Exploration of Conditions Affecting Cytokine Production in Experimental type 1 Diabetes Mellitus

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Abstract


Cytokines are soluble signalling mediators within the immune system, and have been shown to be of importance in the development of type 1 diabetes (T1D). This thesis studied the production of cytokines in experimental models of T1D and during transplantation of insulin-producing islets of Langerhans.

We have demonstrated that the transcriptional TNFα-inhibitor MDL 201,449A, previously shown to reduce immune-mediated diabetes induced in mice by multiple low doses of streptozotocin, was not TNFα-specific, but also inhibited IFNγ and IL-10 in spleen cells. Furthermore, when the inhibitor was removed from in vitro cultures, a rebound phenomenon of increased cytokine secretion occurred.

The thesis also investigated whether plastic adhesion, a method generally employed to deplete macrophages, influenced cytokine production in spleen cells. We observed that plastic adhesion increased TNFα, IFNγ and IL-10 release, and decreased IL-4 secretion. Plastic adhesion depleted only ~30% of the macrophages, but as much as ~60% of the regulatory T cells.

Thirdly, we found that “control” treatments for islet transplantations, i.e. syngeneic and sham transplantations, exerted a clear effect on cytokine production from spleen cells, possibly due to a decrease in regulatory T cells that may be caused by the surgery and/or anaesthesia. Moreover, spleen cells from mice exposed to surgery exhibited a decreased proliferative capacity to concanavalin A stimulation. We also perceived a marked difference in cytokine response depending on the mouse strain used in the experiments.

Finally, we aimed to elucidate if, besides autoimmune activities, also high glucose- and free fatty acid concentrations as seen in diabetes could cause changes in cytokine production. We observed that spleen cells cultured in varying glucose concentrations had different cytokine production profiles. The free fatty acid palmitate might also influence cytokine release, but this effect was obscured by the cytokine-suppressive action of the ethanol used to dissolve the palmitate.

Keywords: Cytokine, Diabetes, Tumor necrosis factor-α, Interferon-γ, Interleukin-4, Interleukin-10, Transplantation, Pancreatic islet, Glucose, Regulatory T cell

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


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Abbreviations

ANOVA  Analysis of variance
APC  Antigen presenting cell
CD25  Cluster of differentiation 25
COX  Cyclooxygenase
EAE  Experimental autoimmune encephalomyelitis
ELISA  Enzyme-linked immunosorbent assay
ER  Endoplasmic reticulum
FACS  Fluorescence activated cell sorter
FCS  Fetal calf serum
FFA  Free fatty acids
FoxP3  Forkhead box protein 3
G6PDH  Glucose-6-phosphate dehydrogenase
IFNγ  Interferon gamma
IL  Interleukin
i.p.  Intraperitoneal
MLDSTZ  Multiple low dose streptozotocin
NF-κB  Nuclear factor kappa B
NO  Nitric oxide
NOD  Non-obese diabetic
PBS  Phosphate buffered saline
ROS  Reactive oxygen species
RT-PCR  Reverse transcriptase polymerase chain reaction
s.c.  Subcutaneous
SCMC  Sodium carboxymethylcellulose
STZ  Streptozotocin
TGF-β  Transforming growth factor beta
Th1  T helper 1
Th2  T helper 2
TNFα  Tumour necrosis factor alpha
TLR  Toll-like receptor
Introduction

Diabetes mellitus is a disease characterised by elevated blood glucose levels, increased urinary production, thirst and fatigue. The disease is ultimately caused by inability of the insulin-producing $\beta$-cells in the islets of Langerhans in the pancreas to produce sufficient insulin and can be fatal if left untreated [1]. Serious complications, such as cardiovascular disease, renal failure, retinal and neural damage may arise from long-term diabetes.

There are two major types of the disease. In type 1, or insulin-dependent diabetes mellitus, the insulin-producing $\beta$-cells are destroyed by immune cells, which lead to a subsequent total insulin deficiency. The patients, who usually are young when the disease breaks out [2], are entirely dependent on exogenous insulin treatment for survival. Type 2 diabetes, or non-insulin-dependent diabetes mellitus, is caused by a decreased $\beta$-cell sensitivity to insulin and insufficient insulin secretion, and has a strong connection to obesity, lack of exercise and other environmental factors [3]. It usually has a slower course of events and is more common among overweight adults. Type 2 diabetes can often, at least initially, be treated with diet and exercise.

Unfortunately, type 1 diabetes is increasing worldwide. In Sweden, the incidence between 1990 and 1999 was 30 per 100 000/year, which is the third highest in the world after Finland and Sardinia, and it increased by 3.6% annually [4]. There is a clear genetic factor in the susceptibility of the disease, with a 20 to 40% concordance in monozygotic twins [5, 6], but different environmental factors, such as viruses [7], are also believed to be of importance.

Years before the clinical onset of type 1 diabetes, signs of inflammation such as autoantibodies can be evident in individuals with a high genetic risk of diabetes [1]. Insulitis, i.e. infiltration of immune cells in the islets, is believed to be responsible for the selective destruction of the $\beta$-cells. The causes for this autoimmune process are not as yet clarified, but cytokines have been postulated to have an important role in disease development [8, 9]. Much of the knowledge about type 1 and type 2 diabetes originates from studies in animal models, which are often used to investigate mechanisms for the pathogenesis and prevention of the disease.
The Multiple Low Dose Streptozotocin Model

Streptozotocin (STZ) was originally isolated from fermentation broth from the bacterial strain *Streptomyces achromogenes* variant 128 in 1959 [10, 11]. It exhibited good antibacterial activity against both gram-positive and gram-negative bacteria and was initially used as an antibiotic. However, the compound was soon found to induce diabetes in rats and dogs [12] and was eventually also shown to be carcinogenic [13]. The diabetogenic properties led to its use as an animal model for human diabetes, by injecting mice or rats with a single dose of the substance, which results in a β-cell specific toxic effect, thus inducing hyperglycaemia.

The multiple low dose streptozotocin (MLDSTZ) model for human type 1 diabetes was first described in 1976 by Like and Rossini [14]. It was shown that by injecting mice with five daily low (40 mg/kg b.w.) doses of streptozotocin, the mice developed a delayed hyperglycaemia and infiltration of immune cells into the pancreatic islets after 10-14 days, symptoms and signs that are also seen in human type 1 diabetes. It was subsequently demonstrated that male mice had a much higher susceptibility to the diseases [15-17], which seemed to correlate with testosterone levels.

STZ is taken up into the cells by the glucose transporter GLUT2. It brings about β-cell death by alkylating the DNA, resulting in DNA fragmentation and excessive DNA repair leading to NAD depletion. STZ also generates reactive oxygen species and act as a nitric oxide (NO) donor, which may contribute to the DNA fragmentation [18].

There is a strong genetic component in the susceptibility to the disease, demonstrated by the fact that only certain strains of mice are susceptible to disease induction, primarily the CD-1 and C57BL/Ks mouse strains [19]. Both MHC and non-MHC genes appear to be of importance for disease development [20, 21]. There is also a prominent immune component of the response. The MLDSTZ model is dependent on T cell signalling via CD28, as CD28-deficient mice have been demonstrated to be protected from MLDSTZ-induced diabetes [22]. Moreover, the depletion of T cells by the administration of an anti-Thy-1.2 antibody also protect against MLDSTZ [23], and T cell-deficient mice do not develop diabetes [24]. In a study by Kim and Steinberg, T cells from MLDSTZ diabetic mice could not transfer the disease to healthy recipients unless the recipients had been pre-treated with a single low dose of STZ, so this effect seems to be dependent on modifications of STZ on the β-cell [24]. However, some β-cell aggression and also hyperglycaemia can be transferred by mononuclear spleen cells from MLDSTZ-treated mice [25-27]. There is also evidence supporting a critical role of macrophages in disease development. Silica treatment, which is specifically toxic to macrophages, has been demonstrated to prevent hyperglycaemia and insulitis [23, 28].
TNFα

The cytokine tumor necrosis factor alpha (TNFα) was first described in 1975 as a cytotoxic agent [29], and was eventually also found to induce fever [30]. It has been shown in vitro that heat-induced apoptosis is mediated by TNFα through the p55 TNFα receptor [31].

TNFα belongs to the TNF superfamily, which consists of at least 18 members, with implications in numerous conditions such as rheumatoid arthritis, inflammation, septic shock, viral replication, tumorigenesis, transplant rejection and diabetes [32, 33]. TNFα is secreted from macrophages, monocytes, neutrophilic granulocytes and Th1 cells [34] and it induces the expression of e.g. IL-1β and IL-6 [35, 36].

Drugs counteracting TNFα activity, e.g. Etanercept and Infliximab, are used to treat autoimmune conditions such as rheumatoid arthritis, ankylosing spondylitis and Crohn’s disease [37-40]. The treatment often improves the quality of life and slows down the disease. Unfortunately, an increased susceptibility to infections, in the case of Infliximab especially tuberculosis, is a common side effect. However, trials to investigate the possibility of using TNFα inhibitors to treat other inflammatory diseases are ongoing.

Expression of TNFα in pancreatic islets of non-obese diabetic (NOD) mice (another model of type 1 diabetes [41]) leads to a massive infiltration of immune cells (Th1, Th2 cells and B cells) into the pancreatic islet, but the insulitis does not progress to overt diabetes [42, 43]. Several attempts have been made to decrease diabetes development by regulating TNFα. The TNFα inhibitor troglitazone has been shown to inhibit diabetes development in both MLDSTZ-induced diabetes [44] and NOD diabetes [45]. Also, the transcriptional TNFα inhibitor MDL 201,449A decreases MLDSTZ-induced hyperglycaemia in mice [46]. NOD mice deficient in TNFα receptor are protected against disease [47] and administration of soluble TNF receptor decreases NOD diabetes [48]. It has also been observed that macrophages from diabetes prone BB rats secrete higher levels of TNFα than diabetes resistant or normal Wistar rats [49]. TNFα has also been shown in humans to inhibit the suppressive function of both naturally occurring CD4+CD25+ regulatory T cells and TGF-β1-induced CD4+CD25+ regulatory T cells. Treatment with Infliximab increased the FoxP3 expression and restored the suppressive function of the regulatory T cells [50].

MDL 201,449A

MDL 201,449A (9-[(1R, 3R)-trans-cyclopentan-3-ol] adenine), is an adenosine analogue that function as a transcriptional inhibitor of TNFα by binding the adenosine A₃ receptor. It thereby interrupts the endotoxin CD14 receptor signal transduction pathway, which blocks induction of TNFα [51, 52].
MDL 201,449A has been shown to reduce mRNA levels of TNFα by nearly 50% [53]. Administration of MDL 201,449A has in previous studies been shown to decrease MLDSTZ diabetes in mice [46]. Moreover, MDL 201,449A reduces autoimmune disease and joint destruction experimentally induced by injection of staphylococcal enterotoxin B [54].

Th1/Th2 Cytokines

T helper cells (Th cells) can be divided into different subsets, depending on the category of cytokines they produce. Th1 cells predominantly secrete IFNγ, TNFα and IL-2, whereas Th2 cells mainly release IL-4, IL-5 and IL-10. There are also Th3 cells, producing TGF-β, that act as regulators of effector T cells [55]. Th1 and Th2 cells are derived from naïve CD4+ (helper) T lymphocytes, which differentiate into an uncommitted cell type designated Th0 upon the contact with antigen-presenting cells (APC) under the appropriate co-stimulatory conditions. The cytokines present in the environment then influence the polarization of the Th0 cells into Th1 or Th2 cells. Th1 cytokines induce IL-12 production from APCs, which induce the further polarization into Th1 cells, while IL-4 produced by mast cells and eosinophils induce polarization into Th2 cells. The Th1 and Th2 cells also cross-regulate each other in that IL-10 release from Th2 cells down-regulate the polarization into Th1 cells and IFNγ release from Th1 cells inhibit Th2 polarization. There is also an autocrine regulation, whereas Th1 cells produce IFNγ, which further stimulate the Th1 differentiation, and Th2 cells secrete IL-4, that induce a Th2 polarization [8, 56].

The different cell subsets have different effects on diseases. A preponderance of Th1 cytokine secretion has been connected to development of autoimmune diseases such as type 1 diabetes and EAE (experimental autoimmune encephalomyelitis), whilst a Th2 profile has been observed in e.g. helminthic infections, systemic lupus erythematosus and allergy. Adoptive transfer of Th1 polarized cells cause accelerated diabetes and EAE compared to transfer of Th2 polarized cells [57-60]. In autoimmune diabetes, it has been observed that a β-cell destructive insulitis is connected to a Th1 cytokine profile, while a non-destructive insulitis and disease protection is associated with Th2 cytokine secretion [61]. It has also been shown that susceptibility to MLDSTZ in various mouse strains is linked to regulation of Th1/Th2 balance in the respective strain. C57BL/6 mice, in which MLDSTZ treatment induced a Th1 cytokine profile, are susceptible to disease, while the same treatment induced a Th2 response in diabetes-resistant BALB/c mice [62]. Various treatments that shift the cytokine profile in MLDSTZ from Th1 to Th2 has reduced diabetes incidence and decreased immune cell infiltration of the pancreatic islets [63-67]. It has been suggested that the higher diabetes incidence in female NOD mice could be a result of their
higher IFNγ production, while male NOD mice, that have a lower diabetes incidence, have a higher production of IL-4 [68].

However, there are studies indicating a greater complexity to the Th1/Th2 paradigm. The manner of cytokine administration seems to be of great importance to the effect it generates. Constitutive expression of IL-10 has been observed to result in a more severe diabetes in NOD mice and to induce immune cell infiltration into the pancreas of healthy mice [69, 70], whereas a regulated T cell specific expression of IL-10 provides protection from the disease [71]. IFNγ also has conflicting effects in different studies as a disruption of the IFNγ signalling pathway by inactivating the gene for interferon regulatory factor-1 results in an increased susceptibility to MLDSTZ-induced diabetes in mice.

Macrophages

One of the key cell types in development of autoimmune diabetes is believed to be the macrophages. These cells play an important role in the first line of defence against pathogens [72] and function as a link between innate and adaptive immunity. In insulitis development, macrophages are the first cells detected to infiltrate the islets, and depletion of macrophages can abort the following infiltration of T cells, NK-cells and B-cells [73, 74].

Macrophages are both antigen presenting cells and phagocytes, and exert effects e.g. by production of IL-12, TNFα and IFNγ [75]. By secreting IL-12, they regulate the Th1/Th2 cytokine balance towards a Th1-profile, and induce a β-cell-destructive insulitis. Macrophage depletion in NOD mice induces a shift towards Th2 cytokine production and prevents diabetes development [76]. Furthermore, macrophage secretion of cytokines might provoke free radical production from β-cells [77].

Depletion of macrophages has been shown to inhibit the development of autoimmunity in several animal models of diabetes. Administration of macrophage-toxic silica prevents insulitis and hyperglycaemia in NOD mice [78], BioBreeding (BB) rats [79] and MLDSTZ diabetic mice [23]. Moreover, macrophage depletion by administration of liposome-encapsulated dichloromethylene diphosphonate prevent diabetes in Kilham-virus induced autoimmunity in a diabetes-resistant strain of BB rats [80].

Regulatory T cells

Regulatory T cells are a subtype of T cells that have an essential role in the immune system by suppressing the function of effector T cells. Some autoreactive T cells always escape the selection in the thymus, and regula-
tory T cells suppress the activity of these cells, thereby preventing autoimmunity [81].

The regulatory T cells have received increasing attention since 1995, when Sakaguchi et al. demonstrated that depletion of CD4+ (helper) T cells expressing the IL-2 receptor protein CD25 induced a variety of autoimmune diseases in mice [82]. However, CD25 is also expressed on activated effector T cells, and therefore a more specific marker for T cells with a regulatory function was needed. In 2003 several groups simultaneously discovered that the Forkhead box protein 3 (FoxP3) could be used as such a marker [83-85]. FoxP3 is essential for the development and function of regulatory cells. A mutation in FoxP3 has been found to be the cause of the human IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X linked) syndrome [86]. Correspondingly, the disruption of this transcription factor induces a fatal lymphoproliferative disorder in mice, creating the scurfy mouse strain [87].

There are several populations of regulatory T cells. They can be produced as a functionally distinct population in the thymus, which often express FoxP3 and are referred to as the natural regulatory T cells [88]. There is also a large variety of regulatory T cells that can be induced in the periphery from naïve T cells, not all of which are expressing FoxP3 [89]. Research in this area is advancing very rapidly, and new knowledge about different subtypes of regulatory T cells is currently emerging month by month.

A possible role for regulatory T cells in disease control has been suggested in various conditions such as asthma, allergy [59] and type 1 diabetes [90]. However, these data are far from conclusive, and often suffer from the use of CD25 as a regulatory T cell marker. More recent studies using the FoxP3 marker have reported no difference in regulatory T cell populations between diabetic and healthy subjects [91]. Nevertheless, there are studies pointing to functional defects in CD4 CD25+ regulatory T cells in various human autoimmune diseases [92-94]. Also, siblings of diabetic patients carrying high-risk HLA alleles have decreased numbers of CD4+CD25+ T cells [95], so there is still hope that expansion of regulatory cells might be a way to ameliorate these diseases.

It has been shown that CD4+CD25+ T cells inhibit both Th1 and Th2 cytokine production [96], but with a higher suppressive effect on Th1 cells [97]. Few studies has been conducted regarding the role of regulatory T cells in MLDSPTZ, but Anastasi et al. has demonstrated that expression of a constitutively active Notch3 domain enhances the generation of regulatory T cells and protects against MLDSPTZ [98]. Several studies have been performed in NOD mice and it has been shown that these mice have low levels of CD4+CD25+ T cells [99] and also that there is a functional impairment of the FoxP3-expressing CD4+CD25+ cells in NOD mice [100]. The mechanism(s) by which the defective regulatory T cell action are mediated in NOD mice is still the subject of much research, but it has been proposed that a
failure of the NOD antigen-presenting cells to activate the regulatory T cells could be a contributing factor [101].

It has been proposed that regulatory T cells could be a means of inducing tolerance to allografts. If the regulatory T cells could be induced to accept the transplanted graft as self and suppress alloreactive T cell responses, additional immunosuppression might not be needed. There is much research being performed to investigate this possibility. Nanji et. al. has induced tolerance to islet allografts by blocking costimulation through CD40L and ICOS, which lead to increased levels of FoxP3-expressing CD4⁺CD25⁺ T cells [102]. Simultaneous transfer of regulatory T cells together with a heart or islet graft has been shown to prolong graft survival through the induction of tolerogenic CD4⁺CD25⁺ T cells in the recipient [103, 104]. It has also been demonstrated that tolerated heart allografts contain a large number of FoxP3- and CD25-expressing CD4⁺ T cells, and that these cells can confer tolerance to other grafts [105].

Islet Transplantation

Even though insulin treatment in many cases provides a well-functioning treatment for type 1 diabetes, about 3 in 1000 patients have major problems controlling the disease, resulting in recurrent and unpredictable hypoglycaemic episodes often resulting in hospitalization [106]. This leads to a lower quality of life, an increased risk for complications and a shortened life expectancy. These patients would benefit greatly from transplantation of either pancreas or islets of Langerhans. Whole pancreas transplantations usually results in a sustained and durable function, but entail a complicated surgical procedure [107]. Transplantation of isolated islets of Langerhans is a simpler procedure, as the islets are injected into the portal vein with only local anaesthetic. In the years 1990 to 1998 only 8% of all allogeneic islet transplantations resulted in insulin-independence one year post transplantation [108]. In 2000, however, Shapiro et al. published a new protocol for islet transplantation which showed dramatically improved results, with all seven patients still insulin-independent one year post-transplantation [109]. A follow-up study in 2005 reported that 10% of the patients transplanted according to this so-called Edmonton protocol remained insulin-independent after five years [110], and about 80% of the patients had detectable C-peptide levels and much improved glycemic control [111]. This protocol was different from earlier protocols in three major aspects. Firstly, in that it involved a glucocorticoid-free immunosuppressive regime instead of the more aggressive regimes used earlier. Secondly, instead of transplanting islets simultaneously with a kidney, these transplantations were comprised of islets only, and the operation was consequently much smaller and the patients were in a better general condition before the transplant. Lastly, each recipient received islets
from at least two, and sometimes three, donors to compensate for the amount of islets lost after transplantation.

Even though islet transplantation is becoming a more accepted treatment for unstable diabetes, the shortage of donors is limiting its use. There is much research regarding finding an alternate source of islets. A few clinical trials of xenotransplantation of porcine islets have been performed, but the immunological differences between humans and pigs demand strong anti-suppressive therapy, and there are concerns that porcine endogenous retroviruses might cause disease in humans [112, 113]. As an alternative source of insulin-producing cells, embryonic or adult stem cell differentiated in culture might be used, but these studies are still at an experimental stage [112].

The most difficult obstacle to overcome in the course of improving the results of clinical islet transplantation is graft rejection. The immunological barrier between donor and recipient cause the immune system of the recipient to launch an attack on the graft. This process is to a large extent dependent on T cell derived cytokine production. During the allogeneic rejection of a graft, cytokine release leads to activation and recruitment of allogeneic T cells to the graft. IL-2 and IFNγ are generally believed to be the principal cytokines involved in this process, in that IL-2 mediates T cell proliferation and both IL-2 and IFNγ promote the T cell cytokine production towards a proinflammatory Th1 profile [114]. However, there is conflicting evidence regarding the role of these cytokines in allograft rejection as their presence is sometime needed for graft survival [115, 116]. Several other cytokines, e.g. TNFα, IL-1, IL-4, IL-5 and IL-6 has been observed to increase in connection with allogeneic graft rejection [117-120]. Studies with the objective to decrease the levels of cytokines by depletion or inhibition of cytokine-producing Kupffer cells in islet transplantations to the liver [121] or treatment with the anti-inflammatory enzyme activated protein C [122] has reduced the loss of functional islet mass post transplantation.

Anesthesia and Wound Healing

During islet transplantations to mice, the recipients are subjected to several procedures that in themselves may have effects on the immune system and the body in general. The mouse is anaesthetized, a wound is made, surgery is performed, and the wound has to heal.

That anaesthesia has effects apart from the desired ones is well known. For example, Hindlycke and Jansson have observed that rat islet blood flow was affected differently by five different anaesthetic drugs [123]. Furthermore, Brown et al. have studied four anaesthetics; nembutal, pentothal, avertin (tribromoethanol) and ketamine/xylazine, and concluded that all of them increased the blood glucose levels in C56BL/6 mice [124]. Tribromoethanol has also been demonstrated to cause acute peritoneal inflamma-
tion and necrosis in muscles and abdominal organs [125]. These and other adverse effects, such as hepatotoxicity and visceral adhesions, have actually caused recommendations to the effect of restricting the use of tribromoethanol to acute terminal studies [126]. The increased intra-abdominal pressure caused by the intra-peritoneal injections employed when administering certain anaesthetics also increases the plasma concentrations of proinflammatory cytokines, which could cause further damage [127]. Anaesthesia has also long been known to cause modifications of the immune response [128]. For example, thiopentone anaesthesia and surgery has been observed to cause a decrease in lymphocyte proliferation in response to mitogen stimulation, with the depression being more pronounced if the surgical trauma is more extensive. However, five other anaesthetic drugs tested simultaneously did not affect the cellular function [129]. Cytokines, such as IL-6, IL-1α, IL-4 and IFNγ have been demonstrated to be increased in humans by different anaesthetics, whereas all the investigated drugs induced TNFα release [130, 131]. On the other hand, tribromoethanol, ketamine/xylazine and urethane reduced basal TNFα mRNA levels, while splenic IL-1β mRNA expression was decreased by urethane and increased by ketamine/xylazine in rats [132]. The divergent results from these studies suggest that anaesthetics have different immune modulatory effects depending on the drug used and the circumstances under which it is used.

Surgical trauma in itself cause a general suppression of immune responses, which normally is beneficial as it promotes wound healing and prevents the formation of autoantibodies. However, it may also contribute to complications in the form of postoperative infections and spreading of malignant diseases [133]. The postoperative pain could also depress immune functions, and analgesia is known to counteract the surgery-induced immune suppression [134].

The healing of the wound induced by surgery triggers an inflammation at the injury site, with activation of macrophages and infiltration of neutrophils. These cell types secrete several cytokines, e.g. TNFα, IL-1β and epidermal and fibroblast growth factors [135]. This inflammation has previously been assumed to be of essential importance to the wound healing, but recently this view has been challenged by the observation that inflammation in some cases is deleterious to wound healing and causes increased scarring [136]. There are several factors influencing the wound healing. Psychological stress, such as in hostile marital interactions, have been demonstrated to slow down wound healing and decrease cytokine (IL-6, IL-1β and TNFα) production at the wound site, whilst increasing plasma concentrations of IL-6 and TNFα [137]. The cytokine TGF-β has been shown to have both positive and negative effects on wound healing. It is normally increased shortly after injury, but constant overexpression proved to decrease the healing rate [138]. NO is induced during the inflammatory phase of wound repair, and seems to play a key role in this process by increasing angiogenesis, initiating inflam-
mation, increasing the collagen content of the wound and affecting the function of several cell types involved in wound healing [139]. Moreover, NO is one of the factors that may be involved in the upregulation of the suppressor of cytokine signaling-3 that has been observed during the inflammatory phase of skin repair [140]. Furthermore, an important contribution of cyclooxygenase (COX)-1 and 2 and subsequent prostaglandin synthesis have been discussed, as both COX-1 and COX-2 are increased in connection to wound healing [141-143].

Metabolic Factors in Type 1 Diabetes

Some of the characteristic features of both type 1 and type 2 diabetes is the presence of elevated concentrations of free fatty acids (FFA) and glucose in the blood of the patients [144]. Both FFA and glucose may induce β-cell toxicity if they are present in high concentrations [145]. Glucose has been shown to have DNA-damaging effects through the generation of reactive oxygen species (ROS) [146] and may in itself lead to β-cell apoptosis [147].

There is a multitude of FFAs that exert pleiotropic effects on β-cells. Generally, acute exposure to FFA increases insulin release, while chronic exposure results in desensitization and a suppressed insulin response [148]. Although physiological levels of FFA are important for normal β-cell function, increased plasma concentrations of FFA have been observed in the development of MLDSTZ diabetes [149] and fatty acids have been observed to potentiate the β-cell toxic effects of IL-1β [150]. However, FFA and cytokines do not induce β-cell apoptosis through the same mechanisms, as cytokines (TNFα and IL-1β) activate NF-κB and induce endoplasmic reticulum (ER) stress through nitric oxide (NO) formation, whilst FFA (in this case oleate and palmitate) trigger ER stress independently of NO and NF-κB [151].

The elevated glucose and FFA concentrations in diabetes is in part an effect of the immune attack on the islets, but several studies indicate that elevated glucose and/or free fatty acids in themselves may have effects on the immune system. Maedler et al. have shown that high glucose levels induce IL-1β production by human islets [152], and there is a correlation between poor glucose control and a higher susceptibility to infections in diabetic patients [153]. Also, culture of peripheral blood mononuclear cells at high glucose leads to an increased TGF-β production that in turn decrease the production of IL-2, IL-6 and IL-10 [154]. TNFα and IL-1 also have been demonstrated to be increased by high glucose concentrations in rat serum [155], and glucose loading in rabbits led to a fourfold increase in TNFα plasma concentrations [156]. Culture of islets of Langerhans has shown that they are more sensitive to STZ-induced damage when pre-cultured in 11.1 mM glucose than in 5.6 or 28 mM glucose [157]. Moreover, decreased glucose con-
centrations can also have effects on the cytokine production, as IFNγ gene expression is inhibited by glucose deprivation [158].

Macronutrient intake has been shown to induce oxidative stress and pro-inflammatory changes in plasma and peripheral blood mononuclear cells [159] and fatty acids have been demonstrated to have various effects on the immune system, acting as intra- and intercellular mediators [160]. The saturated free fatty acid palmitate has been demonstrated to induce the production of the transcription factor NF-κB and the cytokines IL-6 and TNFα in adipocytes [161], and of IL-6 in endothelial cells [162]. It also increases TNFα [163], and IL-6 expression in skeletal muscle [164].

Ethanol and Immunity

Ethanol is widely used in experimental circumstances to dissolve substances before administration. Because of the problem of ethanol abuse in humans much research has been performed concerning the effects of ethanol on the immune system. The length of ethanol exposure has a great impact on the cytokine response. Studies of human monocytes revealed opposite cytokine effects by 5-7 days of ethanol treatment (“chronic”) and shorter time periods (“acute”). Furthermore, the co-stimulatory signals present and the activation state of the monocytes influences the result of ethanol exposure [165].

Acute ethanol exposure inhibits the activation of proinflammatory cytokines like IL-1β and TNFα, whilst it augments inhibitory cytokine production such as IL-10 and TGF-β [166] in human monocytes. There are several possible ways that the decrease in cytokine response might be mediated. Toll-like receptors (TLR) could play an important part in determining the inflammatory response, including lowering the resistance to infections by decreasing cytokine production [167, 168]. A possible mechanism for this effect is that a reduction in TLR4 by acute ethanol has been demonstrated to decrease p38 and ERK1/2, thereby inhibiting TNFα and IL-6 production in macrophages [169, 170]. The phosphorylation of the regulatory protein IκB is also retarded by acute ethanol, which results in the inhibited activation of the transcription factor NF-κB, and reduced production of TNFα and IL-1β from monocytes, and augmentation of IL-10 [171, 172].

Chronic ethanol exposure results in a general immunosuppression as seen by decreased numbers of lymphocytes [173] that may be an effect of apoptosis [174], and reduced IL-17 and antibody production from T cells. Rat studies have demonstrated that ethanol suppresses chemokine activity in the lung, thereby decreasing neutrophil recruitment. This leads to an increased bacterial burden in response to Streptococcus Pneumoniae infection and a higher mortality. This phenomenon might explain the increased incidence of pneumonia in alcoholics [175]. However, the proinflammatory cytokine responses are enhanced by chronic alcohol exposure, with increased produc-
ton of TNFα, IL-1 and IL-6 [165]. Chronic alcohol exposure also increases superoxide generation [176].
Aims

The general aim of this thesis was to investigate how cytokine production is regulated in experimental type 1 diabetes and islet cell transplantation, and more specifically:

1. To investigate how reduction of MLDSTZ-induced diabetes by MDL 201,449A treatment influences cytokine secretion in vitro from spleen cells.

2. To examine the influence of cell depletion by plastic adhesion in vitro on cell distribution and cytokine production from spleen cells of MLDSTZ-treated mice.

3. To study multiple “control” treatments in islet cell transplantation, to be able to distinguish effects on cytokine production and regulatory T cell activation by the operating procedures themselves versus reactions induced by islet graft rejection.

4. To analyse whether the diabetes-like environment (elevated concentrations of glucose and free fatty acids) in itself also could affect cytokine production.
Materials and Methods

The MLDSTZ Diabetes Model

In this animal model of autoimmune diabetes, mice of susceptible strains (in our case C57BL/Ks) were injected intraperitoneally with 40 mg/kg body weight of streptozotocin (STZ; Sigma Chemicals, St Louis, MO, USA) or saline vehicle for five consecutive days. Blood glucose levels were measured in tail vein blood on days 0, 3, 7, 10 and 14 using a blood glucose meter (Medisense, London, UK). Blood glucose values above 11.1 mM were considered diabetic. The mice usually became hyperglycaemic between day 10 and 14. Mice were weighed before the experiment (day 0) and on day 14, before killing the mice by cervical dislocation.

In paper I, mice were given either daily s.c. injections of 100 mg/kg body weight (bw) MDL 201,449A (100 μl) or 100 μl of 0.5% sodium carboxymethylcellulose (SCMC) buffer during a 14-day period. Streptozotocin was administrated during the first 5 consecutive days, 30 minutes before MDL 201,449A / SCMC buffer injections was given. MDL 201,449A was kindly provided and tested for transcriptional inhibition of TNFα mRNA and optical rotation by Dr. Ekkehard H. W. Böhme, Hoechst Marion Roussel Research Institute, Hoechst Marion Roussel, Inc., Cincinnati, OH. MDL 201,449A injections were continued throughout the experiment period, to give a total of 14 daily injections.

Spleen Cell Isolation

As the immune cells of the islets of Langerhans are very difficult to isolate in sufficient quantities for studying immune responses, we have instead chosen to isolate immune cells from spleens, which can be assumed to reflect the general immune state in the mouse.

Spleens were surgically removed from the mice and immediately placed on ice in Hanks’ balanced salt solution (SBL Vaccin, Stockholm, Sweden) with 50 U/ml benzylpenicillin and 0.05 mg/ml streptomycin (Roche Diagnostics Scandinavia, Bromma, Sweden). They were mechanically disrupted to release the spleen cells. The spleen capsule was removed and the cell suspension centrifuged. The erythrocytes were lysed by suspending the cell pellet in 1–2 ml 0.19 M NH₄Cl for 10 min at 4°C.
In paper I all samples were depleted of plastic-adherent cells as described below. In paper II we divided the cell suspensions into two equal fractions. One fraction of spleen cells was kept on ice in 15 ml Falcon tubes while the other fraction was incubated in tissue culture dishes at 37°C (air + 5% CO₂) for 60 min to allow cells to adhere to the bottom. The non-adherent free-floating cells were collected and both fractions were counted. In paper III and IV all cells populations were used without plastic adhesion. Cells at a density of 5 x 10⁶ cells/ml were seeded in 3 ml RPMI 1640 (Sigma Chemicals Co, St Louis, MO, USA) with 10% foetal calf serum (FCS) (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml benzylpenicillin and 0.1 mg/ml streptomycin (Roche) in six-well free-floating culture plates. The remaining cells were in paper II lysed and frozen for analysis by RT-PCR. The cells were left overnight and then stimulated with concanavalin A (con A; 2 µg/ml; Sigma). In paper II, the cells that had adhered to the plastic were also maintained in culture for examination after 48 hrs.

Islet Isolation

Pancreatic islets were isolated by collagenase digestion [177] and islets were hand-picked with a braking pipette. The islets were cultured in 5 ml RPMI 1640 (Sigma Chemicals Co, St Louis, MO, USA) with 10% foetal calf serum (FCS) (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml benzylpenicillin and 0.1 mg/ml streptomycin (Roche Diagnostics, Mannheim, Germany) for 5-7 days before use.

Islet Transplantation

The mice were anaesthetized and 500 islets of Langerhans were transplanted under the left kidney capsule [178] of MHC-mismatched BALB/c or C57BL/6 mice to induce an allogeneic rejection of the graft. Other experimental groups were comprised of syngeneic islet transplantation, i.e. BALB/c islets grafted to BALB/c mice, and sham-operated BALB/c mice. Sham-operated or transplanted mice were killed by cervical dislocation 3 days after the operations, and grafts and spleens were removed. Untreated BALB/c or C57BL/6 mice were killed and the spleen cells isolated and used to get a baseline level of cytokine, FoxP3 and COX-2 production.
Figure 1. Islets are transplanted under the left kidney capsule of an anaesthetized NOD mouse. A glass bar is used to make a space under the kidney capsule. The islets are collected in a braking pipette and inserted into the space. Courtesy by Dr. A Börjesson.

Cytokine ELISA

Supernatant samples were analysed in duplicate for murine TNFα, IFNγ, IL-10 and IL-4 concentrations using DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturers’ instructions. Absorbance values were read using a Labsystems integrated EIA Management System iEMS ELISA reader and analysed with DeltaSoft3 software (Dr E Bechtold and BioMetallics ©).

mRNA Isolation and cDNA Synthesis

To isolate RNA from spleen cells, we employed the “Spin Protocol for Isolation of Total RNA from Animal Cells” in the RNeasy Mini Kit (or the RNeasy Plus Mini Kit in the case of the ethanol dose-response study of paper IV) from Qiagen (Maryland, USA). DNase digestion (RNase-Free DNase Set, Qiagen) was used with the RNeasy Mini Kit to decrease DNA
contamination. 50 x 10⁶ cells were lysed in Lysis buffer RLT/RLT Plus with 1% β-mercaptoethanol added, and subsequently stored in -70ºC.

Transplants were stored in RNA later RNA Stabilization Reagent (Qiagen) immediately after isolation and frozen at -70ºC as soon as possible. RNA was isolated using the “Total RNA Isolation from Fibrous Tissues” protocol from the RNeasy Micro Kit (Qiagen) with DNase digestion.

cDNA was synthesised from the RNA using the Reverse Transcription System from Promega Corporation (Madison, USA) or, in the etanol dose-response study of paper IV, the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer’s instructions.

Real-Time RT-PCR Assay

RT-PCR was performed using the Lightcycler instrument and the FastStart DNA Master SYBR Green I Kit (Roche, Bromma, Sweden) in paper II. For FoxP3 and COX-2 analysis in papers III and IV the SYBR Green JumpStart Taq ReadyMix kit (Sigma) was used. Primers were purchased from and designed by TIB Molbiol Syntheselabor (Berlin, Germany). The primers (Genebank accession number included) were:

- **G6PDH**: 5’-ATTGACCACCTACCTGGGCAA-3’,
  5’-GAGATACACTTCAACACTTGGACCT-3’, #NM 008062,
- **TNFα**: 5’-GACCCTCACACTCAGATCATCTTC-3’,
  5’-CGCTGGCTCAGCCACTCC-3’, #NM 013693,
- **IFNγ**: 5’-CATCACGCAACAACATAAGCGTCA-3’,
  5’-CGCTGGACCTGTGGGTT-3’, #XM 125899,
- **IL-10**: 5’-CATTCAATGGCCTTGTAGACACC-3’,
  5’-CTTAATGCAGGACTTTAAGGG TT-3’, #NM 010548,
- **IL-4**: 5’-ACAGGAGAAGGGACGCCAT-3’,
  5’-TGCAGCTTATCGATGAATCCAG-3’, #NM 021283

RT-PCR reactions were performed at 50µM in a 10 µl volume, containing 1 µl cDNA sample. The reaction was initiated by activating the polymerase by a 10 min pre-incubation at 95ºC. Amplification was performed by 40–45 cycles of 15 s denaturation at 95ºC, 10 s annealing at 65–67ºC and 10 s annealing at 72ºC. The program was concluded by a melting curve analysis with a temperature transition rate of 20ºC/s.

G6PDH was used as an internal control and run in parallel to the cytokines. Negative controls (reactions without cDNA or cDNA reactions without mRNA) were included in each run. A difference by 1 in cycle threshold (CT) values signifies a doubling of the amount of RT-PCR product. To compensate for this, the CT values are recalculated to allow for an exponential rate between the product and the housekeeping gene and expressed as 2^−ΔCT [179].
Hybridization Probe Real-Time RT-PCR

RT-PCR for detection of TNFα, IFNγ, IL-10 and IL-4 mRNA expression in transplants and spleen cells in paper III were performed using the Lightcycler instrument and the Lightcycler FastStart DNA Master Hybridization Probes Kit (Roche). Primers and hybridization probes were purchased from and designed by TIB Molbiol Syntheselabor (Berlin, Germany). The primers (GenBank accession number included) were:

**G6PDH:** 5´-ATTGACCACACTACCTGGGCAA-3´,
5´-GAGATACACTTCAACACTTTGACCT-3´, #NM 008062,

**TNFα:** 5´-GACCCTCACACTCAGATCATCTTC-3´,
5´-CGCTGGCTCAGCCACTCC-3´, #NM 013693,

**IFNγ:** 5´-CATCAGCAACAACATAAGCGTCA-3´,
5´-CGCTGGACCTGTGGGTT-3´, #XM 125899,

**IL-10:** 5´-CATTCTAGCCCTTTGTAGACACC-3´,
5´-CTTAATGCGAAGCTTTAAAGGGTTA-3´, #NM 010548,

**IL-4:** 5´-ACAGGAGAAGGGACGCCAT-3´,
5´-TGCGAGCTTATCGATGAATCCAG-3´, #NM 021283.

The hybridization probe sequences were:

**G6PDH:** GTTGCTCTCGATTCCAGATGGGTCC—FL,
LC Red705-AGATCCTGTTGGCAACACCTAGCA—PH,

**TNFα:** CAAGCCTGTAGCCACGTCGTA—FL,
LC Red640-CAGGCGACCCACGTCGTA—PH,

**IFNγ:** TTCAGCAACAGCAAGGCCAAA—FL,
LC Red640-TGCATTCATGAGTATTGCCACCC—PH,

**IL-10:** GAAGCTGAAGACCCTCAGGATGCG—FL,
LC Red640-TGAGGCGCTGTCATCGATTTCTCC—PH,

**IL-4:** GTGGTGTTCTTCTTTGCTGTGAGG—FL,
LC Red640-CGTTTGGCCACATCCATCTCCGT—PH.

RT-PCR reactions were performed in a 10 µl volume containing 1 µl cDNA sample. The reaction was initiated by activating the polymerase by a 10 min pre-incubation at 95°C. Amplification was performed by 45 cycles of 15 s denaturation at 95°C, 10 s annealing at 65°C and 10 s acquisition at 72°C.

G6PDH was used as an internal control and analyzed together with the cytokines using dual colour detection. Colour compensation was performed using the Lightcycler Color Compensation Set (Roche). Negative controls (reactions without cDNA or cDNA reactions without mRNA) were included in each run. Cycle threshold (CT) values were calculated as previously described [179].
Extracellular Flow Cytometry

Freshly isolated spleen cells, untreated or depleted of adherent cells, were treated with 2.4G2 antibody for 15 min to block Fc-receptor binding. Antibodies for CD8-APC, CD4-FITC, CD19-FITC, CD3-PerCP, CD25-PE and GR1-PE were purchased from Pharmingen (Stockholm, Sweden) and F4/80-FITC was purchased from Caltag Laboratories (Burlingame, CA, USA). Cells were incubated with antibodies for 30 minutes and analysed on FACScan (BD Biosciences, Mountain View, CA, USA) using CellQuest software (BD Biosciences).

Intracellular Cytokine Flow Cytometry

10% of the freshly isolated spleen cells were incubated in RPMI 1640 supplemented with 1 μg/ml Brefeldin A (Sigma) for 5 hrs at 37°C (air + 5% CO₂). The cells were fixed in 4% paraformaldehyde in PBS for 5 min. Permeabilisation was performed in PBS with 2% FCS, 0.1% BSA (Sigma) and 0.1% Triton (Sigma). The cells were incubated with antibodies for 30 min at +4°C in the dark. Antibodies used were TNF-FITC (MP6-XT22, Pharmingen), IFNγ-FITC (XMG1.2, Pharmingen) and IL-10-FITC (JES5-16E3, Pharmingen). Analysis was performed on a FACSCalibur flow cytometer (Becton-Dickinson) using the CellQuest software (Becton-Dickinson).

Statistical Analysis

Values were expressed as means ± SEM. Statistical analyses were made using the Sigma Stat™ software. Values were compared using One Way Repeated Measurements ANOVA, unpaired or paired t-tests, or the Mann-Whitney Rank Sum Test depending on the data. Probability values less than 0.05 were considered as statistically significant.
Results and Discussion

Paper I

Administration of the TNF$\alpha$ inhibitor MDL 201,449A has in previous studies decreased hyperglycaemia and insulitis in MLDSTZ diabetic mice [46]. The present study was designed to investigate cytokine production during that process.

The cytokine secretion from spleen cells isolated from MLDSTZ-treated mice displayed a retarded rate of increased TNF$\alpha$ accumulation compared to controls. This could be caused by the diabetic environment in the mice on day 14 when the spleen cells were removed, which might have rendered the spleen cells somewhat less responsive to con A stimulation compared to spleen cells from normoglycaemic mice. Contrary to what might have been expected, TNF$\alpha$ production in MDL 201,449A treated animals did not differ from vehicle treated mice. In interpreting these results, one has to bear in mind that the spleen cells after being isolated no longer are exposed to MDL 201,449A. The lack of TNF$\alpha$ inhibition could be caused by a rebound phenomenon when TNF$\alpha$ production no longer was suppressed by MDL 201,449A.

MLDSTZ treatment increased IFN$\gamma$ production compared to the control group. There was also a trend towards a higher IFN$\gamma$ secretion by cells from MDL 201,449A treated MLDSTZ mice compared to that from cells from vehicle treated MLDSTZ mice. Similarly to TNF$\alpha$ production, there seemed to be a compensatory increase in IFN$\gamma$ secretion when spleen cells were removed from the MDL 201,449A suppression in the mice. We could not detect any significant differences in spleen cell production of IL-10. Levels of IL-10 were very low and not detectable before 48 hrs of culture. In line with findings from other studies, these data point towards an increase in Th1 cytokine production from spleen cells from MLDSTZ diabetic mice.

In order to investigate if there indeed is a rebound effect after MDL 201,449A administration, we cultured spleen cells from untreated mice in the presence or absence of the compound for 96 hours. Secretion of TNF$\alpha$, IFN$\gamma$ and IL-10 was all suppressed in the presence of MDL 201,449A, but the production of all cytokines was increased upon discontinued administration. In the case of TNF$\alpha$, it even exceeded the secretion from cells cultured in the absence of the compound. These data confirms that ceased administration of MDL 201,449A results in a rebound phenomenon. As the cytokine
production was resumed when the administration was disrupted, it is probable that the compensatory reaction originated from a cellular response rather than a cytotoxic effect.

To investigate if the effect of MDL 201,449A on IFN$\gamma$ and IL-10 was caused by changed TNF$\alpha$ concentrations in the media, we cultured cells in the presence of exogenous TNF$\alpha$. As this did not affect IFN$\gamma$ and IL-10 production, we speculate that MDL 201,449A also inhibits these, and possibly other cytokines as well.

**Paper II**

The standard protocol for spleen cell isolation includes a step of cell depletion by plastic adherence. This procedure is assumed to deplete the macrophages in the cell cultures. Loh and Hudson demonstrated in 1979 that more than 50% of plastic adherent cells were macrophages as determined by non-specific esterase staining, and more than 90% had a “typical macrophage morphology” [180]. However, macrophages are important mediators of autoimmunity and have been shown to be of importance in diabetes development. We therefore investigated how plastic adhesion influenced cytokine mRNA expression and secretion and how different cell types are affected by the procedure.

In our study, we could only observe a decrease of 32% in the macrophage population following plastic adhesion, whereas about 60% of CD4$^+$CD25$^+$ regulatory T cells were depleted. Interestingly, this was only observed in cultures from MLDSTZ-treated mice. We therefore hypothesize that MLDSTZ treatment might induce a subpopulation of macrophages with adhesive properties.

We noticed that the population of CD4$^+$CD25$^+$ regulatory T cells was higher in spleens from MLDSTZ-treated mice than in spleens from saline treated controls. This might reflect a mechanism to down-regulate the insulitis in the pancreas and the developing hyperglycaemia. There are not many studies on how MLDSTZ-induced diabetes influences the numbers of CD4$^+$CD25$^+$ regulatory T cells, but Anastasi et al. have demonstrated that MLDSTZ treatment of transgenic mice expressing a constitutively active Notch3 domain were protected from hyperglycaemia, and that the protection coincided with an increase in the number of CD4$^+$CD25$^+$ T cells compared to wild type mice [98]. However, the lack of vehicle alone controls in this study makes it difficult to deduce if there is an effect of the MLDSTZ treatment only. However, it is possible that the CD4$^+$CD25$^+$ regulatory T cells are increased by MLDSTZ as an attempt to counteract the $\beta$-cell damage.

Secretion of TNF$\alpha$, IFN$\gamma$ and IL-10 was elevated after cell depletion in spleen cell cultures from MLDSTZ-treated mice. Possibly, this could be an effect of the reduction in CD4$^+$CD25$^+$ regulatory T cells, as these cells in-
hibit the release of both Th1 and Th2 cytokines [96]. It could also be speculated that the adherent cells represent a cell contingent with a low proliferative capacity regarding con A stimulated cytokine release. Both macrophages and CD4^+CD25^+ regulatory T cells have been reported to be relatively unresponsive to con A stimulation [181, 182]. If so, depletion of these cells would increase the proportion of con A-responsive cells in the cultures.

Spleen cells (both depleted of adherent cells and not) as well as plastic adherent cells from MLDSTZ-treated mice displayed an increased IFNγ production compared to cells from control mice. This is in line with previous studies where MLDSTZ-induced hyperglycaemia coincided with an elevation of the IFNγ production [63, 64]. However, no differences in IFNγ mRNA expression were observed. Possibly, this indicates that in vitro production of IFNγ is mainly post-translationally regulated.

Both protein secretion and mRNA expression of IL-10 were increased as a consequence of MLDSTZ treatment. This is somewhat surprising, as most other studies demonstrate a connection between MLDSTZ diabetes and a decrease in IL-10 production [62, 183], as well as a relation between disease protection and increased IL-10 production [64-66, 98, 184]. However, the increase in IL-10 might reflect an activity aiming to balance the Th1-driven mechanisms leading to β-cell loss and hyperglycaemia, when the cells were no longer subjected to the β-cell destructive environment in the mouse.

Protein levels of IL-4 were not detectable by ELISA under these experimental circumstances. As an alternative, detection by ELISPOT sometimes provides a more sensitive method [60]. Contrary to the other investigated cytokines, the IL-4 mRNA expression levels were decreased in cultures depleted of adherent cells. This might be explained by a production of IL-4 by CD4^+CD25^+ T cells, as some studies have observed IL-4 production from these cells [98, 185]. If that is the case, a decreased number of IL-4-producing cells after adhesion would indeed bring about the observed decrease. IL-4 mRNA expression was inhibited by MDLSTZ both in cultures with and without adherent cells. This correlates well with observations in other studies of a decreased IL-4 production in MLDSTZ-treated animals [62, 63, 183].

To summarize, we have observed that plastic adhesion depletes CD4^+CD25^+ T cells to a larger extent than macrophages. However, the plastic adhesion does not seem to influence the interpretation of the results of the MLDSTZ experiments. Plastic adhesion does influence cytokine production, but the effect of the MLDSTZ treatment surpasses this action. Altogether our data suggest that plastic adhesion may not be an optimal method to deplete macrophages from spleen cell cultures. Furthermore, our results support the view that CD4^+CD25^+ T cells inhibit both Th1 and Th2 cells.
When performing experimental transplantation studies, many groups use syngeneic transplantations or sham-operated animals as controls. However, these manipulations involve anaesthesia, surgery, the formation of a wound and subsequent wound healing, which all might have an effect on the immune system. To investigate if this is the case, and whether cytokine production and/or regulatory T cells are influenced by these procedures, we have performed allogeneic, syngeneic and sham transplantations and investigated the cytokine production, FoxP3 and COX-2 expression in spleen cells and, when possible, transplants from these as well as untreated mice.

Isolating RNA from transplants did initially present us with some problems. The immunological barrier between C57BL/6 and BALB/c mice is very strong [186], and if the transplanted animals were left for longer than three days after the operations, some of them died, probably due to systemic effects when a strong rejection occurred. After this short time, the transplant had not been much infiltrated by connective tissue, and it was difficult to remove it without losing some of the islets. We therefore had to increase the size of the transplant to 500 islets in order to recover enough graft material to isolate RNA. Also, some degradation occurs during the time between extraction of the transplant and freezing the sample. This problem was solved by immediately submerging the transplant in RNAlater buffer, which preserves the RNA until isolation. We also changed isolation method and used the RNA Micro Kit for isolation, as this was better suited for small amounts of material. To increase the sensitivity of the RT-PCR, we successfully tried the Hybridization probes technique, in which a specific DNA probe binds to the gene in question.

We observed an increase in cytokine production from spleen cells of sham-operated and syngeneically transplanted mice. We hypothesize that there might be a connection between these results and the low levels of FoxP3 mRNA expression in these experimental groups. As regulatory T cells decrease the production of both Th1 and Th2 cytokines [96] there could be an association between fewer regulatory T cells (as demonstrated by decreased FoxP3 mRNA expression) and increased cytokine production. This might be an effect of the anaesthesia and/or wound healing induced by the operation. We used tribromoethanol as anaesthetic agent, which has been shown to reduce TNFα mRNA expression in rat spleen cells [132]. It has also been observed to have other effects on the immune system, such as a dampening of the natural killer cell activity [187, 188]. Furthermore, an inflammation is induced by the wound healing after the operation, and the cytokine production triggered by this inflammation might influence the regulatory T cell activity and cytokine balance at the injury site [136]. This, in turn, could cause the increased COX-2 expression observed in the sham-transplanted groups, as certain proinflammatory cytokines are known to in-
duce COX-2 expression [179, 189, 190]. The cytokine response induced by allogeneic rejection might be strong enough to counteract the effect of anaesthesia and/or wound healing in allogeneically transplanted groups. After 48 hrs of culture, the cytokine production in spleen cells from sham-operated and syngeneically transplanted mice was no longer elevated, indicating that the effect was induced in vivo and did not persist during culture in vitro.

We observed quite different results in groups transplanted to BALB/c and C57BL/6 recipients. In many cases the cytokine production was the opposite in the two strains, which indicates a difference in regulation of cytokine production, similarly to the difference in cytokine profile observed during Leishmania infections in these mouse strains [191-193]. FoxP3 and COX-2 mRNA expressions also differ in BALB/c and C57BL/6 mice, indicating that the strains also vary in the regulation of regulatory T cell and COX-2.

After 48 hrs of culture, spleen cells from the untreated group had increased protein secretion and mRNA expression of cytokines as well as increased FoxP3 and COX-2 mRNA expressions compared to the other experimental groups. This is the only group not subjected to surgery. Therefore, we hypothesise that the surgery results in a general suppressive effect on the spleen cells, which interferes with the cells’ response to con A in the in vitro situation in all groups except the untreated controls. This finding is in line with the immunosuppression that has been observed in PHA-stimulated human monocytes and lymphocytes after surgery [129].

To summarize the findings from this study, we have observed that so-called control treatments such as sham operations and syngeneic transplants influence cytokine production. We hypothesize that this effect is caused by the surgery and/or anaesthesia involved in these treatments, that may decrease the regulatory T cell population, thereby leading to increased cytokine expression. We therefore would like to emphasize the importance of using the appropriate control groups when performing transplantation studies. Another observation of the present study is that spleen cells from surgically manipulated mice exhibit a decreased responsive capacity to con A stimulation when cultured in vitro, compared to spleen cells from untreated animals. Also, there was a distinct difference in the cytokine response from the two mouse strains investigated in the study. The genetic background of both islet donor and recipient seem to be important in determining the magnitude and skewing of the cytokine production, and it is possible that the genetic background might influence the outcome of clinical islet transplantations in humans in a similar way.

**Paper IV**

During type 1 diabetes, several changes in the immune system leading to the autoimmune destruction of the β-cells have been described. One of these
changes is a skewing of the cytokine production towards a proinflammatory profile [9]. However, it is not completely clarified whether this is the direct consequence of the autoimmune attack, or if the diabetic milieu, with increased glucose and free fatty acid concentrations, could be contributing to this change. We have investigated this by culturing normal mouse spleen cells in varying concentrations of glucose and the saturated free fatty acid palmitate, and examined the supernatant secretion and mRNA expression of the cytokines TNFα, IFNγ and IL-10.

In our study, we observed that culture at high glucose concentrations lead to decreased TNFα and IFNγ concentrations compared to culture in low glucose. Also, culture at normal glucose concentrations (11.1 mM) induced the highest levels of all the investigated cytokine production. This is the normal glucose concentration in the RPMI 1640 medium, which was developed for lymphocyte culture [194], and it is likely that the spleen cells would have the best proliferative conditions in response to con A stimulation at this glucose level. Furthermore, the secretion of TNFα and IFNγ from spleen cells cultured at 28 mM decreased during culture, while IL-10 secretion at 11.1 mM increased. This was even more evident at the IL-10 mRNA level, where all 96 h values exceeded the 0 h value. These data suggest that prolonged culture of spleen cells at high glucose might induce a slight shift towards a production of Th2 cytokines.

An elevation of free fatty acid levels have been observed to be part of the diabetic milieu [149, 195], and we wished to investigate if elevated FFA concentrations could influence cytokine production. We therefore studied if the addition of the saturated fatty acid palmitate into the media of cultured spleen cells had an effect on the production of TNFα, IFNγ and IL-10. Palmitate did seem to inhibit cytokine production, but in the case of IFNγ and IL-10 the control group that had been cultured with the addition of ethanol (which was used as a solvent for palmitate) also showed a suppression of cytokine secretion. The combined effect of palmitate and ethanol regarding IFNγ and IL-10 makes it difficult to determine if the inhibition was caused by palmitate or if it mainly originated from the ethanol addition. It is not unlikely that ethanol could have effects on cytokine production, as acute alcohol treatment in vitro has been shown to down-regulate proinflammatory cytokines, while chronic alcohol use has the opposite effect [165, 176]. Palmitate has been observed to increase the expression of TNFα and IL-6 in skeletal muscle cells, adipocytes and endothelial cells [161-163], but as we have observed the opposite effect in our experimental setting, it seems probable that palmitate might not have the same effect on spleen cells, or that this effect, if present, is overcome by the stronger suppression by the ethanol. However, this might explain why the suppressive action we noticed was more pronounced for IFNγ and IL-10, as TNFα could have been slightly activated by the palmitate.
We also noticed that the suppression of cytokine production by ethanol and/or palmitate was abolished by culture in high (28 mM) concentrations of glucose for TNFα and IFNγ, but not IL-10. In order to further investigate this, we performed a dose-response study of the effect of different concentrations of ethanol on spleen cells cultured at 11.1 or 28 mM glucose. We observed a similar effect on the cytokine production as in the experiments using palmitate. Ethanol suppressed the secretion of all three cytokines, but with the most pronounced effect on IFNγ and IL-10, when cultured in normoglycaemic conditions. The suppression, especially of IL-10, seemed to be dose-dependent. Similarly to the studies using palmitate, culture at high glucose levels counteracted the suppression of TNFα and IFNγ observed at normal glucose concentrations. There could be several explanations to this finding. Ethanol is known to have immunosuppressive properties mediated through various mechanisms, for example by down-regulating toll-like receptors (TLR) by limiting p38 and ERK1/2 activation, which in turn leads to suppression of cytokine (TNFα, IL-6, IL-10 and IL-12) responses [168, 170]. There are changes in the expression of TLRs in the liver, kidney and brain of streptozotocin-induced diabetic rats [196], and p38 and ERK1/2 has been observed to be induced by high glucose in mesangial cells [197], indicating that an effect on TLRs or p38 and ERK1/2 of high glucose could be involved in counteracting ethanol-induced immune suppression.

Conclusively, our data suggest that high glucose levels could be a factor influencing the immune deviations during type 1 diabetes. It is also possible that palmitate could be of importance, but our experiments could not conclusively determine that due to the practical difficulty of having to dissolve palmitate in ethanol for it to be in liquid form, and we have shown that ethanol in itself have a suppressive effect on cytokine secretion. This observation emphasizes the importance of using the proper control groups when employing substances dissolved in ethanol, to rule out possible confounding effects of the vehicle. Also, the cytokine inhibition by ethanol and/or palmitate was counteracted by culture in high glucose, although the mechanism underlying this effect needs to be further investigated.
Conclusions

Paper I
- The TNFα transcription inhibitor MDL 201,449A inhibits IFNγ and IL-10 as well as TNFα, and may not be an entirely specific TNFα inhibitor.
- Discontinued administration of MDL 201,449A leads to a rebound phenomenon of increased cytokine levels.

Paper II
- Plastic adhesion depletes CD4⁺CD25⁺ regulatory T cells to a larger extent than macrophages.
- CD4⁺CD25⁺ regulatory T cells may inhibit both Th1 and Th2 cells.
- MLDSTZ treatment might increase the CD4⁺CD25⁺ regulatory T cell content in spleen cell populations.

Paper III
- Anaesthesia and/or surgery affect cytokine production, regulatory T cells and COX-2 expression.
- Mouse strains differ in responsiveness to transplantations with regard to cytokines, regulatory T cells and COX-2.
- Surgery may render spleen cells less responsive to con A stimulation in vitro.

Paper IV
- A diabetes-like environment in vitro, characterized by high glucose and free fatty acid levels, influences cytokine production in spleen cells.
- Ethanol and/or palmitate suppress cytokine production at normal glucose concentrations (11.1 mM), but not at high glucose concentrations (28 mM).
Målsättningen med avhandlingen har varit att studera hur cytokinutsöndring påverkas i experimentella modeller för typ 1 diabetes samt vid transplantation av Langerhanska öar.

**Delarbete 1: Thorvaldson L, Holstad M, Sandler S. Cytokine release by murine spleen cells following multiple low dose streptozotocin-induced diabetes and treatment with a TNFα transcriptional inhibitor. (Int Immunopharmacol. 3:1609-17 (2003).)**

I det första delarbetet undersökta vi om MDL 201,449A, en transkriptionell inhibitor av TNFα, påverkar cytokinutsöndringen i odlade mjältceller. Tidigare försök har visat att administrering av MDL 201,449A motverkar diabetesutveckling hos möss behandlade med multipla låga doser av streptozotocin (MLDSTZ). TNFα-utsöndringen i mjältceller från djur behandlade med MLDSTZ och MDL 201,449A var fördöjd jämfört med den från djur som inte fått MDL 201,449A, men IFNγ och IL-10 sekretionen påverkades inte. Vi misstänkte att detta kunde bero på att mjältcellerna under odlingen inte längre exponerades för MDL 201,449A. Försök in vitro påvisade att MDL 201,449A som tillsatts under cellodlningen inhiberade samtliga de undersökta cytokinerna, men vi noterade att nivåerna återgick till de normala eller steg över de normala då exponeringen avbröts. Vi drog därför slutsatsen att MDL 201,449A inte är en specifik TNFα-inhibitor, utan även inhiberar IFNγ och IL-10. Vi observerade även att inhiberingen följs av en höjd cytokinproduktion då cellerna inte längre är i kontakt med MDL 201,449A.


Det andra delarbetet behandlade plastadhesion, en metod som traditionellt används för att reducera makrofaginnehåll i mjältcellskulturer. Vi ville undersöka dels hur detta påverkar cytokinproduktionen från cellerna och dels vilka celltyper som verkligen påverkades av behandlingen, då det inte finns några moderna studier av detta. Våra försök visade att plastadhesionen bara tar bort ca 30% av makrofagerna, men däremot ca 60% av de regulatoriska T-cellerna, vilket i sin tur leder till ökad sekretion av TNFα, IFNγ och IL-10 och minskade mängder av IL-4. Slutsatsen av våra experiment är att plastad-
hesion inte är en särskilt effektiv metod för att minska makrofaginnehållet i en cellkultur, eftersom även regulatoriska T-celler fastnar på plasten, och att detta till viss grad påverkar den resulterande cellkulturens egenskaper.

**Delarbete 3: Thorvaldson L och Sandler S. Factors influencing the regulation of cytokine balance during islet transplantation in mice. (Insänt för publikation.)**

I det här delarbetet undersökte vi hur olika typer av transplantationer av Langerhanska öar påverkar cytokinproduktionen i mjältceller. Vi upptäckte att s.k. shamtransplantationer, d.v.s en operation utan insättning av öar, och syngena transplantationer (mellan genetiskt identiska djur) gav en tydlig effekt på cytokinproduktionen i mjältceller jämfört med celler från obehandlade djur. Vi såg en minskning av de regulatoriska T-cellerna och en ökad cytokinproduktion i dessa grupper. Detta kan bero på att operationen eller anestesin minskar de regulatoriska T-cellerna och att denna minskning ger utrymme för en ökad cytokinutsändring. Vi noterade också att mjältceller från opererade djur svarar sämre på stimulering i odling än celler från obehandlade djur. Förmodligen har dessa celler förlorat en del av sin svarskapacitet och har därmed inte samma möjlighet att reagera på stimuleringen. Vi utförde transplantationer av öar både från BALB/c möss till C57BL/6 möss och i motsatt riktning och observerade olika cytokinsvar beroende på vilken stam som användes som mottagare. Samma behandling ger olika respons beroende på vilken genetisk bakgrund mottagaren har, vilket är en lärdom som kan vara intressant att föra över på den kliniska situationen.

**Delarbete 4: Thorvaldson L, Stålhammar S och Sandler S. Effects of a diabetes-like environment in vitro on cytokine production by mice splenocytes. (Manuskript.)**

Det sista delarbetet behandlar hur olika omgivningsfaktorer som karakteriseras som ett diabetesstillstånd, såsom höjda nivåer av glukos och fria fettsyror (i det här fallet palmitat), påverkar cytokinproduktionen hos odlade mjältceller. Vid odling i olika koncentrationer av glukos noterade vi att cytokinproduktionen var högst i celler odlade i 11.1 mM glukos och av detta drar vi slutsatsen att glukos kan påverka cytokinbalansen. Odling med palmitat motverkar cytokinutsändring, men den effekten sågs också i kontrollgruppen som bara odlats i etanol (vilket användes för att lösa upp palmitatet) och således är det svårt att avgöra om cytokininhiberingen beror på palmitatet eller etanolen som den lösts i. Denna effekt motverkades dock av odling vid höga koncentrationer av glukos, då cytokinnivåerna normaliserades. Mekanismerna för denna effekt är inte känd och behöver undersökas vidare.
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