of mouse protein was also reported to reduce inflammation and joint bone erosion in mice with collagen-induced arthritis (77). Furthermore, in a small, initial phase I trial of a human IL-1 trap, patients displayed a dose-dependent fall in serum C-reactive protein, an inflammatory marker of RA (78). In this study the administration of the IL-1 trap was well tolerated and pharmacokinetic analysis
indicated a half-life of 7.5 days, which is compatible with administration once a week or less.

![Figure 2. Schematic outline of an IL-1 trap (modified from ref. 77).](image)

**Th1/Th2 cytokines**

In 1986, Mossman et al. suggested a division of Th cells into two populations, Th1 and Th2, with contrasting and crossregulating cytokine profiles (79). The Th1 and Th2 cells patterns of cytokine production were originally described in mouse CD4$^+$ T cell clones (79, 80) and later among human T cells (81). Depending on their co-stimulatory conditions, naïve CD4$^+$ T cells can differentiate into Th1 or Th2 cells. In mice, Th1 cells produce cytokines like IL-2, IFN-γ and IL-12, while Th2 cells produce e.g. IL-4, IL-5 and IL-10 (56). Human Th1 and Th2 cells produce similar patterns, but some, like IL-2 and IL-10, are not as tightly restricted to a single subset as in mouse T cells (82).

Th1 effector response is considered to be involved in disease progression in NOD mice, while Th2 effector response is believed to help suppress the diabetes development (83, 84). Although this is perhaps an oversimplification of existing data, many studies in NOD mice have demonstrated that factors that help promote Th1 over Th2, or vice versa, can directly affect diabetes susceptibility. For example, the cytokine IL-12, one of the most important inducer of Th1 responses, has been shown to accelerate diabetes onset in NOD mice (85). Also, injection of IL-4 or other Th2 cytokines or overexpression of Th2 cytokines in the islets can help protect against diabe-
tes in NOD mice (86). However, there are some contradicting data, such as the fact that transfer of Th2 polarized cells into lymphopenic NOD SCID recipients can still induce diabetes (87).

Simvastatin

Simvastatin (Figure 3) belongs to a class of drugs called 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, a.k.a. statins. Statins effectively lower serum cholesterol levels and are widely prescribed for the treatment of hypercholesterolemia. They are known to block the conversion of HMG-CoA to mevalonate, the rate limiting step in the cholesterol synthesis pathway, by inhibiting the enzyme HMG-CoA reductase. This leads to reduced synthesis of cholesterol and also to a decreased post-translational lipid modification (prenylation) of a variety of proteins such as small GTP-binding proteins (88).

![Chemical structure of simvastatin](image)

*Figure 3. Chemical structure of simvastatin.*

Anti-inflammatory effects of statins

Clinical trials show that statins reduce the risk of stroke even though high cholesterol is not considered to be a risk factor for stroke (89). Statins have also been shown to reduce the incidence of acute rejection episodes and to significantly improve the long term survival rates of heart, as well as kidney, transplant recipients (90–92). Detailed analysis of clinical trials with patients, with or without coronary artery disease and with or without elevated cholesterol levels, show that statins can reduce cardiovascular-related mor-
bidity and mortality, and that the clinical benefits of statins manifest much earlier than the effects of other cholesterol-lowering therapies (e.g. ileal by-pass surgery) although similar reductions in plasma cholesterol levels are achieved (93–97). Since inflammatory processes are thought to play a key role in the pathogenesis of coronary heart disease, stroke and transplant rejection, it has been hypothesized that statins have direct anti-inflammatory effects that are independent of their effects on plasma cholesterol levels. Several studies in animal models have also showed that statins exert anti-inflammatory effects by interfering with endothelial adhesion and transendothelial migration of leukocytes to sites of inflammation (98–102). These effects have been shown to be both of an HMG-CoA reductase dependent and, more recently of an, HMG-CoA reductase independent nature.

The HMG-CoA reductase dependent effects could either be attributed to the reduced prenylation of intracellular proteins or to the reduction of intracellular cholesterol and they have been shown to be reversible by the addition of isoprenoids (necessary for prenylation of proteins) or mevalonate (98, 100, 103–108). Some of these effects that statins have been shown to exert include inhibition of e.g.; chemokine (MCP-1) expression in activated leukocytes and endothelial cells (103); integrin (LFA-1, Mac-1 and VLA-4) expression (104–106) and integrin (VLA-4) activation in leukocytes (107); and ICAM-1 expression in stimulated endothelial cells (108) and on monocytes (106).

Lovastatin, and later also simvastatin and mevastatin, were found to inhibit LFA-1 by an allosteric mechanism (109, 110). The inhibition is caused by binding to the lovastatin-site (L-site), a hitherto unknown site in LFA-1 located distant from the ICAM-1-binding site, which most likely results in a stabilization of the receptor in an inactive conformation thus preventing the conformational transition to its high-affinity ligand-binding form. This inhibition results in decreased LFA-1-mediated leukocyte adhesion to ICAM-1 as well as decreased T-cell co-stimulation.
Aims

The general aim of this thesis was to study possible strategies to prevent type 1 diabetes mellitus in different experimental models and more specifically:

1. To study if the highly selective iNOS inhibitor 1400W could prevent interleukin 1β (IL-1β) induced suppression of pancreatic islet function \textit{in vitro} and MLDS-induced diabetes \textit{in vivo}.
2. To test a new high-affinity blocker of IL-1 action, so-called IL-1 trap \textit{in vitro} in preventing suppressive and cytotoxic effects to rat pancreatic islets induced by IL-1β alone or in combination with TNF-α and IFN-γ.
3. To examine if administration of a murine IL-1 trap could prolong islet graft survival in the ROD model in NOD mice.
4. To investigate the effect of administration of the HMG-CoA reductase inhibitor simvastatin in protection against MLDS-induced diabetes and prolongation of islet graft survival in the ROD model in NOD mice.
Materials and methods

Animals

Adult, male Sprague Dawley rats (Biomedical Centre, Uppsala, Sweden), C57BL/Ks mice (Biomedical Centre, Uppsala, Sweden), CD-1 mice (Charles River, Uppsala, Sweden) and NOD mice (Biomedical Centre, Uppsala, Sweden) were used. The animals had free access to tap water and pelleted food throughout the study. All experiments were approved by the local animal ethics committee for Uppsala University.

Islet isolation and culture

Pancreatic islets from rats and mice were isolated by a collagenase digestion procedure. The pancreas was removed and distended by injection of Hanks’ balanced salt solution (HBSS; SBL vaccin, Stockholm, Sweden) and cut into small pieces. The pancreatic tissue was then transferred to vials, containing collagenase, and vigorously shaken until the tissue was disintegrated. The digest was then washed 3 times with HBSS and the islets subsequently handpicked under a stereo microscope using a braking pipette. Groups of 150 islets were cultured free-floating in 5 ml medium RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10 % foetal calf serum (FCS) (vol/vol; Sigma-Aldrich), L-glutamine (Sigma-Aldrich), benzylpenicillin (100 U/ml; Roche Diagnostics Scandinavia, Bromma, Sweden) and streptomycin (0.1 mg/ml; Sigma-Aldrich). Islets were precultured for 5–7 days for the in vitro experiments (study I, II and IV), and 3–5 days for the transplantation experiments (study III and IV), with medium changes every second day. All islet cultures and incubations were kept at 37 °C in humidified air + 5 % CO₂ in these studies, except when noted otherwise.

Islet treatments

In study I and II, we cultured groups of 50 Sprague Dawley rat islets in a volume of 2.5 ml for 48 h as described above, with different additives. Islets cultured without additives were used as control. After this 48 h culture medium samples were collected and analyzed for nitrite and insulin contents (as
described below) while the islets were subjected to one or more of the following experiments (as described below): Determination of islet glucose oxidation rate, glucose induced insulin release, insulin content, DNA content, islet (pro)insulin biosynthesis and total protein biosynthesis.

In study I, three of the groups were cultured with 1400W (1, 10 and 50 μM; Alexis Biochemicals, Lausen, Switzerland), one with IL-1β (25 U/ml; PeproTech, London, U.K.), and another three groups with IL-1β and the different concentrations of 1400W.

In study II, one group was cultured with IL-1β (150 pM corresponding to 25 U/ml), three of the groups were cultured with different concentrations of IL-1 trap (150, 1500 and 15000 pM corresponding to 1, 10 and 100 times the IL-1β concentration), one group was cultured with Fc protein (150000 pM corresponding to 1000 times the concentration of IL-1β), three groups were cultured with a combination of IL-1β and the different IL-1 trap concentrations, and one group was cultured with IL-1β and the Fc protein. In another experimental setup in this study, one group was cultured with a cytokine mix, consisting of IL-1β (25 U/ml), TNF-α (1000 U/ml) and IFN-γ (1000 U/ml), one group with the cytokine mix and IL-1 trap (100 times the concentration of IL-1β) and one group with the cytokine mix and the Fc protein (1000 times the concentration of IL-1β). The IL-1 trap and Fc protein were kindly provided by Dr. Margaret Karow and colleagues (Regeneron Pharmaceuticals, Tarrytown, NY).

In study IV we incubated groups of 35 CD-1 mouse islets in sterile Krebs-Ringer buffer supplemented with 10 mM HEPES (Sigma-Aldrich, Stockholm, Sweden) (KRBH), 5.6 mM glucose and 2 mg/ml bovine serum albumine (BSA) (ICN Biomedicals, Aurora, OH, USA), first, for 30 min, with or without simvastatin (60 and 600 μg/ml) and then, for another 30 min, with saline or STZ dissolved in saline (to a final concentration of 1.8 mM). After rinsing and removal of the medium the islets were cultured overnight in medium RPMI 1640 supplemented with 10 % FCS. The islets were then subjected to glucose induced insulin release experiments and analyzed for insulin content (as described below).

Analysis of medium nitrite content

In study I, a volume of 10 μl of freshly prepared Greiss reagent (0.5 % naphtylethlenediamine dihydrochloride, 5 % sulfanilamide and 25 % H₃PO₄) was added to 100 μl samples of culture medium and incubated for 2 min in a water bath at 60 °C. The light absorbance of the reaction products were then measured by a spectrophotometer (λ = 546 nm) and the nitrite concentrations were calculated by the use of standard curves. All determinations (including the standard curves) were performed in triplicates.
In study II, a volume of 100 μl of reagent solution, made from 10 ml 100 % acetic acid, 300 μl 10 % sulfanilamide (dissolved in acetone), and 30 μl naphtylendiamine (dissolved in redistilled H₂O) was added to 250 μl samples of culture medium and incubated for 15 min at room temperature (20 °C). The light absorbance of the reaction products were then measured at 540 nM in a Labsystems iEMS spectrophotometer and the nitrite concentrations were calculated by the use of standard curves. All sample determinations were performed in duplicates and the standard curve in triplicates.

Determination of islet glucose oxidation rate

In study I and II, groups of 10 islets were incubated in glass vials containing 100 μl of KRBH supplemented with D-[U-¹⁴C]glucose (Amersham-Pharmacia Biotech, Amersham, UK) and non-radioactive glucose to give a final concentration of 16.7 mM. The vials were placed in glass scintillation vials, with their shafts squeezed through rubber membranes, gassed with 95 % O₂ and 5 % CO₂ and sealed tightly. The vials were then incubated in a slow-shaking water bath (30 strokes/min) at 37 °C for 90 min before the glucose oxidation was terminated with 100 μl of 0.05 mM antimycin A (Sigma-Aldrich) (dissolved in 99.9 % ethanol), injected through the membrane into the inner vial. In the same way 100 μl of 0.4 M Na₂HPO₄ (pH 6.0) was injected to release the CO₂ formed. The CO₂ was then trapped by injection of 250 μl Hyamine 10-X (New England Nuclear, Boston, MA, USA) into the outer vial during incubating for another 2 h in a water bath at 37 °C. Finally, the inner vial were removed and 5 ml of Ultima Gold scintillation liquid (Packard Instrument, Meriden, CT, USA) was added to the outer vial before the radioactivity was measured by liquid scintillation counting. Triplicate samples were used for all determinations, and vials without islets were used as blank.

Determination of glucose induced insulin release, medium insulin accumulation, islet insulin and DNA content

In study I we used a rat insulin ELISA (Mercodia, Uppsala, Sweden) for all insulin determinations. To determine the glucose induced insulin release we incubated duplicate groups of 10 islets in 100 μl KRBH, supplemented with 16.7 mM glucose and 2 mg/ml BSA, for 90 min before the incubation medium was collected for insulin determination. After the incubation the islets were pooled to groups of 20 and ultrasonically disrupted in 200 μl redistilled water.
In study II and IV we used a high range rat insulin ELISA (Mercodia, Uppsala, Sweden) for all insulin determinations. To determine the glucose induced insulin release we incubated triplicate groups of 10 islets in 100 μl KRBH, supplemented with 2 mg/ml BSA and 1.7 mM glucose for 1 h, collected the incubation medium for insulin determination and transferred the islets to 100 μl KRBH, supplemented with BSA and 16.7 mM glucose for another 1 h. Again, the incubation medium was collected while the islets were pooled in groups of 30 and ultrasonically disrupted in 200 μl redistilled water.

In these studies, we then mixed a 50 μl volume of the islet homogenate with 125 μl of acid ethanol (0.18 M HCl in 96 % (vol/vol) ethanol) and the islet insulin content was extracted at 4 ºC overnight. In study I we also used another two 50 μl aliquots of the homogenate to determine the DNA contents of the islets by a flophosphometric method (111).

Determination of islet (pro)insulin biosynthesis and total protein biosynthesis

In study I, duplicate groups of 10 islets were incubated for 2 h in 100 μl KRBH, supplemented with 2 mg/ml BSA, 16.7 mM glucose and 50 μCi/ml L-[4,5-3H]leucine (Amersham-Pharmacia Biotech, Uppsala, Sweden). After incubation the islets were washed in HBSS containing 10 mM non-radioactive leucine and ultrasonically disrupted in 200 μl redistilled water.

From each sample 4 x 10 μl aliquots of the homogenate were transferred to Eppendorf tubes for (pro)insulin analysis according to Halban et al (112). We added 100 μl of glycine buffer, with Triton X-100, to the tubes before adding 10 μl of guinea pig anti-bovine insulin serum (ICN Biochemicals, Cosa Mesa, CA, USA) to two of the tubes and 10 μl of guinea pig serum (for non-specific antibody binding) to the other two. The tubes were then mixed vigorously and incubated at room temperature for 1 h. The tubes were then mixed again and 100 μl of protein A-Sepharose (Amersham-Pharmacia Biotech, Uppsala, Sweden) were added before incubating for another 15 min during slow shaking. The samples were centrifuged (30 s at 500 g) and washed twice with 0.5 ml glycine buffer, containing Triton X-100 before the pellets were resuspended in 2 x 250 μl acetic acid solution and transferred to scintillation vials. We added 4 ml Ultima Gold scintillation liquid and counted the samples in a liquid scintillation counter. A mean was calculated for the two tubes with added guinea pig serum and subtracted from the calculated mean for the two tubes with added anti-insulin serum to obtain a measure of the (pro)insulin biosynthesis.

For the analysis of total protein biosynthesis, we transferred 2 x 10 μl aliquots from the islet homogenate to Eppendorf™ tubes. We added 250 μl of
glycine solution and 250 μl of trichloroacetic acid and mixed vigorously. The samples were centrifuged (10 min at 2000 rpm), the supernatant removed and the pellets dissolved in 2 x 250 μl of 0.15 M NaOH and transferred to scintillation vials before 4 ml Ultima Gold scintillation liquid was added and the samples counted in a liquid scintillation counter.

**Determination of total plasma nitrite and nitrate in vivo**

In study I, male and female C57BL/Ks mice, 10–17 weeks old, were injected intraperitoneally with either saline (0.2 ml) or lipopolysaccharide (LPS) (500 μg; 0.2 ml) dissolved in saline. Three hours later, the mice received another intraperitoneal injection with either saline or 1400W (14 mg/kg body weight; 0.2 ml) dissolved in saline. After another 3, 6 or 10 h, retro-orbital venous blood samples were collected before the mice were killed by cervical dislocation. Samples were centrifuged at 4000 g for 5 min and plasma collected. Total nitrite and nitrate levels were determined by the use of the spectrophotometric assay kit BIOXYTECH NO-540 (Oxis Research, Portland, OR, USA) according to the manufacturer’s instructions. This assay kit uses cadmium to reduce nitrate to nitrite before using the Greiss reaction and measuring the light absorbance at 540 nm.

**Cell viability assessment by flow cytometry**

In study II, we did additional experiments in which islets in groups of 50 were cultured for 48 h, as described above. One group with a cytokine mix, consisting of IL-1β (25 U/ml), TNF-α (1000 U/ml) and IFN-γ (1000 U/ml), one group with the cytokine mix and IL-1 trap (100 times the concentration of IL-1β), one group with the cytokine mix and the Fc protein (1000 times the concentration of IL-1β) and one group without additives were used as control. After this, the islets were incubated at room temperature (20 °C) with propidium iodide for 15 min and then dissociated with 0.05 % trypsin in HBSS. Changes in light scattering and fluorescence emission were determined by flow cytometry with a Becton Dickinson FACSCalibur equipped with CellQuest software (Becton Dickinson, San Jose, CA, USA). A total of 5000–10000 cells per sample were analyzed by exciting the cells at 488 nM and examining fluorescence at 650 nm. Propidium iodide stains apoptotic and necrotic cells, the latter with high intensity. As a cell shrinks or loses volume, forward-scattered light decreases. Untreated cells were gated on a forward-scatter versus propidium iodide fluorescence dot plot, and the same gates were then applied to cytokine-treated cells for calculation of dead islet cells, i.e. the fractions of apoptotic and/or necrotic cells.
Multiple low dose streptozotocin (MLDS)

C57BL/Ks (study I) and CD-1 mice (study IV) were injected intraperitoneally with either 40 mg/kg body weight streptozotocin (STZ) or vehicle once a day for 5 consecutive days. In all the MLDS studies performed the time for the first STZ injection constituted day 1. STZ was dissolved in either citrate buffer (study I) (pH 4.5; 0.2 ml) or saline (study IV) (0.2 ml) immediately before administration.

In study I we injected the mice intraperitoneally with vehicle (saline; 0.2 ml) or 1400W, dissolved in saline (5.9 mg/kg body weight; 0.2 ml), either once a day or twice a day (14 mg/kg body weight; 0.2 ml/injection) for 10 consecutive days, starting treatment on day 1. The injections with 1400W/vehicle were administered 30 minutes after injections with STZ/vehicle. In the case when 1400W were given twice a day, there were ~10 hours between the first and the second injection.

In study IV we injected the mice intraperitoneally with vehicle (0.2 ml) or simvastatin, dissolved in vehicle (30 mg/kg body weight; 0.2 ml), once a day. The treatment was started on day 4 and continued until day 14. Simvastatin was dissolved in vehicle as described by Leung et al (113). Briefly, simvastatin was dissolved in 1 part ethanol and 1.5 part 0.1 N NaOH and incubated at 50 °C for 2 h. The pH was adjusted to 7 with HCl and the volume adjusted to a total of 10 parts by adding saline. The simvastatin/vehicle was, when administered the same day as the STZ/vehicle injections, injected 30 minutes after STZ/vehicle.

The mice were killed for morphological examination either on day 14 (study I and IV) or on day 35 (study IV). Blood glucose measurements were performed, on blood from tail vein of non fasting mice, on days 0 (before any injection), 3, 7, 10 and 14 (study I and IV) and on days 17, 21, 24, 28, 31 and 35 (study IV). Blood glucose was measured with a blood glucose meter (Medisense, London, UK). The mice were considered hyperglycemic with a blood glucose concentration ≥11.1 mM. The mice were weighed on days 0, 7 and 14 (study I and IV) and on day 35 (study IV).

Recurrence of disease (ROD)

In study III and IV, pancreatic islets from normoglycemic male NOD mice were isolated and precultured as described above, after which 600 islets were transplanted under the left kidney capsule to newly diabetic female NOD mice. The islet recipients were anesthetized with an intraperitoneal injection of Avertin (a 2.5 % (v/v) solution of 10 g 97 % 2,2,2-tribromo-ethanol in 10 ml 2-methyl-2-butanol; Kemila, Stockholm, Sweden). Treatments started on day 0, the day before transplantation, and were then administered once a day, until ROD occurred.
In study III, the mice were treated with subcutaneous injections of either mouse IL-1 trap (30 mg/kg bodyweight; 0.1 ml), or an equimolar dose mouse Fc-control protein (8.4 mg/kg bodyweight; 0.1 ml), or saline (0.1 ml).

In study IV, the mice received intraperitoneal injections with vehicle (0.2 ml) or simvastatin (30 mg/kg bodyweight; 0.2 ml) dissolved in vehicle.

The treatments were maintained until ROD, i.e. until the transplanted mice was hyperglycemic (blood glucose >11.1 mM) for two consecutive days, at which time the mice were killed and, in the case of study III, the spleens and the graft bearing kidneys were removed.

Morphologic examination of pancreatic glands
In study I and IV, the pancreatic glands were removed, fixed in 10% formalin for 24 h, embedded in paraffin and cut into 5 μm thin sections. The sections were stained with hematoxylin and eosin and examined under a light microscope. The pancreatic islet histology was ranked into four arbitrary classes, where class A denotes normal islet structure; class B mononuclear cell infiltration in the peri-insular area; class C heavy mononuclear cell infiltration into a majority of islets (i.e. insulitis); and class D only a few residual islets present, often showing an altered architecture and pyknotic cell nuclei (114). The examiner was unaware of the origins of the sections.

Morphologic examination of islet grafts
In the ROD experiment, in study III, the graft bearing kidneys were fixed in 10 % formalin for 24 h, embedded in paraffin and cut into 5 μm thin sections. The sections were stained with hematoxylin and eosin, and some also for insulin, and then examined under a light microscope. The grafts were examined concerning immune cell infiltration and insulin-positive staining. The islet graft morphology was ranked into four arbitrary classes and in the case of immune cell infiltration 0 denotes a graft with no apparent infiltration of mononuclear cells, + denotes a graft with infiltration in <1/3 of the sectioned graft area, ++ denotes infiltration in 1/3-2/3 of the sectioned graft area and +++ denotes infiltration in >2/3 of the sectioned graft area. In the case of insulin-positive staining, 0 denotes absence of insulin staining in the sectioned graft area, + denotes <25 % of insulin-positive cells in the sectioned graft area, ++ denotes 25–50 % of insulin-positive cells in the sectioned graft area and +++ denotes >50 % of insulin-positive cells in the sectioned graft area. The examiner was unaware of the origins of the sections.
Spleen cell preparations

After removal of the spleens, in study III, they were placed in cold HBSS. The spleens were punctured repeatedly with a pair of forceps to free the spleen cells. The resulting cell suspensions were centrifuged and the cell pellets resuspended in 1 ml 0.19 M NH₄Cl in 4 ºC for 10 min to lyse the erythrocytes before 5 x 10⁷ cells were prepared for RNA isolation.

RNA isolation and cDNA synthesis

In study III, spleen cells were lysed in RLT Lysis buffer from the RNeasy Mini Kit (Qiagen, KEBO, Spånga, Sweden) supplemented with 1 % (vol/vol) β-mercaptoethanol and subsequently stored at -70 ºC. The cell lysates were then further homogenized by the use of Qia Shredder (Qiagen) before total RNA was isolated from the samples using the “Spin Protocol for Isolation of Total RNA from Animal Cells” in the RNeasy Mini Kit (Qiagen) with RNase free DNase (Qiagen).

Conversion of mRNA to cDNA was performed with Reverse Transcription System (Promega, SDS, Falkenberg, Sweden) using 9 μl RNA. The reaction volume for each cDNA synthesis was 20 μl and contained 5 mM MgCl₂, reverse transcription buffer (10 mM Tris-HCl, 50 mM KCl, 0.1 % Triton® X-100), 1 mM dNTP mix, 1 U/μl recombinant Rnasin® ribonuclease inhibitor, 15 U/μl AMV reverse transcriptase, and 0.5 μg oligo(dT)₁₅ primer. The reactions were incubated for 60 min at 42 ºC followed by 5 min at 99 ºC after which the samples were stored in -20 ºC.

RT-PCR assay

The LightCycler System (Roche) was used for semi-quantitative analyses of cDNA in real time with the SYBR® Green JumpStart™ Taq ReadyMix™ used for detection. RT-PCR reactions were performed in a total volume of 10 μl, containing 1μl cDNA sample, 2.4 μl sterile H₂O, 5μl SYBR Green JumpStart Taq ReadyMix (containing Taq DNA Polymerase, dNTP mix, JumpStart Taq antibody and SYBR Green I dye), 0.5 μM each of forward and reverse primer, and 0.6 μl of a 25 mM MgCl₂ stock solution. Each RT-PCR run started with a 10 min period at 95 ºC to activate the Taq polymerase. The amplification was done for 40 cycles, where each cycle consisted of a 15 s denaturation period at 95 ºC, a 10 s annealing period at 60 ºC, and a 15 s elongation period at 72 ºC. In the end of each RT-PCR run, after amplification, a slow heating from 60 ºC to 95 ºC were used for melting curve analysis. All samples were run in duplicates with TATA-binding protein (TBP), or when we analyzed the TNF-α mRNA levels with glucose-6-
phosphate dehydrogenase (G6PDH), used as house-keeping gene. To screen for possible contamination, two negative controls were included in each PCR-run, one for the house-keeping gene and one for the cytokine being studied. All primers used were purchased from TIB MOLBIOL Synthese-labor (Berlin, Germany). The primer sequences were:

**IL-1β** forward: 5’-AGC CCA TCC TCT GTG ACT CAT G-3’, reverse: 5’-GCT GAT GTA CCA GTT GGG GAA C-3’

**IFN-γ** forward: 5’-CAT CAG CAA CAA CAT AAG CGT CA-3’, reverse: 5’-CGC TGG ACC TGT GGG TT-3’

**IL-4** forward: 5’-ACA GGA GAA GGG ACG CCA T-3’, reverse: 5’-TGC AGC TTA TCG ATG AAT CCA G-3’

**TBP** forward: 5’-ACC CTT CAC CAA TGA CTC CTA TG-3’, reverse: 5’-ATG ATG ACT GCA GCA AAT CGC-3’

**TNF-α** forward: 5’-GAC CCT CAC ACT CAG ATC ATC TTC-3’, reverse: 5’-CGC TGG CTC AGC CAC TCC-3’

**G6PDH** forward: 5’-ATT GAC CAC TAC CTG GGC AA-3’, reverse: 5’-GAG ATA CAC TTC AAC ACT TTG ACC T-3’

Cycle threshold (CT) values (i.e. the cycle number when the fluorescence exceeds the baseline) were used to calculate $2^{-ΔCT}$, as previously described (115), in order to compensate for the doubling of PCR product at each cycle and present relative mRNA levels.

**Statistical analysis**

Data are presented as means ± S.E.M. In the *in vivo* experiments, groups of data were compared by one-way ANOVA, with subsequent all pairwise comparison procedures by Bonferroni t-test or Student-Newman-Keuls method. We used Fisher’s exact test to compare the proportion of hyperglycemic mice in the different experimental groups. In the *in vitro* experiments, groups of data were compared by one-way repeated measurement ANOVA, with subsequent all pairwise comparison procedures by Bonferroni t-test or Student-Newman-Keuls method. Cumulative islet graft survival was analyzed using the Kaplan-Meier survival curve and Logrank test, while data for mRNA levels were analyzed using one-way ANOVA with subsequent all pairwise comparison procedures by Student-Newman-Keuls method. A p-value of less than 0.05 was considered statistically significant. In the glucose-induced insulin release experiments, where islets were incubated in duplicates (study I) or triplicates (study II and IV), a mean was calculated for each experimental group and considered as one separate observation. Statistical analysis was performed using SigmaStat® (SPSS Inc., Chicago, IL, USA).
Results and discussion

Study I

It has previously been reported that iNOS -/- mice displayed a reduced sensitivity against MLDS induced diabetes (50). Earlier studies with administration of iNOS inhibitors in this T1DM mouse model that have been able to prevent diabetes onset (116, 117), have employed NOS inhibitors that are not particularly selective (45). This study was therefore designed to examine if the administration of a highly selective iNOS inhibitor, called 1400W, could prevent IL-1β induced suppression in vitro and protect against MLDS in vivo.

We show in this study that the highly selective iNOS inhibitor 1400W, at the highest concentration used (50 μM), could counteract the suppressive effects of IL-1β on rat islet function in vitro. The inhibitor fully prevented the suppression of glucose oxidation rate, (pro)insulin biosynthesis and the rise in nitrite accumulation caused by IL-1β. In spite of the latter, the IL-1β induced suppression of medium insulin accumulation and glucose induced insulin release was only partly counteracted by 1400W, which suggest that this cytokine damage to the insulin release mechanism is in part NO independent. This is in line with earlier findings that cytokines can cause NO independent damage in both human, rat and mouse β-cells (47, 118–120). An NO independent impairment of insulin release capacity could also explain the increased insulin content seen in islets treated with IL-1β and 1400W compared to control treated islets. If the iNOS inhibitor can not prevent the IL-1β damage to the insulin release mechanism, but prevents other suppressive effects of IL-1β, this could result in an increased accumulation of insulin in islet β-cells.

Despite our promising results in vitro, 1400W administration was not able to prevent MLDS-induced diabetes in vivo and it did not prevent the infiltration of mononuclear cells into the pancreatic islets in C57BL/Ks mice. Not even with our second protocol used in which we increased the daily dose of the inhibitor almost 5 times, administered as two separate injections per day, MLDS could be prevented. The reason why we in our second protocol gave the injections twice daily was that a study by Parmentier et al came to our attention (121). In this study they used 8 h intervals between subcutaneous injections of 1400W to rats. The treatment was based on experiments in which the reducing effect of 1400W on ischemia-induced iNOS activity
lasted for 6 h, but not 9 h, after injection. In our study we show that 1400W can inhibit LPS-induced nitrite and nitrate production in vivo at 3, 6 and 10 h after administration, although, at the latest time point one might observe a tendency towards a less effective inhibition. However, the reason for the negative results in our in vivo assays should not be the pharmacokinetic properties of 1400W since we compensated for this in our second protocol, which was also confirmed by the LPS experiments just mentioned. As an alternative explanation, we therefore suggest either that NO, produced by iNOS, does not have a decisive role in this T1DM model, or that the bioavailability of 1400W in β-cells is not sufficient. The latter has to our knowledge never been studied in vivo, thus we can not exclude the possibility that our MLDS experiments failed due to an insufficient uptake of 1400W within the islet β-cell. The role of NO in this and other T1DM animal models has been much debated with reports of evidence both in favor of (48–50, 116, 117, 122–124) and against (125–127) its involvement in β-cell destruction.

Study II

It was recently reported about the development of a new high-affinity blocker of cytokine action, called cytokine traps (77). These are a kind of soluble receptors for cytokines such as IL-1 that use two distinct receptor components.

We wanted to test the efficacy of such a trap for IL-1 in counteraction of suppressive and cytotoxic effects after exposure of rat pancreatic islets in vitro to IL-1β. This could form a basis for testing it in a relevant animal model for T1DM. The cytokine trap we used in this in vitro study was made of human protein components, since we frequently use human recombinant IL-1β in our in vitro studies.

The suppressive effects induced by IL-1β on the medium insulin accumulation, glucose oxidation rate and medium nitrite accumulation, was fully prevented with the use of the IL-1 trap at 10-fold and 100-fold molar excess compared to the IL-1β concentration, but not when we used the trap at the same concentration as IL-1β. When we analyzed the insulin release in islets that had been cultured with the different additives for 48 h, we found no significant differences between the groups at low glucose. Still, there was a trend towards a decreased insulin release in islets treated with IL-1β. At high glucose, however, there was a marked decrease in insulin secretion in IL-1β treated islets and this was prevented with the IL-1 trap at all tested concentrations. Even though, with the lowest concentration (1 x IL-1β), the protection tended not to be complete.

It might have been expected that this concentration (1 x IL-1β) would have protected better against the suppressive effects of IL-1β. However,
previous results using a human MRC5 fibroblast cell line (77) indicate that when the IL-1 trap is given at a concentration 4 times that of IL-1β, about 5% of the IL-1β bioactivity remain. Thus, it is possible that at the 1:1 ratio we used, a small fraction of free IL-1β still exists and causes the suppressive effects seen in our experiments. In fact, a 10–20 times lower concentration of IL-1β than we used, has previously been shown to cause suppression of rat pancreatic islet function (128).

We also tested the efficacy of the IL-1 trap in preventing cell death induced by a combination of cytokines (IL-1β, TNF-α and IFN-γ). The cytokine mixture caused a 4 times increase in islet cell death compared to untreated islets during a 48 h culture and this was completely prevented by the addition of IL-1 trap (100 x IL-1β). This suggests that it might be adequate to block the effect of IL-1β alone to prevent cell death induced by a combination of cytokines.

We also did similar 48 h cultures, after which we analyzed islet function as before. Here, the cytokine mixture caused a decrease in medium insulin accumulation, affected the glucose stimulated insulin release (increased the insulin release at low glucose and decreased the insulin release at high glucose), and decreased glucose oxidation rate. Effects that were completely prevented with the addition of IL-1 trap, although, the decrease in insulin released at high glucose incubations tended not to be completely prevented. Furthermore, the cytokine mixture increased the medium nitrite accumulation, which was only partly prevented with the addition of IL-1 trap. The fact that the IL-1 trap tended not to fully prevent the effect of the cytokine mixture on glucose induced insulin release, could be explained by the fact that we observed this small increase in nitrite production in this group.

In all of these experiments, cytokine trap or Fc protein, alone, at the different concentrations used, did not have any effect on the islet functions analyzed.

The addition of Fc protein, to islets treated with IL-1β, did not protect against any of the IL-1β - or cytokine mixture - induced suppressions reported here. This suggest that the protective effects of the IL-1 trap that we have seen here corresponds to its specific inhibition of IL-1β, rather than the addition of a nonspecific protein or via cytokine binding to the Fc part of the cytokine trap.

Study III

Based on the results in our previous study with the IL-1 trap, we decided to test a murine IL-1 trap on the ROD model in NOD mice. We found, with the Kaplan-Meier survival curve and Logrank test that the islet graft survival in mice treated with IL-1β was significantly prolonged compared to mice treated with Fc protein and also tended to be different compared to saline
treated mice. When the two control groups were merged the IL-1 trap still significantly prolonged islet graft survival. In mice treated with IL-1 trap the mean onset of ROD tended to be delayed compared to mice treated with saline or mice treated with Fc protein. However, in the IL-1 trap treated group there were three mice that distinguished themselves in that they lasted much longer until ROD (28–33 days) compared to the others (3–12 days), which suggest an “all or nothing” effect of the IL-1 trap in this model.

A recent report by Gnacadja et al. (129) could offer one possible explanation for an “all or nothing” effect. In this article they start by laying down the facts that IL-1β can bind to both its receptor (IL-1RI) and IL-1 trap, and that IL-1Ra can bind to both IL-1RI and the IL-1 trap, which results in four possible binding combinations. After this, they present mathematical evidence, which is also confirmed experimentally in vitro, that given a certain relationship among the concentrations of these different proteins and the equilibrium constants of the possible binding reactions between them, the ability of the IL-1 trap to bind IL-1β is reduced by the ability of IL-1Ra to bind to IL-1 trap, resulting in an increased concentration of IL-1β, free to exert its effect in vivo. According to Gnacadja et al. the conditions necessary for this to occur fall within a physiologically realistic range. Since we do not know the concentrations of the involved proteins in and around the transplanted islets in our ROD study, we can not exclude this possible explanation. Also, it has been reported that increased levels of soluble interleukin-1 receptor type II (sIL-1RII), was higher in patients with nondestructive arthritis than in patients with destructive arthritis (130). Perhaps this endogenous regulator of IL-1 activity could also have a role in the outcome of our study as well. Thus, at a given moment, IL-1 activity in vivo may be regulated by a number of molecules and balances, and a possible therapeutic result following IL-1 trap administration may be difficult to predict (Figure 3).

Another potential explanation for this effect could be differences in general health of the animals, depending on e.g. duration of hyperglycemia prior to islet transplantations and differences in phenotypes despite similar genotypes, which could be of relevance for e.g. the severity of the disease.

When we performed morphological examinations of the grafts retrieved after ROD onset we saw no obvious differences in insulin positive staining or immune cell infiltration between the different treatment groups. The heavy mononuclear cell infiltrations seen in most of our histological preparations were consistent with the fact that all mice had returned to their hyperglycemic condition when the grafts were retrieved.
Figure 3. Schematic diagram of four possible binding reactions involving IL-1, IL-1 trap, IL-1RI and IL-1Ra. As Gnacadja et al. (129) reported, at a certain relationship between the concentrations of the different agents and the equilibrium constants for their binding reactions, it is possible that IL-1Ra by binding to IL-1 trap could reduce the amount of IL-1 binding to IL-1 trap so that more IL-1 would be able to exert its effect \textit{in vivo}. This, and perhaps also the presence of soluble IL-1RII molecules, an endogenous IL-1 regulator, could have a role in the outcome of study III.

In this study we also removed the animals’ spleens after ROD onset, in order to isolate mononuclear cells from them and analyze their mRNA levels with RT-PCR. Here we found that the relative mRNA level of IL-4 was increased in mice treated with IL-1 trap compared to mice treated with saline or Fc protein. This increase suggests a shift towards Th2-cytokine production in these transplanted mice and if so, could be a contributing factor to the increase in graft survival seen with the IL-1 trap treatment.

If the spleens had been removed, and perhaps islets grafts as well, and analyzed for mRNA levels prior to ROD onset, it can not be excluded that additional differences in relative levels of cytokine mRNA would have been seen. Also, morphological examinations of grafts, at a time point prior to ROD onset could perhaps result in differences in insulin staining and/or mononuclear infiltration between the different treatment groups.
Study IV

Simvastatin is a frequently prescribed cholesterol lowering drug (e.g. Zocor®) that has been suggested to have anti-inflammatory properties independent of its cholesterol-lowering effects (110) and has been reported to markedly inhibit both developing, and a clinically evident, collagen induced arthritis in DBA/1 mice (113), a Th1-driven model of murine inflammatory arthritis. Since there are evidence that suggest that both NOD diabetes development and MLDS induced diabetes are Th1-driven (82, 128), we wanted to investigate the effect of simvastatin administration on MLDS induced diabetes in CD-1 mice as well as on the ROD model in NOD mice.

We found that simvastatin could delay, and in some mice prevent, the onset of hyperglycemia induced by MLDS in CD-1 mice. MLDS treatment induced an increase in mean blood glucose concentrations from day 10 onwards, compared to control, which, in part, was counteracted by the treatment with simvastatin. All mice (n=12) treated with STZ + vehicle were hyperglycemic on day 14, while 5 out of 14 mice treated with STZ + simvastatin were still considered normoglycemic and the proportions of hyperglycemic mice between these two groups were significantly different when compared with Fisher’s exact test. When we did a morphological examination of the pancreas on day 14, we could see a tendency towards less mononuclear cell infiltration in mice treated with STZ + simvastatin (2 ranked C, 4 ranked B and 2 ranked A) compared with mice treated with STZ + vehicle (4 ranked C and 2 ranked B).

In another set of experiments we investigated if this protective effect could be maintained after the administration of simvastatin was discontinued. The mice were kept untreated for 21 days after the treatment with simvastatin had ended. Here the protective effect remained until day 28 when the mean blood glucose levels of mice treated with STZ + simvastatin were no longer significantly different from that of mice treated with STZ+ vehicle. However, it should be noted that 2 out of 6 mice in the STZ + simvastatin group were still normoglycemic, at the end of the experiment on day 35, while all the mice in the STZ + vehicle group were hyperglycemic at day 14 onwards. Morphologic examination of the pancreas on day 35 showed, once again, a tendency towards less mononuclear cell infiltration in mice treated with STZ + simvastatin (3 ranked D and 3 ranked A) compared with mice treated with STZ + vehicle (5 ranked D and 1 ranked A).

Our experiments in vitro show that simvastatin could not prevent the inhibition of glucose induced insulin release of mouse pancreatic islets, caused by a high dose of streptozotocin. At first sight it might appear that simvastatin could partly prevent the effects of streptozotocin on insulin release at 16.7 mM glucose, but this is most likely due to leakage of insulin from damaged islet β-cells. This since the ratio of relative stimulation of insulin release differs between the control group and the group treated with STZ +
simvastatin (low dose), but not between the latter and the group treated with STZ only. Also, when we examined the islets under stereomicroscope, after the incubations, it was clear that simvastatin itself caused a dose-dependent destruction of the islets and that the destructive effect was even augmented when simvastatin and streptozotocin was combined. This was also in line with the results from the analyzed islet insulin contents.

Mice that were treated with simvastatin in the ROD model showed a delayed onset of ROD (13.1 ± 2.6) compared to mice treated with vehicle (7.2 ± 0.5). On day 7 in this experiment, 5 of 8 vehicle treated mice displayed ROD, but none of the simvastatin treated mice (n=8) did (a significant different when compared with Fisher’s exact test). In the simvastatin treated mice, the last mouse to become hyperglycemic again reverted on day 29.

The results from our in vitro study suggest that the protective effect of simvastatin in vivo is of an immunomodulatory nature rather than the result of a direct effect on the streptozotocin induced β-cell damage. This view was further reinforced by the results from the ROD study.

The apparently toxic effect of simvastatin to pancreatic islets in vitro was not evident in our in vivo studies with the protocols we used in the MLDS or ROD model. However, in a pilot study, using a protocol adopted from a study in a rheumatoid arthritis DBA/1 mouse model by Leung et al (113), in which we used a higher dose of simvastatin (40 mg/kg body weight and the mice were treated from day 1–14), about 1/3 of the animals treated with simvastatin displayed swollen abdomens before treatment was ended. As these mice were killed we observed enlarged intestines as well as adhesions between intestines. The mice that did not show signs of swollen abdomens during treatment, when killed on day 14, did display similar intestinal changes, although, of milder forms. Since Leung et al did not report any such toxicity we thought it possible that CD-1 mice were more sensitive to simvastatin than DBA-1 mice. However, in a study by Palmer et al. (131) in which they reproduce the protective effects of simvastatin in the collagen-induced arthritis model in DBA-1 mice, they also report of severe side effects of the treatment with similar symptoms as in our pilot study (described above). Autopsies showed macroscopic signs of severe peritonitis in simvastatin treated mice and histologic examination revealed severe subacute inflammation of the peritoneum and infiltration of inflammatory cells into the parenchyma of the liver and spleen. These side effects led to somewhat increased serum levels of glucocorticoids, which according to the authors would explain the beneficial effects of simvastatin in this model. Hence, although we lowered the dose of simvastatin, from 40 to 30 mg/kg body-weight, and did not see any obvious signs of toxicity, we can not exclude the possibility that the effects seen in our models were due to the same side effects.
Conclusions

Study I
- The highly selective iNOS inhibitor 1400W could fully, or partly, prevent IL-1β induced suppression of rat pancreatic islet function \textit{in vitro}. The latter suggests NO independent damage by IL-1β.
- 1400W was not able to protect against MLDS induced diabetes in C57BL/Ks mice with the protocols used, even though it could reduce LPS induced NO production in the same strain.

Study II
- The human version of the recently developed IL-1 trap, a high-affinity blocker of IL-1, could prevent the suppressive effects of human recombinant IL-1β on rat pancreatic islet function in vitro.
- The IL-1 trap could also protect against rat pancreatic islet cell death, induced by a combination of IL-1β, TNF-α and IFN-γ, and fully, or partly, prevent the suppressive effects on rat pancreatic islet function induced by the same cytokine combination.

Study III
- The murine version of the IL-1 trap could prolong islet graft survival in the ROD model in NOD mice.
- Mice treated with IL-1 trap also displayed a higher relative mRNA level of IL-4, compared to control treated mice, in isolated spleen cells retrieved after ROD onset. This suggests a shift towards Th2 cytokine production, which may partly explain the protective effect of the IL-1 trap.

Study IV
- CD-1 mice treated with the HMG-CoA reductase inhibitor simvastatin showed a delayed onset of MLDS induced diabetes.
- Simvastatin appeared toxic to mouse pancreatic islets \textit{in vitro}, and this effect was augmented by the addition of streptozotocin, which suggest that the protective effect of simvastatin in the MLDS model was not due to a direct effect on streptozotocin induced β-cell damage.
- Simvastatin was also shown to prolong islet graft survival in the ROD model in NOD mice.
- Pilot experiments with a higher concentration of simvastatin caused toxic effects in CD-1 mice with or without MLDS treatment.
Målsättningen med doktorsavhandlingen har varit att i olika experimentella modeller för typ 1 diabetes studera olika möjliga sätt att förhindra denna sjukdom.


I detta arbete undersökte vi om 1400W, en ny selektiv hämmare av inducerbart kväveoxid syntas (iNOS), kunde förhindra skadliga effekter orsakade av interleukin (IL)-1β på isolerade Langerhanska öar från råtta. Vi fann att 1400W, vid en koncentration på 50 μM, fullständigt kunde förhindra både minskningen i metabolisering av glukos och (pro)insulinbiosyntes samt högningen av nitritackumulering i odlingsmediet. Vidare kunde vi notera att 1400W endast delvis kunde förhindra de hämmande effekterna på glukos-stimulerad insulinfrisättning och insulinackumulering i odlingsmediet. Detta stämmer väl med tidigare rapporter att IL-1β kan orsaka kväveoxidoberoende skador på Langerhanska öar. Vi använde oss också av en väletablerad djurmodell för typ 1 diabetes, kallad MLDS, för att testa effekten av 1400W in vivo. I denna djurmodell ger man 5 på varandra dagliga injektioner av en låg dos streptozotocin, i vårt fält, C57BL/Ks möss, för att inducera en autoimmun diabetes inom 14 dagar. Vi fann att administrering av 1400W ej kunde förhindra uppkomsten av diabetes, inte med något av de protokoll vi använde. Detta trots att 1400W kunde förhindra ökningen av nitrit och nitrat i plasma som inducerats av LPS i samma musstam. Slutsatsen i denna studie är att antingen har inte kväveoxidbildningen den betydelse vi trodde för uppkomsten av diabetes i denna modell eller så är biotillgängligheten av 1400W otillräcklig i och runt om de Langerhanska öarna.


I detta delarbete undersökte vi om en ny högaffinitetsblockerare av IL-1, en s.k. IL-1-fångare, kunde förhindra, såväl IL-1β-inducerade skadliga effek-
ter på isolerade Langerhanska öar från råtta, som celldöd och skadliga effekter på isolerade råttöar orsakade av en kombination av IL-1β, tumor necrosis factor (TNF)-α och interferon (IFN)-γ. Våra resultat visade att IL-1-fångaren, vid 10 gånger den molära koncentrationen av IL-1β, helt kunde förhindra de skadliga effekter vi annars såg med IL-1β. Vidare kunde IL-1-fångaren även helt förhindra celldöd i de Langerhanska öarna, vilket visar att det kan vara tillräckligt att blockera IL-1β för att förhindra celldöd som orsakas av en kombination av cytokiner. IL-1-fångaren tenderade att inte helt kunna förhindra skadorna på glukosstimulerad insulinrättning på grund av cytokinkombinationen. Denna tendens kan förmodligen hänföras till IL-1-fångarens förmåga att endast delvis skydda mot cytokinkombinationens ökning av nitritackumulationen i odlingsmediet.

Delarbete 3: Rydgren T, Börjesson A, Sandler S. Administration of a murine interleukin-1 cytokine trap counteracts the recurrence of disease following syngeneic islet transplantation to diabetic NOD mice. (Manuscript)

I det tredje delarbetet behandlar vi frågan om subcutana injektioner av en gnagarvariant av IL-1-fångaren kan påverka transplantatöverlevnaden i ROD (recurrence of disease)-modellen i NOD (nonobese diabetic)-möss. I denna modell transplanteras friska Langerhanska öar från NOD-hannar in under den vänstra njurens bindvävskapsel i, nyligen insjuknade, diabetiska NOD-möss. Vi visar att IL-1-fångaren kunde förlänga transplantatöverlevnaden i transplanterade NOD-möss och vidare att den relativa mRNA nivån av cytokinen IL-4 var förhöjd i dessa möss. Det sistnämnda tyder på att cytokinproduktionen verkar ha tagit en Th2 (T-hjälparcell typ 2)-inriktning, vilket delvis skulle kunna förklara den skyddande effekten av IL-1-fångaren.


I det sista delarbetet har vi undersökt möjligheten att med hjälp av simvastatin, en HMG-CoA reduktashämmare, förhindra uppkomsten av diabetes i MLDS-modellen och/eller förlänga gräftpoverlevnaden i ROD-modellen. Simvastatin är ett väletablerat läkemedel för behandling av hyperkolestero-lemi som på senare tid även har visat sig ha anti-inflammatoriska egenskaper, t.ex. genom att påverka leukocytes transendoteliala migration ut till inflammationsområdet. Vi visar i detta arbete att administrering av simvastatin kan försvaga, och i vissa fall helt förhindra, uppkomsten av diabetes i MLDS-modellen. Vi visar också att denna skyddande effekt av simvastatin inte beror på en direkt effekt på streptozotocinets β-cellsskadande verkan, utan snarare p.g.a. en påverkan på immunsvaret som uppkommer i denna modell. Denna teori var också förstärkt av resultatet av den andra delen av studien.
där vi visar att simvastatin även kan förlänga tiden för graftöverlevnad i ROD-modellen.
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References

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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”.)