TYPE 1 DIABETES - THE AUTOIMMUNE PROCESS AND ISLET TRANSPLANTATION

Stella Jacobson
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Till Johan
Type 1 diabetes (T1D) is an autoimmune disease characterized by the selective loss of the insulin-producing \( \beta \)-cells residing in the islets of Langerhans in the pancreas. Cytokines are involved in diabetes development in the nonobese diabetic (NOD) mouse model. NOD mice over-expressing the suppressor of cytokine signaling (SOCS-1) specifically in the \( \beta \)-cells are protected from T1D. Previous studies showed that immune cells infiltrated the pancreas of SOCS-1-transgenic (tg) mice, however, infiltrating T cells were less pathogenic than those infiltrating the islets in non-tg NOD mice. In this thesis one of the aims was to further dissect the infiltrating T cell populations in SOCS-1-tg mice in order to gain further insight into the mechanisms behind disease protection. In paper I the main finding was that specific autoreactive T cells were strongly reduced in the pancreas of SOCS-1-tg mice compared to non-tg mice. Previous studies have shown that autoreactive T cells are recruited to the pancreas by cytokine-induced CXCL10 expression by the islets. The receptor for CXCL10 is CXCR3, which was more frequently expressed on autoreactive T cells than bulk T cells. Since SOCS-1-tg mice have reduced expression of CXCL10, autoreactive T cells are less likely to migrate to the pancreas of these mice and pose one possible explanation for this finding. This study shows that the \( \beta \)-cell response to cytokines plays a major role in the accumulation of autoreactive T cells to the pancreas.

Blood glucose metabolism in patients with T1D can only be restored by islet transplantation. Unfortunately, the benefits of islet transplantation are only short-term since the graft is lost over time. Therefore, exposing T1D patients to the risks associated with the immunosuppressive therapy cannot be motivated in most cases. In the second part of this thesis the aim was to evaluate new methods to prevent islet allograft rejection. In paper II it was shown that the mesenchymal stromal cell (MSC)-line MBA-1 suppressed T cell proliferation in vitro and slowed down rejection of allogeneic islets in Balb/c mice. This indicates the possible use of MSCs as cell therapy in islet transplantation. Another method to avoid immunosuppressive treatment is to encapsulate the islet allografts inside immunoprotective membranes (TheraCyte™ devices) preventing immune cells from interacting with the grafts. In paper III it was shown that the TheraCyte™ device completely protected islet allografts from rejection in both naive and immunized recipient rats. This is an important finding since many patients are sensitized prior to transplantation for example due to a previous transplant. Finally, graft loss is difficult to study in humans and small animal models do not always reflect the human situation. Therefore, the final aim of this thesis was to evaluate so-called humanized mice for their potential use to study human islet rejection mechanisms. In paper IV human immune system (HIS) mice were established and transplanted with human islets. However, no signs of rejection were detected in the HIS mice questioning the usefulness of this model as a tool to study human islet transplantation. This highlights the need for more robust humanized mouse models.
LIST OF PUBLICATIONS

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


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LIST OF ABBREVIATIONS

APC  Antigen presenting cell
APS-1  Autoimmune polyendocrine syndrome-1
ATP  Adenosine triphosphate
BLT  Bone marrow-liver-thymus
BM  Bone marrow
C-peptide  Connecting peptide
CSF  Colony stimulating factor
CTL  Cytotoxic T cell
CTLA-4  Cytotoxic T lymphocyte antigen-4
CXCL10  Chemokine (C-X-C motif) ligand 10
CXCR3  Chemokine (C-X-C motif) receptor 3
DC  Dendritic cell
DMEM  Dulbecco’s modified eagle medium
DMSO  Dimethyl sulfoxide
DTH  Delayed type hypersensitivity
EBV  Epstein Barr virus
FCS  Fetal calf serum
GAD  Glutamic acid decarboxylase
GFP  Green fluorescent protein
GM-CSF  Granulocyte macrophage colony stimulating factor
GVHD  Graft-versus-host disease
HGF  Hepatocyte growth factor
HIS  Human immune system
HIV  Human immunodeficiency virus
HLA  Human leukocyte antigen
HSC  Hematopoietic stem cell
HSV-2  Herpes simplex virus type-2
I.h.  Intrahepatic
I.p.  Intraperitoneal
I.v.  Intravenous
IA-2  Islet antigen-2
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<tr>
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<tr>
<td>IBMIR</td>
<td>Instant blood-mediated inflammatory reaction</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<td>IFIH1</td>
<td>Interferon-induced helicase C domain-containing protein</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IGRP</td>
<td>Islet specific glucose 6-phosphatase catalytic subunit-related protein</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IPEX</td>
<td>Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>JDRF</td>
<td>Juvenile diabetes research foundation</td>
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<tr>
<td>LN</td>
<td>Lymph node</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MBA-1</td>
<td>Mouse bone marrow adherent cells-1</td>
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<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
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<td>Metalloproteinase</td>
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<td>MS</td>
<td>Multiple sclerosis</td>
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<td>MSC</td>
<td>Mesenchymal stromal cell</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NOD</td>
<td>Nonobese diabetic</td>
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<td>nPOD</td>
<td>Network for pancreatic organ donors</td>
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<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
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<tr>
<td>PLN</td>
<td>Pancreatic lymph node</td>
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<td>PP</td>
<td>Pancreatic polypeptide</td>
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<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase, non-receptor type 22</td>
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<td>RA</td>
<td>Reumatoid arthritis</td>
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<td>RAG</td>
<td>Recombination-activating gene</td>
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<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>SOCS-1</td>
<td>Suppressor of cytokine signaling-1</td>
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<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>T1D</td>
<td>Type 1 diabetes</td>
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<td>T2D</td>
<td>Type 2 diabetes</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>Tg</td>
<td>Transgenic</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>Tr1</td>
<td>Type 1 regulatory T cell</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WF</td>
<td>Wistar Furth</td>
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<tr>
<td>WHO</td>
<td>World health organization</td>
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<tr>
<td>Znt8</td>
<td>Zink transporter 8</td>
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1 INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease where the insulin producing β-cells are mistakenly seen as foreign and destroyed by the immune system. This process results from both genetic risk factors in combination with environmental triggers. The details of this process are unclear and limit the development of preventative treatments. In this thesis I have studied the autoimmune process in order to understand events in the pancreas supporting the autoimmune activities associated with disease progression. β-cells reside in so-called islets of Langerhans in the pancreas and an attractive method to cure T1D patients is to transplant islets from brain-dead donors. However, islet transplantation faces several major challenges. I have therefore evaluated new methods to improve the success rate of islet transplantation. Finally, it is difficult to study interactions between the human immune system and human islets after transplantation. To overcome this problem, I have generated so-called humanized mice and evaluated their potential use as new tools to study human islet rejection mechanisms. To better understand the concepts discussed in this thesis, a brief overview of this field of study is presented in this section.

1.1 TYPE 1 DIABETES

1.1.1 The islets of Langerhans

The pancreas consists of exocrine and endocrine tissues and is one of the organs contributing to the digestion of food and regulation of glucose metabolism. The exocrine tissue secretes digestive enzymes whereas the endocrine tissue secretes hormones that regulate the glucose level in the blood [1]. The endocrine cells are assembled into cell clusters entitled the islets of Langerhans that are scattered throughout the exocrine tissue. The number of islets in the human pancreas are approximately 1.5 millions corresponding to 1-2% of the total pancreatic mass. The size of the islets varies between approximately 20-250 µm in diameter with the majority of islets being <100 µm [2].

1.1.2 The β-cell

The pancreatic islets consist of β-cells producing insulin, α-cells secreting glucagon, δ-cells secreting somatostatin and pancreatic polypeptide (PP)-producing cells [1]. To a lesser extent, stromal cells, blood vessels, neurons and immune cells, such as dendritic cells (DCs), reside in the islets. The hormones insulin and glucagon are responsible for maintaining blood glucose homeostasis. When blood glucose levels rise after food intake, insulin is secreted by the β-cells and stimulates the cells in the body to take up glucose. Instead, when blood glucose levels decrease, glucagon is produced and stimulate glucose release from the liver [1]. In order to produce ATP (adenosine triphosphate) and secrete insulin, the β-cells consume large amounts of oxygen and requires oxygen tension close to venous blood (40 mmHg) [3]. For this reason the islets
are highly vascularized and β-cells are sensitive to hypoxic conditions that may occur during isolation and after transplantation.

The insulin precursor proinsulin consists of an A and B-chain separated by a connecting peptide (C-peptide) [4]. During insulin biosynthesis proinsulin is cleaved into insulin and C-peptide. C-peptide is commonly used as a diagnostic marker for insulin secretion, for example after islet transplantation since it is detected at higher concentrations in blood than insulin. In addition, C-peptide can be used as a marker for endogenous insulin production in T1D patients treated with insulin.

1.1.3 Type 1 diabetes

Diabetes mellitus is a heterogeneous group of disorders all characterized by disturbances in blood glucose metabolism due to lack of or resistance to insulin. The most common form of diabetes is Type 2 diabetes (T2D) affecting approximately 220 million people worldwide according to the World Health Organization (WHO). T2D is characterized by resistance to insulin in tissues leading to elevated blood glucose levels and β-cell apoptosis [1, 5]. The most severe form of diabetes is T1D, which accounts for approximately 5-10% of all diabetes cases worldwide and usually appears during childhood [6]. In T1D the β-cells are lost due to an autoimmune process and, depending on the age, approximately 40-85% of the islets are lost at time of diagnosis [7]. T1D results from insufficient or absent production of insulin leading to high blood glucose levels (hyperglycemia) that can only be controlled by life-long intake of exogenous insulin. Although insulin treatment may seem uncomplicated, some patients have difficulties in controlling blood glucose levels [6]. Unstable blood glucose control can lead to life-threatening ketoacidosis and hypoglycemia, as well as long-term complications such as blindness, kidney failure, nerve damage and vascular disease. There is no cure for T1D and at present the only way to restore insulin production is by the transplantation of isolated islets.

1.1.4 Etiology of T1D

The etiology of T1D is poorly understood but both genes and environmental factors are associated with the disease. The major genetic risk factor is polymorphisms in the human leukocyte antigen (HLA)-DQ on chromosome 6. Almost 90% of children diagnosed with T1D have haplotype DQ8, DQ2 or DQ2/8 [8, 9]. Polymorphisms in these HLA regions create an unusual binding pocket, which may alter presentation of β-cell autoantigens to T cells. Non-HLA genetic risk factors for T1D are for example the insulin gene as well as genes involved in immune system functions such as Ptpn22, Ctda-4, interleukin (IL-2) receptor and Ifih-1, a gene encoding the viral sensing protein MDA-5 [8-12]. Genetic susceptibility cannot solemnly explain the development of T1D for several reasons. For example, the concordance rate in monozygotic twins is approximately 65% over time and only 10% of individuals with HLA risk haplotypes develop T1D [13, 14]. Moreover, the incidence of T1D differs dramatically between countries. China has the lowest incidence of T1D (0.57/100,000 per year) whereas Finland and Sardinia have the highest (48-49/100,000 per year), closely followed by Sweden (25.8/100,000 per year) [6, 15]. Finally, the incidence of T1D increases, particularly in younger children. In children aged 0-4 years in Europe, the annual
increase in incidence was 6.3% compared to 3.4% in the overall population [15]. For these reasons, environmental factors are believed to be involved in the triggering of T1D, however, it is not clear which factors are involved and how they contribute to disease development. Factors that have been proposed to be important for T1D development are dietary factors, infections and altered intestinal microbiota [16-19]. It has been suggested that several events are needed for the triggering and/or continuation of the islet autoimmune process [19].

1.2 IMMUNOLOGY

1.2.1 The immune system

The immune system has evolved to protect an individual from invasive pathogens and tumors and is divided into two arms: the innate and adaptive immune systems. The innate immune system consists of epithelial barriers and chemical substances produced at epithelial surfaces, the complement system as well as different myeloid cell lineages such as phagocytic cells (neutrophils and macrophages) and DCs. The innate immune system is the first line of defense against infections and surveys the blood and tissues for the presence of invading microbes. Innate immune cells become activated rapidly upon encounter with pathogen-associated molecular patterns (PAMPs) binding to Toll-like receptors (TLRs) on the surface of innate cells [20]. Upon activation, innate leukocytes (i.e. macrophages and natural killer (NK) cells) produce cytokines, which recruit additional macrophages and neutrophils and stimulate phagocytosis of microbes [21]. The adaptive immune system consists of T and B lymphocytes, which recognize distinct structures on microbes due to their highly diverse antigen receptors. Adaptive immune cells also generate immunological memory enabling a quicker response to repeated exposure to the same microbe. Adaptive immunity is activated later in an infection to assist innate immune cells in pathogen clearance.

1.2.1.1 T cell maturation and activation

All hematopoietic cell lineages are generated from self-renewing hematopoietic stem cell (HSC) progenitors in the bone marrow (BM). Myeloid cell progenitors develop into innate cell lineages including erythrocytes and platelets whereas lymphoid progenitors generate T, B and NK cells (Figure 1) [21]. Early T cell progenitors migrate from the BM to the thymus and acquire expression of antigen specific T cell receptor (TCR) molecules in a random manner. This is achieved by recombination of gene segments mediated by proteins encoded by recombination-activating gene RAG1 and RAG2. Therefore, mice deficient in RAG-1 or 2 proteins fail to produce mature T cells. As T cell maturation progress, associated TCR molecules (co-receptors) CD3, CD4 and CD8 are expressed on the cell surface. Double-positive CD4⁺CD8⁺ T cells undergo positive and negative selection in the thymus to ensure that the produced T cells are self-restricted but not autoreactive. This is achieved by the binding of CD4⁺CD8⁺ T cells to major histocompatibility complex (MHC) class I and II expressed on thymic epithelial cells. T cells binding to MHC molecules with low affinity receive a survival signal (positive selection) and mature into naive single-positive CD4⁺ or CD8⁺ T cells [20]. Instead, T cells that fail to recognize MHC molecules are subjected to apoptosis (>95% of double-positive thymocytes). T cells can recognize an enormous variety of
antigens including self-antigens (so-called autoreactive T cells), which can lead to autoimmune diseases if not controlled. Therefore, T cells with strong binding to MHC molecules presenting self-peptides undergo apoptosis (negative selection) [22]. The expression of self-peptides in the thymus is controlled by the autoimmune regulator (Aire) gene.

After the selection process, naive single-positive CD4+ and CD8+ T cells circulate secondary lymphoid organs (lymph nodes (LNs), spleen and mucosal lymphoid tissues). Naive T cells become activated upon encounter with their cognate peptide presented on MHC class I and II molecules by antigen presenting cells (APCs) such as DCs. Activation of T cells involves intracellular phosphorylation cascades leading to gene expression of different cytokines [21]. T cells are only activated if co-stimulatory molecules are simultaneously expressed on the activated APCs. This includes the expression of B7-1 (CD80) and 2 (CD86) and CD40, which bind to CD28 and CD40 ligand expressed on T cells.

1.2.1.2 T cell subsets

CD4+ T cells are considered MHC class II restricted and are referred to as T helper (Th) cells as they support activation of CD8+ T cells, macrophages and B cells. MHC class II is mainly expressed by APCs. Depending on the co-stimulation, different subsets of CD4+ T cells expand with different cytokine profiles fine-tuning the immune response towards the specific pathogen (Figure 1) [21]. Activated CD4+ T cells are often divided into Th1 and Th2 depending on the type of cytokines they produce. Th1 cytokines (for example interferon (IFN)-γ and IL-2) activate macrophages and CD8+ T cells, which effectively eliminate intracellular pathogens. Instead, Th2 cytokines (IL-4, IL-5 and IL-10) shift the immune response towards combating extracellular pathogens by inducing B cell activation and antibody production [20, 21]. A recently described novel subset of Th cells is the IL-17 producing Th17 cells, which are involved in inflammation and recruitment of leukocytes (reviewed in [23]). Th17 cells are induced by a combination of IL-6 and transforming growth factor (TGF)-β (but not TGF-β alone), which simultaneously suppress the generation of regulatory T cells important for controlling autoimmunity [24]. Th17 cells have been shown to participate in the development of various inflammatory and autoimmune diseases including T1D [25, 26].

In contrast to CD4+ T cells, CD8+ T cells are restricted to MHC class I, which is expressed by all nucleated cells. Therefore, cells infected with an intracellular pathogen can signal to already activated CD8+ T cells by presenting pathogen-derived peptides on MHC class I molecules. CD8+ T cells thereafter induce apoptosis in the infected cell by granzymes and perforin secretion or Fas/Fas ligand (FasL) engagement. Activated CD8+ T cells are also referred to as cytotoxic T cells (CTLs).
1.2.2 Autoimmunity

Approximately 3-8% of the human population in the industrialized world suffers from autoimmune diseases [27]. Such diseases result from a breakdown in the mechanisms controlling autoreactive T cells, leading to immune responses directed at self-peptides. The mechanisms controlling autoreactive T cells are divided into central and peripheral tolerance. Central tolerance occurs in the thymus where negative selection eliminates autoreactive T cells as described previously. Peripheral tolerance controls autoreactive T cells escaping central tolerance. Peripheral tolerance suppresses T cell responses by various mechanisms that will be described later in this section.

The initial trigger of autoimmunity as well as the autoimmune process is poorly understood. In single-gene disorders, autoimmunity results from mutations in genes important for maintaining tolerance such as the Aire gene leading autoimmune polyendocrine syndrome (APS-1). APS-1 is characterized by an autoimmune attack against, for example, endocrine organs and skin [28]. Moreover, mutations in the Foxp3 gene expressed by regulatory T cells leads to IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) [20]. IPEX is characterized by multi-organ autoimmunity, including T1D [11, 29, 30]. In contrast, common autoimmune diseases such as T1D, rheumatoid arthritis (RA) and multiple sclerosis (MS) are believed to result from a combination of multiple genetic and environmental components, which are not fully identified. Gene polymorphisms contributing to autoimmune diseases are often associated with immune system functions [31].
Autoimmune diseases are divided into organ-specific or systemic depending on the distribution of the self-antigen/s recognized by the autoreactive T cells. Organ-specific diseases are for example T1D, Addison’s and thyrotoxicosis whereas systemic diseases are systemic lupus erythematosus (SLE) and RA [32]. Some autoimmune diseases, such as MS, are more difficult to classify into organ-specific or systemic. In addition, many patients suffer from multiple autoimmune disorders for example RA and SLE. Cytokines are often central in the pathogenesis of autoimmunity and several therapeutic agents targeting cytokines are currently evaluated in clinical trials for the treatment of RA, SLE and MS [25-27].

1.2.3 Transplantation immunology

Organs or cells transplanted from one individual to another are subjected to rejection. Rejection is divided into hyperacute, acute and chronic rejection [21]. Hyperacute rejection is characterized by the binding of pre-formed antibodies to donor blood group antigens and subsequent complement activation and thrombosis. This reaction does not normally pose a clinical problem since donors and recipients are blood type-matched. Hyperacute rejection can create a problem if the recipient is sensitized due to a previous transplant. Therefore patients are usually screened for the presence of alloreactive antibodies prior to transplantation. A acute rejection involves adaptive immune responses and occurs after the first week of transplantation. Chronic rejection, on the other hand, develops over an extended period of time. The mechanisms behind chronic rejection are not clear but characteristic for such rejection is the growth of fibrotic tissue and a gradual loss of graft function due to wound healing or cytokine production.

A acute rejection is the result of adaptive immune responses targeted against donor cells. These responses occur as an organ or cells transplanted from one individual to another of the same species are seen as foreign by the recipient’s immune system. Rejection results from T cells binding to allogeneic MHC molecules. This is possible as T cells with a high affinity for allogeneic MHC molecules survive selection in the thymus. A reason why allografts cause a very strong immune reaction is that approximately 2% of an individual’s circulating T cells recognize foreign MHC molecules [21]. The allogeneic MHC molecules are presented to the recipient T cells in two ways, namely via direct or indirect presentation. Direct presentation involves activation of T cells as a result of direct binding to foreign MHC molecules [21, 33]. Indirect presentation involves uptake and processing of foreign MHC molecules by the recipient APCs at the graft site. The APCs then migrate to LNs and present the peptides to T cells like conventional foreign antigens.

CD4+ T cells participate in graft destruction by delayed type hypersensitivity (DTH) reactions involving cytokine production and macrophage activation. CD4+ T cells are also helper cells for B cells leading to the production of alloreactive antibodies that can bind to the graft and in turn activate the complement system. Activated CD8+ T cells that directly bind to allogeic cells induce apoptosis by the secretion of granzyme B and perforin. CD8+ T cells activated by the indirect pathway are unable to kill the graft cells since they are self-restricted. Therefore, the direct pathway requires cell-cell contact between T cells and allogeic cells whereas the indirect pathway does not
require cell-cell contact and only involves CD4+ T cells. The relative importance of the different pathways for allograft rejection will be discussed later in this thesis.

1.2.4 Immunomodulation

Immune responses are highly potent and must be tightly regulated in order to limit undesired cell damage after an infection. Also, some autoreactive T cells escape negative selection in the thymus and can be potentially harmful if activated. To reduce damage after pathogen clearance as well as autoreactivity, various natural mechanisms control activated immune cells. Moreover, it is also crucial to suppress immune responses after allogeneic cell or organ transplantation to prevent acute rejection of the graft. This is obtained by life-long intake of immunosuppressive drugs.

1.2.4.1 Natural mechanisms modulating immune responses

Autoreactive T cells present in lymphoid organs are controlled by peripheral tolerance mechanisms leading to anergy (functional unresponsiveness), deletion (apoptosis) and immunosuppression by regulatory T cells [20]. T cells interacting with MHC-peptide complexes without co-stimulation result in a state of anergy. Anergic T cells are unable to become activated even upon encounter with their cognate peptide presented by APCs expressing co-stimulatory molecules. CTLA-4 expressing T cells can also induce T cell anergy. CTLA-4 binds to CD28 and delivers inhibitory signals [21]. Immune responses can be regulated by deletion of T cells by apoptosis in response to heavy activation, so-called activation-induced cell death. Moreover, several subsets of regulatory T cells participate in suppressing T cells (Figure 1). Th3 and Type 1 regulatory T cells (Tr1) secrete TGF-β and IL-10, respectively. TGF-β inhibits T cell proliferation whereas IL-10 inhibits the expression of Th1 cytokines by macrophages. IL-10 also inhibits the expression of co-stimulatory molecules and MHC class II on macrophages and DCs [21]. Finally, so-called natural regulatory T cells expressing the transcription factor Foxp3 (CD4+CD25+Foxp3+: Tregs) are important in controlling autoreactive T cells by mechanisms that are not fully understood but cell-cell contact and membrane-bound TGF-β have been proposed (reviewed in [20]). In addition, Tregs have been shown to down-modulate co-stimulatory molecules on DCs via CTLA-4 [34]. The importance of Tregs in controlling tolerance is clearly evidenced by deficiency of Foxp3 in mice and humans leading to IPEX.

1.2.4.2 Immunosuppression to prevent allograft rejection

Life-long immunosuppressive drug treatment is the main strategy to prevent acute rejection of allografts. Several agents are used to block T cell proliferation and cytokine production (cyclosporine, azathioprine, mycophenolate mofetil and rapamycin) as well as anti-inflammatory agents blocking cytokine synthesis and secretion (corticosteroids) [21]. Antibodies targeting CD3 and IL-2 are also potent in inducing graft survival. Immunosuppressive drugs target T cells in a non-specific manner and therefore side effects such as increased susceptibility to infections and tumor development are associated with this therapy. Since organ transplantation is most often life-saving, the use of these drugs is motivated. One strategy to avoid the side effects of immunosuppressive drugs is by co-stimulation blockade using CTLA-4 antibodies and anti-CD40 ligand antibodies. In experimental models co-stimulation blockade has been demonstrated to promote islet xenograft survival [35].
1.2.4.3 Immunomodulation by mesenchymal stromal cells

Mesenchymal stromal cells (MSCs), also referred to as mesenchymal stem cells or BM stromal cells, were first described 40 years ago as fibroblast-like, multipotent cells with the capacity to differentiate into bone, cartilage, adipose, tendon, muscle, and marrow stromal tissues (reviewed in [36]). MSCs can be isolated from various tissues such as BM, liver, adipose tissue, lung, placenta and various fetal tissues [36-38] and provide the microenvironment supporting the development of HSC [39]. Although these cells only constitute approximately 0.001%-0.01% of the nucleated cells in the BM [36] they adhere to plastic and expand relatively easily in culture. There is no specific marker for MSCs, instead they are usually characterized by the expression of different cell surface markers, their ability to differentiate into for example adipocytes or osteocytes as well as their immunosuppressive ability in vitro.

MSCs seem to home to injured tissue and participate in tissue regeneration [40]. Studies have demonstrated a promising usage of MSCs as cellular therapy in a variety of disease conditions. MSCs support cardiac repair and HSC transplantation and they have also been successfully tested in the treatment of bone disorders [41-46]. In addition, MSCs home to the pancreas and kidney and improve blood glucose levels and renal functions after streptozotocin-induced diabetes in mice [47-49]. MSCs have been regarded as non-immunogenic and to escape allore cognition, however, some studies show that these cells can be rejected in vivo [50-52]. The therapeutic effect of MSCs seems to be associated with the production of factors capable of stimulating survival and function of injured tissue rather than cell replacement. Interestingly, MSCs produce TGF-β, hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) [53, 54], which have been shown to enhance survival of transplanted islets [55-59].

Another characteristic of MSCs is their ability to inhibit immune responses (reviewed in [60]). The suppressive effect of MSCs on T cell proliferation and IFN-γ production in vitro has been shown in numerous publications [38, 43, 61-63]. The immunosuppressive effect has also been demonstrated in vivo as they prolong heart and skin transplants and support BM engraftment [50, 61, 64-66]. The perhaps most striking effect by MSCs was demonstrated by LeBlanc et al who used MSCs to treat a patient with lethal grade IV acute graft-versus-host disease (GVHD) after a HSC transplantation [67]. Remarkably, the patient rapidly recovered and was still well one year after treatment. These results have since been repeated and been tested in a phase II trial [68]. Moreover, murine MSCs have been shown to ameliorate disease in various models for autoimmunity, including reducing the incidence of diabetes in NOD mice [69, 70]. The mechanisms behind the immunosuppressive effect by MSCs remain to be fully established. Several soluble factors have been proposed to be involved, such as IL-10, TGF-β and indoleamine 2,3-dioxygenase (IDO). However, results are contradictory most likely due to differences in sources and species of MSCs, culture conditions and T cell stimuli (reviewed in [37]). One candidate that has been shown to be important for the immunosuppressive effect of MSCs in several studies is prostaglandin E₂ (PGE₂) and recently nitric oxide (NO), both of which has been demonstrated to inhibit T cell proliferation [71, 72]. Blocking these molecules partially restored T cell proliferation suggesting that several factors may be involved [37, 38, 73,
Interestingly, proinflammatory cytokines enhance the immuno-suppressive effect by MSCs by inducing the production of soluble factors [38, 75-77]. This indicates that a proinflammatory milieu stimulate MSCs to regulate an ongoing inflammation in vivo.

1.2.4.4 Mechanical immunoprotection – the TheraCyte™ device

A method to avoid the use of immunosuppression after cell transplantation is to isolate the graft. For islet transplantation this means that single or small clusters of islets are trapped inside a gel capsule (micro-encapsulation) or the whole islet graft inside a chamber with semipermeable membranes (macro-encapsulation), both of which allow for the transport of oxygen, nutrients, glucose and insulin but protect the graft from immune cell entry and destruction. Major limitations of micro-encapsulation are insufficient encapsulation and instability of the capsule, which can lead to graft rejection over time. This can be overcome by macro-encapsulation of the graft inside diffusion chambers such as the TheraCyte™ (TheraCyte Inc, Irvine, CA, USA) device used in this thesis. The advantages of this device are that it requires minor surgery and can be retrieved if needed. The TheraCyte™ device is a planar diffusion chamber shaped as a teabag (Figure 2). The device consists of a polytetrafluoroethylene bilayered membrane. The inner membrane of the TheraCyte™ device has a pore size of 0.45µm, which enables the diffusion of nutrients and oxygen but not cells. Laminated on top of the inner membrane is the outer membrane, which has a pore size of 5µm suitable for the induction of neovascularization. To improve stability, the device is further covered with an inner non-woven polyester mesh and an outer woven polyester mesh. The graft tissue is inserted in one end, which is sealed after cell implantation. Diffusion devices are usually implanted in the peritoneal cavity, the omentum or subcutaneous fat.

Figure 2. The TheraCyte™ device. The device has the form of a teabag and is loaded with cells via the entry seen on the right hand side.

The inner membrane of the TheraCyte™ device prevents immune cells from entering the lumen of the device. The inhibited cell entry blocks the direct antigen presentation pathway since it requires cell-cell contact and allografts survive inside such devices [78, 79]. This was further evidenced by holes made in the membranes and thus enabling cell entry, which resulted in rapid graft rejection [78]. In contrast, xenogeneic cells were rejected when transplanted to intact devices [78, 80-82]. Encapsulated grafts can activate the indirect pathway due to diffusion of donor antigens across the membrane, which are presented to CD4⁺ T cells by DCs. Studies have shown that the presence of CD4⁺ T cells alone but not CD8⁺ T cells, antibodies or complement, induced the accumulation of local inflammation and reduced vascularization around the device leading to xenograft destruction [81]. This indicates that the direct pathway is
the main cause of allograft rejection whereas the indirect pathway is more important for xenograft rejection [33, 83].

Nevertheless, the release of antigens from encapsulated allografts could potentially activate CD4+ T cells and antibody production via the indirect pathway, leading to some level of activation without affecting survival of the recipient. Indeed, production of anti-donor antibodies after encapsulated islet allograft transplantation was demonstrated in 1/10 or 3/8 recipient rats, depending on the number of islets transplanted [84]. However, when receiving a subsequent heart graft none of the rats displayed accelerated rejection against the heart graft regardless of antibody status. This suggests that encapsulated islet allografts can lead to some level of immune activation via the indirect pathway in some but not all recipients, however, these antibodies does not seem to be of any clinical relevance in this experimental setting. This study points out to the safety of transplanting a second graft, which is of importance in the clinical setting.

1.2.5 Novel mouse models to study the human immune system

Animal models are not always suitable for studies on the human immune system due to species differences. Also, studies on immune cell development or interactions with pathogens are difficult or even impossible to perform in humans. Therefore, attempts have been made to produce so-called humanized mice, which harbor human immune cells as a result of the transfer of human stem cells or human PBMCs. The field started in the 1980s with the discovery of severe combined immunodeficiency (SCID) mice. These mice are deficient in mature T and B cells and were engrafted with human peripheral blood leukocytes and hematopoietic cells from human fetal tissues in 1988 [85, 86]. However, engraftment was low in these mice and primary immune responses were rarely detected. Xenoreactivity to the host by the infused human leukocytes also posed a problem. Depletion of the host’s adaptive immune system and substantial reduction in innate immunity is crucial for successful human cell engraftment [87] and the field saw new light in the beginning of the year 2000 when NOD/SCID and Balb/c/Rag2-/- mice lacking a functional IL-2 receptor common gamma chain (γc) were produced. The γc is required for the functional signaling via various cytokine receptors such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, which are crucial for T and NK cell development. Such mice were shown to be particularly permissive hosts for the engraftment of human HSCs due to their additional lack of NK cells [88]. Today several humanized mouse models on different background strains and infusion protocols are used. The humanized mouse models used in this thesis are described below.

1.2.5.1 The human immune system (HIS) mouse model

Human immune system (HIS) mice are generated by the injection of human cord blood HSCs into the liver of sub-lethally irradiated neonatal Balb/cRag2-/-γc-/- mice. Traggiai et al reported that human B cells were detected in the BM, spleens, LNs and blood [88]. Human IgM was present in serum and over time IgG was also detected demonstrating class-switching. Mostly single-positive CD4+ and CD8+ T cells were detected in spleens, mesenteric LNs and BM whereas thymi contained both double-positive and
single-positive T cells. In addition, DCs were detected in lymphoid organs, which stimulated allogeneic T cells in vitro.

Several studies have shown that HIS mice are permissive to infections by the human pathogens Epstein Barr virus (EBV) [88, 89], human immunodeficiency virus type-1 (HIV-1) [90-96] and herpes simplex virus type-2 (HSV-2) [97]. Antibody responses against these viruses are detected in some mice although the levels are lower than in humans. Human T cells in HIS mice proliferated when stimulated with human allogeneic DCs, but not host mouse DCs in mixed lymphocyte reactions (MLR) [98, 99]. Moreover, T cells in HIS mice infected with EBV responded to EBV-transformed B cells in vitro [88]. One major concern with the HIS mouse model is the development of T cells in the mouse thymus in a murine MHC context. This may affect the function of the T cells and explain the overall weak T cell responses in vitro and in vivo in this model (reviewed in [100]).

1.2.5.2 The bone marrow-liver-thymus (BLT) model

Another recently described humanized mouse model is the bone marrow-liver-thymus (BLT) model [101]. In order to generate these mice, human fetal thymic and liver tissues are transplanted under the kidney capsules of adult mice in order for the human T cells to develop on human thymic tissue instead of in the mouse thymus. After three weeks, autologous human fetal liver CD34+ cells are injected intravenously (i.v.). Melkus et al reported that BLT mice on the NOD/SCID background developed high percentages human cells consisting of B cells, T cells, monocytes, macrophages and DCs in lymphoid and non-lymphoid tissues. Peripheral blood and spleens from such mice contained human single-positive CD4+ and CD8+ T cells with a broad TCR repertoire and double-positive T cells in the human thymic tissue. In functional assessments the human T cells isolated from EBV-infected BLT mice responded to EBV-transformed autologous fetal liver B cells in a human MHC restricted manner [101]. The BLT mice are readily infected with HIV-1 and mount antibody responses against HIV-1 and different vaccination treatments [102-105]. Recently, the BLT mice has also been reported to reject skin [106] and islet xenografts [107] indicating functional T cell responses in vivo.

1.3 THE AUTOIMMUNE PROCESS OF T1D

The complexity and heterogeneity of T1D as well as the difficulties in obtaining human biopsies hampers our understanding of the autoimmune process in humans. Our knowledge is restricted to pancreases obtained from autopsies and analysis of serum and PBMCs from patients. The autoimmune process in humans is usually slow and preceded by an asymptomatic period characterized by an increase in numbers of anti-islet autoantibodies (insulin, GAD65, IA-2 and Znt8) sometimes several years before disease onset. Presence of increasing numbers of autoantibodies is a strong prediction for the development of T1D in humans, however, their role in pathogenicity is still unclear [108]. The long-standing hallmark for human T1D is reduced β-cell mass and islets infiltrated by immune cells (insulitis) (reviewed in [109]). Infiltrated islets express increased levels of MHC class I, which may participate in presentation of islet peptides to autoreactive T cells [110, 111]. The selective loss of β-cells in islets has been
demonstrated by the presence of α-cells, δ-cells and PP-cells but lack of β-cells [112]. Pancreas biopsies have shown that most islets in patients with recent onset T1D are deficient of insulin and to a lesser extent, insulin-containing islets are detected [112]. A minor fraction of the islets containing β-cells were inflamed in contrast to insulin deficient islets, which were devoid of infiltrating immune cells. Similar results have been shown in long-standing T1D patients using pancreas material obtained from the Juvenile Diabetes Research Foundation (JDRF)-sponsored nPOD program [111]. Data from human pancreases have also shown that insulitis mainly consists of CD8+ T cells and to a lesser extent macrophages, CD4+ T cells and B cells suggesting that CTLs are important in human T1D [18, 109, 113]. Peripheral CTLs isolated from T1D patients recognize various islet epitopes, for example proinsulin, GAD and islet specific glucose 6-phosphatase catalytic subunit-related protein (IGRP) [114-116].

1.3.1 Prevention strategies

Today, the development of T1D cannot be prevented. However, since it can be predicted with a certain degree of accuracy, attempts are made to slow down disease progression in T1D patients upon diagnosis or in persons at high risk of developing T1D. Some studies have indicated a delay of β-cell loss by treatment with for example oral insulin, GAD65 vaccination, anti-CD3 antibody treatment or depletion of B cells with anti-CD20 antibody ([117]) and reviewed in [11, 118]). Clinical trials will further test the long-term efficacy and safety of these therapies. Moreover, co-stimulatory blockade using CTLA-4 immunoglobulins is currently tested in human trials [119].

1.3.2 Mechanisms behind T1D in the nonobese diabetic (NOD) mouse model

The failure of tolerance to islet autoantigens is not fully understood and much of our knowledge comes from studies in the nonobese diabetic (NOD) mouse, which shares some similarities with human disease. For example, the major genetic risk factor in NOD mice is MHC class II, namely the H-2^g7 haplotype, which display a similar aminoacid substitution as seen in the human HLA-DQ, altering the peptide repertoire binding to this allele [120]. In addition, other genetic risk loci affecting T cell functions and regulation are associated with disease development in NOD mice.

Female NOD mice spontaneously develop T1D from 12-14 weeks of age, most likely as a result of several defects in tolerance mechanisms. The priming of autoreactive T cells occurs in the pancreatic LNs (PLNs) before 3-4 weeks of age leading to immune cell infiltration around the islets (peri-insulitis) generally referred to as checkpoint 1 [120, 121]. The initial trigger of autoreactive T cells is not fully understood but a postnatal wave of β-cell apoptosis occurs around two weeks of age in mice, which may be part of normal pancreas remodeling [122]. NOD mice have defective clearance of apoptotic cells [123] and uncleared dead cells can undergo so called secondary necrosis and activate the immune system. A study by Kim et al showed that DCs pulsed with secondary necrotic insulinoma-cells primed autoreactive T cells. This suggests that defective clearance of apoptotic β-cells may initiate the activation of autoreactive T cells [124]. By 10 weeks of age insulitis invades the islets (checkpoint 2) and shortly thereafter, overt diabetes develops. Infiltrating cells include CD4+ and CD8+ T cells.
recognizing for example insulin, GAD and IGRP epitopes. To a lesser extent, macrophages, NK cells, B cells and DCs are found in insulitic lesions.

T1D in NOD mice is regarded as T cell dependent since both CD4+ and CD8+ T cells from NOD mice transfer T1D and therapies targeting T cells can inhibit disease [120]. In addition, athymic NOD mice are protected from T1D. T cells are believed to contribute to β-cell destruction by the production of cytokines (Th1 and Th17 cells) and cell-mediated killing (CTLs). As mentioned previously, Tregs are important for peripheral tolerance. Tregs have been shown to be functional in NOD mice, interact with DCs in PLNs and participate in regulation of autoreactive T cells [125-127]. Despite the ability to suppress autoreactive T cells in NOD mice in the early phase, and perhaps causing the slow progression from peri-insulitis to invasive insulitis, the Tregs are ultimately unable to control the situation. Moreover, Tregs in NOD mice have been demonstrated to have a declined function over time [125-128].

1.3.3 The role of cytokines in β-cell destruction - The SOCS-1-tg mouse model

Cytokines are involved in the killing of β-cells in NOD mice. This was first shown in vitro as a combination of IL-1, IFN-γ and TNF-α caused β-cell destruction [129]. To further dissect the direct effect of cytokines on the β-cells, our group previously produced a transgenic (tg) mouse model on the NOD background over-expressing the suppressor of cytokine signaling (SOCS)-1 specifically in the β-cells. SOCS-1 inhibits cytokine signaling by blocking the JAK/STAT signaling pathway. Thus, β-cells over-expressing SOCS-1 are unresponsive to cytokines (i.e. IFN-α and IFN-γ). Interestingly, SOCS-1-tg mice were protected from the development of spontaneous diabetes [130]. This was not due to altered central tolerance or early T cell recruitment to the pancreas. However, pancreas-infiltrating T cells from 18 weeks old SOCS-1-tg mice were less pathogenic since they transferred disease in NOD/SCID mice to a lesser extent than non-tg littermates. In summary, this study indicated that the β-cell influences the autoimmune process in NOD mice, however the mechanisms for this were not clear.

1.4 PANCREATIC ISLET TRANSPLANTATION

Transplantation of the whole pancreas organ or isolated islets poses an attractive cure for T1D patients. In the first attempts to transplant whole pancreas in the 1960s and 1970s the mortality rate was more than 60% (reviewed in [131]). Despite improvements over the years, whole pancreas transplantation is a riskful procedure due to the major surgery required. The technique for isolating human islets from the pancreas was developed 30 years ago and was a breakthrough for the potential use of islet transplantation to treat T1D patients [132]. The first patient to reach insulin independence after islet transplantation to the liver was reported in 1990, however, after 22 days the islet graft was lost [133]. The following first attempts to transplant islets were unsuccessful and in many cases even fatal due to hepatic infarction and portal vein thrombosis. Despite protocol improvements less than 12% of patients were insulin independent one year after transplantation between the years 1990-2000 [131].
1.4.1 The Edmonton protocol

In the year 2000 Shapiro et al reported on successful islet transplantation in seven patients who all became insulin independent and remained insulin independent with sustained C-peptide production throughout the one-year follow-up [134]. These results have since been repeated and the Edmonton protocol is now standardized in islet transplantation [135]. More than 300 allogeneic islet transplantations have been performed world-wide between the years 1999-2008, as reported by the Collaborative Islet Transplant Registry [136]. Behind the success of the Edmonton protocol was the use of a refined immunosuppressive therapy. In the early days of islet transplantation, a combination of cyclosporine, azathioprine and glucocorticoids was used. This treatment resulted in low graft survival, toxic effects on islets and short window for additional islet transplantations [137]. Instead, the Edmonton group changed the immunosuppressive drugs to a combination of daclizumab (anti-IL2 receptor antibody), sirolimus and low-dose tacrolimus. This treatment could be used over an extended period of time allowing several infusions of freshly isolated islets, thus increasing the numbers of transplanted islets. Islet transplantation is a relatively safe procedure due to technical improvements over the years, and is associated with low procedure-related complications (i.e. intraperitoneal (i.p.) bleeding, portal vein thrombosis and gall bladder puncture) [138].

Despite the encouraging results presented by the Edmonton group, in the five-year follow-up only 10% of patients were insulin independent [135, 139]. However, some graft function seems to remain since 80% of the patients are still C-peptide positive five years after transplantation [139]. This partial function of the islet graft improves metabolic control, quality of life, production of C-peptide and episodes of severe hypoglycemic [136]. Unfortunately, the side effects associated with the immunosuppressive drugs are severe such as kidney dysfunction and infections [136, 139]. The burden of the immunosuppressive drugs overshadows the benefits of the islet graft and therefore islet transplantation cannot be motivated for most T1D patients. Today, islet transplantation is mainly available to patients with severe hypoglycemic episodes and uncontrollable blood glucose levels. Most patients also receive, or has previously received, a kidney transplant and are therefore already on immunosuppressive therapy [136, 139].

1.4.2 The implantation site

In humans, islets are infused via the portal vein into the liver. The rationale for this was that insulin is normally secreted into the portal vein from the pancreas and in early animal experiments the liver was shown to be the optimal site [131, 138]. It may seem puzzling that the islets are not implanted to the pancreas, which is their normal location in the body, however, due to the risks of digestive enzyme leakage the pancreas is preferentially avoided in surgery. The advantages of the liver as an implantation site are that the islets are infused into oxygenated blood and the implantation procedure is non-invasive. On the negative side, islets are destroyed by the so-called instant blood mediated inflammatory reaction (IBMIR) when islets come in contact with blood. In addition, the surviving islets are exposed to high levels of immunosuppressive drugs and lower oxygen tension than in the pancreas (5-10 mmHg vs. 40 mmHg). The islets
are also scattered throughout the liver making biopsies difficult. In a mouse study by Lau et al, severe metabolic dysfunctions and altered β-cell gene expression was demonstrated in intrahepatic islets compared to endogenous islets [140]. Alternative sites for islet graft implantation are currently being evaluated. For example, successful implantation of islets to the omental pouch and intramuscular sites has been reported [3, 138, 141].

Liver transplantations are technically challenging in rodents and it is difficult to biopsy the grafts. Instead, islets are usually implanted under the kidney capsule. Although the kidney capsule may not be optimal due to low vascular supply, the major advantage of this site is the low procedure-associated complications and the possibility to remove the graft for further studies [142].

1.4.3 Immune reactions to islet allografts

The numbers of islets required to reach insulin independence is approximately twice as many islets as are normally isolated from one pancreas [2]. The survival rate of the transplanted islets has been estimated to be 10-20% explaining the large number of islets needed to reach insulin independence. The reasons for islet graft loss are unclear and assessments of the islet graft post transplantation are difficult due to lack of proper monitoring assays. There may be several reasons for failure to reach insulin independence and graft loss over time, for example insufficient numbers of transplanted islets and low quality of the islets caused by hypoxia during isolation and after transplantation. The immunosuppressive drugs used can also lead to impaired engraftment of islets and decreased β-cell functions [138]. In contrast to allogeneic islet transplantation in T1D patients, transplantation of autologous islets in non-autoimmune patients (for example after a pancreatectomy) can result in long-term graft survival and requires less numbers of transplanted islets. This may be because of higher quality of islets as the donors are not exposed to the stress of intensive care and brain death [2]. Moreover, lack of peripheral insulin resistance and/or allo- and autoreactive responses may also result in long-term islet survival.

1.4.3.1 Innate immune responses

Innate immunity is a major contributor to the destruction of the majority of islets immediately after islet transplantation by IBMIR. When islets come in contact with blood, islets are trapped in clots formed by platelets and within minutes the islets are infiltrated by leukocytes. This event is caused by the islet expression of inflammatory mediators such as tissue factor (TF) glycoprotein and monocyte chemotactic protein-1 (MCP-1). The islet expression of these mediators is upregulated due to activation of defense mechanisms in brain-dead donors [143]. TF and MCP-1 activate the complement system leading to the destruction of up to 70% of the transplanted islets [144-146]. Improvements of the Edmonton protocol includes reducing TF by altered islet culturing conditions, administration of anti-inflammatory TNF-α antibody therapy and heparin treatment post infusion [138]).

1.4.3.2 Adaptive immune responses

HLA matching of donor and recipient is not performed in islet transplantation due to the scarcity of islet material. Therefore, acute rejection of islets by the adaptive immune
system may occur if a patient becomes sensitized to the donor/s HLA. Since several islet infusions are required to reach insulin independence, patients risk becoming broadly sensitized. The importance of adaptive immune responses in islet graft failure are difficult to dissect since many factors are involved in graft failure and the islets cannot be retrieved and studied for the presence of immune cell infiltration. Alloreactive T cells did not correlate with graft failure in a study by Huurman et al, which may be explained either by successful immunosuppressive treatment or insufficient T cell assays [147]. The role of alloantibodies in islet graft rejection is not clear. The development of alloantibodies after islet transplantation have been detected in some patients on immunosuppression, which displayed reduced C-peptide levels compared to patients that did not develop alloantibodies [148]. Another study reported that very few patients developed alloantibodies while on adequate immunosuppressive and therefore a definite association between de novo alloantibody production and graft outcome was not determined [149]. Instead, alloantibodies have been demonstrated as a result of discontinued immunosuppressive treatment due to graft failure, which may pose a problem if a future transplant is needed [148, 149]. Indeed, presence of pretransplant alloantibodies is associated with reduced islet graft survival [150].

1.4.3.3 Recurrent autoimmunity
T1D patients receiving an islet allograft do not only face the problem of controlling alloreactions but also recurrent autoimmunity. In contrast to islet alloreactivity, islet autoimmunity is believed to participate in islet graft loss (reviewed in [138]). Preexisting as well as recurrent autoreactive T cells [138, 147, 151] and in some studies autoantibodies [138] have been correlated to loss of or failure to reach insulin independence. The different correlations between islet autoreactivity and alloreactivity to islet graft failure may be that the immunosuppressive drugs used for islet transplantation are more effective against allograft rejection. As mentioned previously, the severe side effects of the immunosuppressive drugs are one of the reasons why islet transplantation is only offered to selected patients. Alternative methods to suppress allo- and autoimmunity in T1D patients after islet transplantation are clearly warranted.
2 AIMS OF THE THESIS

The first objective of this thesis was to understand the role of the $\beta$-cell in regulating the autoimmune process of T1D. The second and third objectives were to develop and evaluate new means to improve the survival of islet allografts and to assess humanized mice as novel experimental models for clinical islet transplantation.

2.1 SPECIFIC AIMS

To understand how the $\beta$-cell response to cytokines affects the diabetogenic process in the NOD mouse model for T1D (paper I).

To examine whether co-transplantation of MSCs can prolong the survival of allogeneic islet grafts (paper II).

To study whether macro-encapsulation of islet grafts protects from allograft rejection in already sensitized recipients (paper III).

To establish humanized mouse models and evaluate whether such models can be used as tools to study human islet graft rejection mechanisms (paper IV and preliminary study I).
3 MATERIALS AND METHODS

All materials and methods used in this thesis are described in the original papers. In the Results and discussion section (4) some unpublished results from paper II (primary MSCs) and preliminary study I are presented. Therefore, materials and methods for those experiments are described in detail below.

3.1 PAPER II (PRIMARY MSC)

3.1.1 Animals

C57BL/6 mice were purchased from Taconic, Denmark and maintained at Karolinska Institutet. All animal experiments were approved by the local ethical committee and conducted in accordance with institutional guidelines for animal care and use.

3.1.2 Isolation and culture of MSCs

MSCs were isolated from the femurs and tibias of C57BL/6 mice and cultured in complete Mesencult® medium (StemCell Technologies, Vancouver, Canada) according to manufacturer’s instructions. Briefly, femurs and tibias were collected and the BM flushed. Single cells were counted and 2.5 x 10^7 cells were seeded in T-25 cm² flasks. Upon 80% confluency, the cells were detached by trypsin-EDTA and reseeded. Cells were used at passages 5-10 to avoid contaminating CD45⁺ cells and differentiation of the cells.

3.1.3 DC cultures and MLR

DCs were generated from the BM of C57BL/6 mice and cultured as previously described [152]. Briefly, BM cells were obtained from the femurs and tibias of C57BL/6 mice and cultured in Dulbecco’s modified eagle medium (DMEM, Invitrogen, Paisley, UK) with glutamax I, 10% fetal calf serum (FCS), 100U/mL penicillin and 100mg/mL streptomycin (all from Invitrogen), supplemented with 10% culture supernatant from a myeloma cell line transfected with murine colony stimulating factor-2 (CSF-2) cDNA recombinant murine granulocyte macrophage (GM)-CSF. At day three of culture, the medium was gently removed and fresh medium supplemented with growth factors was added. After six days of culture, floating and lightly adherent cells were collected and seeded in new tissue culture plates. On the following day, floating and lightly adherent cells were collected and DCs were purified using CD11c microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). A total of 1µg/ml per well LPS (lipopolysaccharide) was added for the final 48 hours of culture.

The MLR was set up as previously described [153]. Cell suspensions were made from the spleens of Balb/c mice. Splenocytes were depleted of CD11c⁺ cells by MACS using anti-CD11c magnetic microbeads (Miltenyi). CD4⁺ T cells were subsequently purified from the CD11c-depleted splenocytes by anti-CD4 magnetic microbeads (Miltenyi
Biotec) according to the manufacturer’s protocol. A total of $1 \times 10^5$ BALB/c CD4$^+$ T cells were seeded in triplicates in 96-well U-bottom microtiter cell plates (BD, Stockholm, Sweden) with increasing numbers of DCs ($2 \times 10^2$, $1 \times 10^3$, $3 \times 10^3$ and $1 \times 10^4$) with or without MSCs (same numbers as the DCs). Prior to seeding, DCs and MSCs cells were irradiated with 20Gy. MLRs were incubated for a total of 96h in humidified, 5% CO$_2$ incubators at 37ºC and for the last 16h, 1µCi [$^3$H]thymidine (MP Biomedicals, CA, USA) was added to each well. The culture medium was RPMI 1640 (Invitrogen) with sodium pyruvate and supplemented with 50µM 2-ME, 100U/mL penicillin, 100mg/mL streptomycin, 2mM L-glutamine and 10% FCS (all from Invitrogen).

3.1.4 Statistics

Statistical analyses were performed using GraphPad Prism version 5 software. Inhibition of T cell proliferation by MSCs compared to control (T cells and DCs) was analyzed by a two-way ANOVA, followed by a Bonferroni post-test. p-values of less than 0.05 were considered significant.

3.2 PRELIMINARY STUDY I

3.2.1 Ethics Statement

The human pancreatic islet material used represented the unavoidable excess of islets generated within the Nordic Network for Clinical Islet Transplantation. Only organ donors that explicitly had agreed to donate for scientific purposes were included. Informed written consent to donate organs for medical and research purposes was obtained from donors, or relatives of donors, by the National Board of Health and Welfare (Socialstyrelsen), Sweden. Permission to obtain pancreatic islet tissue from the Nordic Network for Clinical Islet Transplantation, and the experiments involving human tissue were reviewed and approved by the local ethics committee (Regionala etikprövningsnämnden, Stockholm) in Stockholm, Sweden.

The human fetal tissues used were obtained from donors who had given their informed verbal consent to donate tissues for scientific purposes. Written consent was not required as no information regarding the donors was provided (i.e. the data were analyzed anonymously). The collection and experiments using human tissues reported here were approved by the local ethics committee (Regionala etikprövningsnämnden, Stockholm) in Stockholm, Sweden.

3.2.2 Animals

BALB/cRag2$^{-/-}$γc$^{-/-}$ mice were bred and maintained at Karolinska Institutet, Stockholm, Sweden, under specific pathogen free conditions. All animal experiments were approved by the local ethics committee (Stockholms Norra Försöksdjursetiska Nämnd) and conducted in accordance with institutional guidelines for animal care and use.
3.2.3 Human fetal tissues

Human fetal liver and thymus tissues from 17 (batch nr 1) and 13 (batch nr 2) weeks of gestation were obtained from the Department of Pathology, Karolinska University Hospital Huddinge, with parental informed consent. The fetal liver tissue was disrupted using with 1mg/mL collagenase/dispase (Roche, Mannheim), and 0.5U/mL DNase I (Roche). After Ficoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation, human CD34+ cells were enriched by MACS cell separation system using anti-CD34 microbeads according to the manufacturer’s instructions (Miltenyi). CD34+ cells were counted and purity (80-89%) was evaluated by flow cytometry. Cells were frozen in 90% FCS and 10% dimethyl sulfoxide (DMSO) at -80°C until transplantation.

3.2.4 Generation of humanized mice

Two separate batches of BLT mice (batch 1 and 2) were generated according to the protocol described by Garcia et al [101]. Briefly, adult Balb/cRag2-/-γc-/- mice were transplanted under isofluran (Baxter, Kista, Sweden) anesthesia with approximately 1mm³ pieces of human fetal thymus and liver under the left kidney capsule. Three weeks post transplantation, mice were conditioned with a sublethal whole body irradiation (550cGy) and injected intravenously via the tail vein with 5 x 10⁵ (batch nr 1) or 1.5 x 10⁵ (batch nr 2) human fetal liver CD34+ cells diluted in 100µL phosphate buffered saline (PBS) from the same donor as the human fetal tissues. For unknown reasons, the mice of batch 2 developed massive tumors and were eliminated from the study. The results presented here are therefore based on mice from batch 1.

3.2.5 Human islets

Human islets were purified from one human cadaver donor at the Uppsala University Hospital, as a part of The Nordic Network for Clinical Islet Transplantation, as previously described [154]. Upon isolation the islets were cultured in CMRL-1066 supplemented with 10mM nicotinamide, 10mM HEPES buffer, 0.25µg/mL fungizone, 50µg/mL gentamycin, 2mM L-glutamine, 10µg/mL ciprofloxacin and 10% heat-inactivated human serum. After isolation the islets were divided into separate batches with varying purity. The provided islets had a purity of 35 and 75%, as determined by dithizone staining, and were further purified by hand picking upon arrival at Karolinska Institutet. The quality of the islets was evaluated by insulin release in response to high glucose concentrations. A dynamic perfusion system was used as previously described [155] and the islets responded with a stimulation index of 12. After arrival at Karolinska Institutet the islets were transferred to RPMI-1640 with the same supplements as above but with FCS instead of human serum and without nicotinamide and incubated 14 days prior to transplantation at 37°C and 5% CO².

3.2.6 Islet transplantation

Prior to transplantation recipient control and BLT mice were anesthetized using isofluran inhalation. A total of 300 human islets were packed into a 22GAVenflon™ (BD, Helsingborg, Sweden) and placed under the right kidney capsule.
3.2.7 Immunohistochemistry

Formalin fixed organs were embedded in paraffin and cut in 4µm thick sections. Before paraffin embedding, the graft bearing kidneys were cut in half at the site of the graft. For insulin staining, sections were stained with a guinea pig anti-insulin primary antibody (DakoCytomation, Denmark) followed by a biotinylated secondary antibody (goat anti-guinea pig IgG) in conjunction with Standard Vectastain ABC kit and Peroxidase Substrate kit (all purchased from Vector laboratories, Burlingame, CA).

For detection of human immune cells, sections from spleens and grafted kidneys were stained with mouse anti-human CD3 (Novocastra Laboratories, Newcastle, UK) or mouse anti-human CD19 (DakoCytomation, Denmark) primary antibodies. Prior to antibody staining, antigen retrieval was performed by heating the sections in 10mM Citric acid buffer (pH 6.0) or in 10mM Tris, 1mM EDTA buffer (pH 9.0) respectively. To detect primary antibodies, a biotinylated rabbit anti-mouse IgG secondary antibody (DakoCytomation, Glostrup, Denmark) in conjunction with Elite Vectastain ABC kit and Peroxidase Substrate kit (both purchased from Vector laboratories, Burlingame, CA) were used.Slides were counterstained in Mayer’s Hematoxylin.

3.2.8 Flow cytometry

After organ collection, single cell suspensions of spleens, thymi and organoids were prepared. Prior to staining, unspecific binding of the antibodies to Fc-receptors were blocked by incubation with antibodies to mouse CD16/CD32 and human immunoglobulins (Gammagard®, Baxter, Kista, Sweden). Lymphocytes were subsequently stained with antibodies against human CD45 (PB) (DakoCytomation, Denmark), CD3 (FITC) (BD Pharmingen, Sweden) and CD19 (APC-Cy7) (BD Pharmingen, Sweden). The samples were then analyzed using a CyanFACS Instrument (BD Pharmingen) and FlowJo software (Tree Star, Ashland, OR).

3.2.9 Human C-peptide assay

Serum samples were collected from recipient mice and stored at -20°C until analysis. The levels of human C-peptide were quantified using Mercodia Ultrasensitive C-peptide ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer’s instructions.
4 RESULTS AND DISCUSSION

4.1 PAPER I

Preventative treatments for T1D patients are not available today and intervention therapies rely on a better understanding of the autoimmune process leading to disease. The direct role of cytokines in disease progression was demonstrated in NOD mice over-expressing SOCS-1 (SOCS-1-tg mice) specifically in the \( \beta \)-cells. These mice have a reduced incidence of diabetes compared to non-tg NOD mice [130]. When transferring T cells from diabetic mice (so-called adoptive transfer experiments), SOCS-1-tg mice developed diabetes less frequently than non-tg NOD mice indicating that SOCS-1-tg islets are less susceptible to destruction by diabetogenic T cells. In addition, T cells from the pancreas of prediabetic SOCS-1-tg mice had a reduced capacity to trigger diabetes when transferred to NOD/SCID mice. This indicated that local events in the pancreas, as a result of the \( \beta \)-cell response to cytokines, affect the pathogenicity of infiltrating T cells. In paper I we therefore studied differences in the T cell repertoire and chemokine expression in the pancreas of SOCS-1-tg and non-tg NOD mice.

T cell populations important for disease regulation in NOD mice were analyzed in the pancreas of SOCS-1-tg and non-tg NOD mice by flow cytometry. No differences in frequencies of CD4\(^+\) and CD8\(^+\) T cells or Tregs were detected (Supplementary Figure 1, paper I). Instead, there was a strong reduction in the presence of autoreactive IGRP-specific CD8\(^+\) T cells in the pancreases, PLNs and blood of SOCS-1-tg mice (Figure 2, paper I). The presence of this T cell clone in blood has previously been shown to mirror frequencies in the pancreas and correlate with the risk of diabetes development in NOD mice [156, 157]. The importance of this clone in diabetes development was demonstrated in the 8.3 TCR-tg NOD mice. This strain harbors T cells recognizing the IGRP epitope and displays an accelerated form of diabetes [158]. In addition, SOCS-1-tg mice crossed with 8.3 TCR-tg NOD mice were protected from diabetes (Figure 3A, paper I and [159]). The lower frequency of IGRP-specific T cells in the pancreas of SOCS-1-tg mice were not explained by a lower proliferation of this clone in the PLNs, as evidenced by adoptive transfer of IGRP-specific T cells (Figure 3B, paper I).

To understand the events in the pancreas leading to altered infiltration of autoreactive T cells we studied the chemokine CXCL10. IFN-\( \gamma \) induces the expression of CXCL10, which is produced by islets during diabetes development and participate in the recruitment of lymphocytes to the pancreas [160-162]. CXCL10 has also recently been described in the pancreases of T1D patients [163]. SOCS-1-tg mouse islets have reduced expression of CXCL10 (Figure 1B, paper I) and this may potentially affect lymphocyte infiltration. The receptor for CXCL10 is CXCR3 and this receptor is expressed on the majority of T cells that have migrated to the pancreas of SOCS-1-tg and non-tg NOD mice highlighting the importance of CXCL10 in recruiting CD8\(^+\) T cells (Figure 3E, paper I). CXCR3 is expressed more frequently on IGRP-specific T cells than bulk CD8\(^+\) cells in peripheral blood and PLNs (Figure 3C and D, paper I), thus these cells are more likely to migrate to the pancreas than other T cells. A reduced
CXCL10 expression in the pancreas may therefore, at least in part, explain the reduced infiltration of autoreactive T cells. Preliminary results also showed that homing of transferred IGRP-specific T cells to the pancreas of SOCS-1-tg mice was lower compared to non-tg NOD mice (unpublished data), indicating a reduced retention of the autoreactive T cells. An explanation for this may be expression levels of MHC class I in islets. Studies in different mouse models have shown that MHC class I expression in β-cells is crucial for the accumulation of autoreactive T cells to the pancreas [164, 165]. In addition, over-expression of SOCS-1 prevents MHC class I expression in islets, which results in an inability of autoreactive T cells to recognize the islets [159]. This may explain the lower susceptibility to destruction demonstrated when transferring diabetogenic T cells to SOCS-1-tg mice [130, 159, 166]. Interestingly, high expression of MHC class I by islets have been detected in T1D patients [110, 111].

In summary, the β-cell response to cytokines affects the infiltration of autoreactive T cells to the pancreas and the progression from insulitis to overt diabetes. This is evidenced by over-expression of SOCS-1 in islets leading to a reduced incidence of T1D in NOD mice. Contributing factors to this protection may be a combination of a lower expression of CXCL10 and MHC class I in islets.

4.2 PAPER II

Islet transplantation can restore blood glucose homeostasis in T1D patients and is therefore an attractive method to treat T1D patients [134]. The use of immunosuppressive therapy after islet allograft transplantation is crucial in order to avoid rejection of the transplanted tissue. Unfortunately, the unacceptable side effects of this therapy pose a major obstacle for the development and use of islet transplantation. Replacement of the classical immunosuppressive drugs with alternative methods is therefore highly warranted. Primary MSCs suppress the activation of T cells in vivot and in vivo and possibly participate in tissue regeneration and vascularization by their production of for example VEGF. Therefore, in Paper II we studied whether MSCs can enhance the survival of transplanted islet allografts in a mouse model.

For this study we used the readily accessible cell line MBA-1 (murine bone marrow adherent cells) [167]. To confirm that MBA-1 cells suppress T cell proliferation in vivot, we first performed MLRs. These experiments showed that the MBA-1 cells strongly suppressed CD4+ T cell proliferation (Figure 1A, paper II). Next, we transplanted C57BL/6 mouse islets under the kidney capsules of healthy Balb/c mice with or without co-transplantation of MBA-1 cells. The MBA-1 cells were placed at the site of the graft since we hypothesized that these cells may produce soluble factors beneficial to the graft and to be in close contact with the alloreactive T cells. The kidneys were harvested on days 7, 14 and 21 and remaining insulin-positive area was measured blinded by computerized analysis. No differences in islet mass were detected on days 7 and 14 between the groups, however, mice transplanted with MBA-1 cells demonstrated a significantly larger insulin-positive area on day 21 after transplantation compared to mice receiving islets alone (Figure 3 and Figure 1B, paper II).
Figure 3. Quantitative assessment of remaining β-cell mass under the kidney capsules of mice co-transplanted with or without MBA-1 cells. Healthy Balb/c mice were transplanted with C57BL/6 islets with or without MBA-1 cells under the kidney capsule. Kidneys were harvested on days 7 (-MBA-1, n=3, +MBA-1, n=4), 14 (-MBA-1, n=6, +MBA-1, n=5) and 21 (-MBA-1, n=6, +MBA-1, n=6) after transplantation and sections stained for insulin. The insulin-positive area was measured blinded by computerized image analysis. * p<0.05, two-way ANOVA. Bars represent means ±SD.

The graft area in mice receiving MBA-1 cells seemed to increase over time rather than decrease. Higher numbers of islets have been detected in MSC-treated streptozotocin-induced diabetic rodent models although the mechanisms for this have not been clarified [48, 49]. The increase in islet mass could be speculated to be the result of soluble factors produced by the MBA-1 cells stimulating islet survival either by inducing β-cell proliferation or by enhancing vascularization. The effect by MBA-1 cells is most likely exerted locally at the site of the graft since the LNs draining the grafted kidneys were enlarged with increased cellularity to a similar level in both control mice and mice receiving islets together with MBA-1 cells. This is perhaps not surprising since the MBA-1 cells were placed under the kidney capsules and may not enter the circulation and affect T cell priming in the draining LNs. Detecting the MBA-1 cells in vivo for example by CFSE-labeled or green fluorescent protein (GFP)-transfected cells would be informative to investigate this.

It is important to point out some limitations in this study. Allograft rejection in mice is usually complete within two weeks after transplantation and although there was a slight reduction in graft area in the control mice over time (Figure 3), rejection was not complete for unknown reasons. It is possible that other mouse strains than Balb/c mice would be better suited for allograft rejection studies. Moreover, in this study we used non-diabetic recipient mice, as we had not optimized the technique for transplanting islets to diabetic mice at this time. The use of diabetic mice would allow us to study the function of the transplanted grafts, i.e. whether the differences in remaining islet graft would also affect the curative capacity of the graft. Despite these limitations, the preliminary results presented in paper II suggested to us that further studies on the role of MSCs in islet transplantation should be continued.
4.2.1 Primary MSCs

In paper II we showed for the first time that MSC-like cells can interfere with islet allograft rejection. Since a cell line would not be used in a clinical setting and in order to work with more well-studied stromal cells, we isolated primary BM MSCs from C57BL/6 mice. The expanded primary MSCs inhibited T cell proliferation in a dose-dependent manner in MLRs (Figure 4). The inhibition by MSCs was statistically significant compared to control (DC + T cells) in all culture conditions except in the 200 and 1000 DC cultures + 200 MSCs ($p<0.05$, two-way ANOVA).

![Figure 4. Primary C57BL/6 BM MSCs inhibit T cell proliferation in MLRs. A total of $1 \times 10^5$ CD4+ Balb/c T cells were cultured with increasing numbers of irradiated C57BL/6 DCs with or without the addition of MSCs. Each value is the mean of three independent experiments. CPM, counts per minute.](image)

Recently, Ding et al demonstrated that diabetic Balb/c/Rag-/-γc-/- mice transplanted with allogeneic islets together with syngeneic MSCs under the kidney capsule and subsequently reconstituted with Balb/c T cells, were completely protected from allograft rejection [168]. This protection was dependent on the secretion of matrix metalloproteinase-2 and 9 (MMP-2 and MMP-9) by cleaving CD25 from the surface of T cells and thereby attenuating the T cell response to IL-2. Blocking of these factors resulted in restored T cell proliferation in vitro and islet rejection in all recipients, suggesting a major role for MMP-2 and 9 in the immunosuppression by MSCs. Solari et al showed that cyclosporine A treated rats receiving allogeneic islets together with MSCs to the omental pouch showed prolonged graft survival in most rats [169]. Interestingly, this effect was only seen when using syngeneic but not allogeneic MSCs further supporting that MSCs are immunogenic. It is still unclear whether MSCs support islet survival in immunocompetent hosts without additional immunosuppression. It may be that the immunosuppressive capacity of MSCs alone is not enough to suppress the strong immune response associated with allograft rejection. However, MSCs may possibly reduce the need for classical immunosuppression.

It is likely that MSCs enhance survival of islet grafts not only by immunomodulation but also by producing soluble factors beneficial for islet function as discussed previously. This was demonstrated in the paper by Solari et al where syngeneic MSCs co-transplanted with marginal mass syngeneic islets in diabetic rats restored blood
glucose levels compared to control rats receiving islets only. In addition, Figliuzzi et al and Sordi et al showed similar results in rodent models and also demonstrated increased vascularization of the islet graft, which was proposed to be due to the production of VEGF by the MSCs [170, 171].

In summary, our preliminary study (paper II) showing enhanced islet allograft survival in non-diabetic mice co-transplanted with MBA-1 cells has now been strongly confirmed by others in diabetic models using primary MSCs. The islet protective effect in vivo by MSCs seem to be the result of suppressing alloreactive T cells as well as promoting increased islet graft survival and function by the production of various soluble factors.

4.3 PAPER III

In paper II we proposed a new method to enhance islet allograft survival without detrimental side effects associated with classical immunosuppressive therapy. Another approach to avoid the need for immunosuppression is by encapsulating the islet graft inside a protective chamber such as the TheraCyte™ device. Allografts transplanted to this device are protected from rejection [78, 79]. However, it had not been studied whether encapsulated islet grafts are rejected in sensitized (i.e. immunized) recipients. The TheraCyte™ device is permeable to low molecular weight molecules such as antibodies, cytokines and NO. Therefore, the islet graft can potentially be exposed to preformed alloreactive antibodies or actions by memory T cells if a patient is already immunized at the time of islet transplantation, for example as a result of a previous kidney transplant. In paper III we therefore examined whether the TheraCyte™ device is protective against islet allograft rejection in immunized recipients. The experimental design for this project is presented in Figure 5.

Figure 5. Schematic overview of experimental design for the study on encapsulated islets. WF rats were pre-implanted with the TheraCyte™ device under the skin (n = 6). Two months later, WF rats were immunized by the transplantation of 1000 Lewis islets under the kidney capsule or by i.p. injection of 2 x 10^6 Lewis splenocytes. After one month, alloantibodies were detected in serum by flow cytometry. Rats positive for alloantibodies were rendered diabetic by streptozotocin and transplanted with 1000 Lewis islets to the device. Blood glucose levels were measured regularly and at the end of the study period graftectomy was performed. Metabolic control by the encapsulated islets was analyzed by oral glucose tolerance test (OGTT) one month post islet re-transplantation.
The alloprotective capacity of the TheraCyte™ device was demonstrated in naive Wistar Furth (WF) rats receiving islets to the device, which all remained euglycaemic. Strikingly, all immunized rats transplanted with encapsulated islets also had normal blood glucose levels throughout the six months study period compared to rats transplanted with free islets under the kidney capsule (both naive and immunized rats) (Figure 2, paper III). At six months, the TheraCyte™ device was removed to determine whether the normal blood glucose levels was the consequence of islet transplantation or restored endogenous insulin production in the pancreas. Graft dependency (i.e. return to hyperglycemia after graftectomy) was confirmed in all but one immunized WF rat transplanted with encapsulated islets, which was excluded from the study.

Alloantibodies produced after immunization were detected prior to islet transplantation. To study whether anti-donor antibodies were maintained in immunized rats, we analyzed serum at the end of the study period. Alloantibodies were present six months after islet transplantation in five out of six immunized rats receiving encapsulated islets (data not shown). None of the naïve rats receiving encapsulated islets produced alloantibodies. We also studied anti-donor responses by T cells to further confirm that immunization was successful and sustained during the study period. Immunized rats transplanted with islets to the TheraCyte™ device produced more IFN-γ than naïve WF control rats and naïve rats transplanted to the device (Figure 4, paper III). As expected, immunized and naïve rats receiving free islets produced high levels of IFN-γ. These results showed that the immunization protocol was successful and that lack of rejection was not the result of failure to immunize the recipients. Moreover, immunized rats receiving free islets rejected the second graft earlier than naïve rats (days 1-9 and 8-13 respectively) (Figure 2, paper III) indicating a secondary response and further confirmed successful immunization.

Immunized rats receiving encapsulated islets were protected from allograft rejection to the same level as naïve recipients transplanted with encapsulated islets. However,
alloreactions by the present alloantibodies and/or T cells may theoretically still occur and reduce islet graft function and viability without affecting the survival of the animal. For example, pre-formed CD4+ T cells could potentially react to alloantigens shed from the device and participate in inflammation around the device. We did not study CD4+ T cell responses *ex vivo* but histological analysis of encapsulated grafts showed no serious inflammation around the device in immunized or naive rats at six months after transplantation. A representative hematoxylin and eosin staining of a section from an immunized rat is shown in Figure 6. Moreover, we did not detect any differences in total volumes of the remaining islet graft tissue or the percentages endocrine and fibrotic tissues inside the devices of naive and immunized recipients using computerized analysis of histology samples (Table 1, paper III). Finally, the response to oral glucose challenge (OGTT) did not differ between naive and immunized rats receiving encapsulated islets (Figure 3, paper III). High variations in blood glucose and plasma insulin values were detected in these groups suggesting different quality and viability of the encapsulated islets. The physiological differences between encapsulated islets and islets located in the pancreas may cause the altered insulin responses to glucose. For example, the encapsulated islets are not in contact with the blood circulation to the same extent as islets situated in the pancreas and insulin released from the pancreas may be consumed quicker in the liver located in close proximity to the pancreas. We also observed extensive overgrowth of fibrotic tissue within the devices. These factors may affect the insulin response to glucose and are important to study further. If such reduced islet graft function and viability of encapsulated islets is also seen in humans, this may lead to long-term loss of graft.

In conclusion, the results presented in paper III demonstrated that immunized recipient rats are completely protected from islet allograft rejection during a six months study period.

4.4 PAPER IV

The monitoring of human islet rejection and evaluation of prevention strategies in humans are difficult. The mouse is usually the animal of choice for studying islet transplantation, however, results obtained in mice cannot always be translated to humans due to species differences [172, 173]. Mice harboring a human immune system pose an attractive model to study the interactions between human immune cells and human islet grafts. The recent description of such mice [88] led us to examine whether the HIS mouse model can reject a human islet graft.
HIS mice were produced by intrahepatic (i.h.) injection of human cord blood CD34+ cells into sub-lethally irradiated newborn Balb/cRag2−/−γc−/− mice. After detection of human cell engraftment, mice were transplanted with human islets and graft rejection was evaluated on days 14 and 35 post islet transplantation.

In paper IV we showed the successful establishment of the HIS mouse model. The experimental design for this project is shown in Figure 7. Human immune cell reconstitution was analyzed by the presence of human CD45+ leukocytes in blood (Table 1, paper IV). Ten reconstituted mice were selected for islet transplantation and at the end of the study period we further characterized the human immune cell reconstitution in these mice (Table 1, and Figure S1, paper IV). High variations in reconstitution levels were detected, which has been reported previously [88, 92] and may be due to the technically difficult procedure. In most mice, B cells were the major human cell population found in the spleens and low levels of CD3+ T cells were detected, as previously described [88, 92]. In the spleens, the CD8+ T cell population was approximately two-fold larger than the CD4+ T cell population (Table 1A), which is opposite to the normal human spleen [21]. Interestingly, the thymi contained exclusively human T cells and T cell generation was evidenced by the presence of double-positive CD4+CD8+ T cells (Table 1B). In contrast, the spleens contained mostly single-positive T cells, resembling the distribution in the human spleen.
Table 1. Percentages human immune cells in the spleens and thymi of transplanted HIS mice detected by flow cytometry. Human CD45+ cells were detected within the lymphocyte gate. Human CD3+ and CD19+ cells were detected within the CD45+ cell population. CD4+ and CD8+ cells were detected within the CD45+CD3+ population. A. Human immune cells in the spleens. B. Human immune cells in the thymi. n.d., not determined.

<table>
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<th>CD19+</th>
<th>CD4+</th>
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The human T cells in the HIS mice are educated in the mouse thymus but the detailed mechanisms for these events have not been described. Recognition of allogeneic human HLA by the T cells is crucial in order to reject human islet allografts. For this reason we studied the ability of the reconstituted T cells to react against allogeneic human DCs. Indeed, CD4+ T cells from HIS mice reacted against allogeneic human DCs in an MLR. The response to host (Balb/c) DCs was low whereas some response to xenogeneic C57BL/6 DCs was detected (Figure 1, paper IV). This indicated that T cells are educated, at least to some extent, on human HLA in HIS mice as well as on mouse MHC. It may be hypothesized that HLA selection occurs on human HSC-derived DCs or by thymocyte-thymocyte interactions [99, 174].

Despite the presence of human immune cells and functional T cell responses in vitro, HIS mice failed to reject human islet grafts during the 14 and 35 days study period. Graft survival in HIS mice was evidenced by immunohistochemistry and measurements of C-peptide levels in serum (Figure 2, paper IV and Table 1, paper IV). In addition, no or very few infiltrating T or B cells were detected around the graft tissue (Figure 2, paper IV and data not shown). Multiple steps are required to take place in order for immune mediated rejection to occur, such as the direct binding of T cells to intact MHC molecules expressed on donor APCs or graft cells (direct allorecognition) or donor antigen presentation by host APCs to T cells (indirect allorecognition). Further, T
cell activation, expansion and migration to the site of graft implantation are required. These steps rely on the presence of secondary lymphoid structures. Peripheral LN development is impaired in the Balb/cRag2\(^{-/-}\);γc\(^{-/-}\) mice, which may hinder an efficient priming and expansion of alloreactive T cells. Moreover, we did not detect any infiltrating human immune cells at the site of graft, possibly resulting from lack of cross-reactivity between human and mouse chemokine/chemokine receptors. However, migration of human PBMCs has previously been demonstrated in SCID mice transplanted with human islets under the kidney capsule [175], suggesting that human T cells can migrate efficiently in a murine environment. Therefore, the explanation to the failure of rejection in the HIS mouse model is likely to be found prior to the T cell migration stage. Poor T cell responses have been described in the HIS mouse model [98, 99] and may be the result of suboptimal T cell education in the thymus. There have been no reports on graft rejection in HIS mice and it is likely that this is due to negative results obtained from such experiments. I believe that negative data on developing models such as humanized mice are equally important as positive results in terms of increasing our understanding of the usefulness as well as the limitations of these models.

In summary, the HIS mouse model failed to reject human islet allografts despite the successful engraftment of human HSCs and presence of human immune cells in lymphoid tissues.

### 4.5 PRELIMINARY STUDY I

During the work on the HIS mouse model, skin xenograft rejection was reported in the BLT mouse model [106]. Since we had already established the technique to transplant tissue under the kidney capsule in murine models, we set out to establish the BLT model and test whether islet allografts were rejected in this model. The BLT study presented here is a pilot study and thus the results are only preliminary. Further studies are required in order to verify the results obtained.

The experimental design for this project is shown in Figure 8. The BLT mice showed lower reconstitution levels compared to the HIS mouse model. A mean of 4.7 ± 7.8% (mean ± S.D.; range 1.3 - 28.4%; n = 12) human CD45\(^{+}\) cells was detected in peripheral blood 4-5 months post human fetal tissue transplantation (data not shown). Four mice were selected for islet transplantation and at the end of the study period of 14 or 35 days, immune reconstitution was further examined. Variable percentages of human CD45\(^{+}\) cells were detected in the spleens (Table 2). Within the human CD45\(^{+}\) cell population the majority of cells were CD3\(^{+}\) whereas no or very low levels of human B cells were found (Table 2 and Figure 9A). In all but one mouse, the majority of T cells were CD4\(^{+}\) or CD4\(^{+}\)CD8\(^{+}\) whereas low percentages CD8\(^{+}\) T cells were detected. Representative immunohistochemical stainings of human CD3 and CD19 in the spleens are shown in Figure 9B-E.
Figure 8. Schematic overview of the experimental design for the study on BLT mice. BLT mice were produced by the transplantation of human fetal liver and thymic tissues under the kidney capsules of adult Balb/c/Rag2⁻/-γc⁻⁻ mice. Three weeks later, autologous CD34⁺ cells isolated from the fetal liver were injected i.v. After detection of human cell engraftment, mice were transplanted with human islets and graft rejection evaluated on days 14 and 35 post islet transplantation.

Table 2. Percentages human immune cell reconstitution in transplanted BLT mice detected by flow cytometry. Human CD45⁺ cells were detected within the lymphocyte gate. Human CD3⁺ cells were detected within the CD45⁺ population. CD4⁺ and CD8⁺ T cells were detected within the CD45⁺CD3⁺ T cell population. n.d., not determined.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>CD45⁺ (blood)</th>
<th>CD45⁺ (spleen)</th>
<th>CD3⁺ (spleen)</th>
<th>CD4⁺ (spleen)</th>
<th>CD8⁺ (spleen)</th>
<th>CD4⁺CD8⁺ (spleen)</th>
<th>Day of sacrifice</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>2.4</td>
<td>0.06</td>
<td>70.0</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>14</td>
</tr>
<tr>
<td>14</td>
<td>7.1</td>
<td>0.5</td>
<td>96.9</td>
<td>45.7</td>
<td>34</td>
<td>1.54</td>
<td>14</td>
</tr>
<tr>
<td>11</td>
<td>28.4</td>
<td>18.2</td>
<td>97.6</td>
<td>50.6</td>
<td>2.8</td>
<td>38.1</td>
<td>35</td>
</tr>
<tr>
<td>13</td>
<td>5.1</td>
<td>15.5</td>
<td>91.2</td>
<td>41.7</td>
<td>1.9</td>
<td>40.8</td>
<td>35</td>
</tr>
</tbody>
</table>

When dissecting the mice we saw substantial growth of the organoids and this tissue was packed with human CD45⁺ cells (mean ± S.D.; 98.7 ± 1.3%; range 97.1-100%; n = 6) (Figure 9F and G), which was also described by Melkus et al. [101]. Within the CD45⁺ cells, 62.1 ± 16.7% (mean ± S.D.; range 44.0 - 92.7%; n = 4) were human CD3⁺ cells. Most of the CD3⁺ T cells were CD4⁺CD8⁺ similar to the HIS mice. To a lesser extent single-positive CD4⁺ and CD8⁺ T cells were detected (data not shown).

Technical issues may explain the low reconstitution levels in blood and spleens. In addition, several mice, which had no or very low levels of human cell engraftment in the periphery, still had enlarged organoids consisting of human CD45⁺ cells. This indicated that the human immune cells were retained inside the organoids and unable to enter the circulation for unknown reasons, which may also explain the low percentages human T cells in the periphery.
Figure 9. Phenotypic analysis of BLT mice. Reconstituted BLT mice were analyzed by flow cytometry and immunohistochemistry for the presence of human CD3⁺ T cells (n=6) and CD19⁺ B cells (n=4). (A) Flow cytometric analysis of splenocytes harvested from BLT mice. Shown is a representative dot-plot for human CD19⁺ (B cells) and human CD3⁺ (T cells) staining of gated human CD45⁺ cells. (B-E) Representative immunohistochemical analysis of the presence of human CD3⁺ (T cells, B, C) and CD19⁺ (B cells, D, E) cells in spleens from BLT (B, D) and non-reconstituted control (C, E) mice. Original magnification, 25x. (F) An organoid harvested 24 weeks after human fetal tissue transplantation. (G) Flow cytometric analysis of thymocytes harvested from the organoid of BLT mice. Shown is a representative dot-plot for human CD45 staining within the lymphocyte gate.

None of the four BLT mice transplanted with human islets showed signs of rejection as assessed by immunohistochemistry (Figure 10A-D) and measurements of C-peptide levels in serum (Figure 11). Although the human serum C-peptide levels were lower in the BLT mice compared to the control mice, C-peptide was present at all different time points measured after transplantation. In addition, human CD3⁺ T cells were absent in and around the islet grafts as assessed by immunohistochemistry (Figure 10E-H).
Figure 10. No signs of graft rejection or infiltrating human CD3+ T cells was detected in BLT mice transplanted with human islets. BLT mice and non-reconstituted Balb/c/Rag2<sup>−/−</sup>γ<sup>−/−</sup> were transplanted with 300 (BLT mice, n = 4; control mice, n = 2) human islets under the right kidney capsules. Graft survival and the presence of graft infiltrating CD3+ T cells were evaluated on days 14 (BLT mice, n = 2; control mice, n = 1) and 35 (BLT mice, n = 2; control mice, n = 1) after transplantation by immunohistochemistry. Representative stainings are presented in A-H. (A-D) Insulin staining in grafts from BLT (A, C) and non-reconstituted control (B, D) mice harvested on days 14 (A, B) or 35 (C, D) after transplantation. Original magnification, 10x. (E-H) Human CD3 staining in grafts from BLT (E, G) and non-reconstituted control (F, H) mice harvested on days 14 (E, F) or 35 (G, H) after transplantation. Original magnification, 25x.
There are several possible explanations for the absence of graft rejection in this study. First, although Balb/cRag2\(^{-/-}\)\(\gamma\)c\(^{-/-}\) mice are particularly permissive hosts for the engraftment of human HSCs when injected as neonates with human CD34\(^{+}\) cells (HIS model), the BLT model has only been reported on the NOD/SCID background. Thus, the supportive ability of Balb/cRag2\(^{-/-}\)\(\gamma\)c\(^{-/-}\) mice for the development of human immune cells in this setting is not clear. Indeed, the background strain has been shown to strongly affect the engraftment levels [87]. Secondly, due to the scarcity of human fetal tissues, a limited number of BLT mice were produced. For unknown reasons the engraftment levels in the BLT mice in our study were overall low further limiting the number of mice available for islet transplantation. Future studies using higher numbers of BLT mice as well as higher levels of human immune cell engraftment are required to firmly determine the lack of allograft rejection in BLT mice on the Balb/cRag2\(^{-/-}\)\(\gamma\)c background. Finally, in a report by Tonomura et al in 2008, rejection of xenogeneic pig islet graft by BLT mice was demonstrated in mice with 5% or more human T cells in blood [107]. Rejection was complete 35 days after islet transplantation and both CD4\(^{+}\) and CD8\(^{+}\) T cell infiltration was detected around the graft as early as two weeks after transplantation. Further, depletion of T cells protected the grafts from rejection. In our study T cell reconstitution in spleens was lower than 5% in the BLT mice analyzed on day 14, which may explain the lack of rejection and infiltration. The mice analyzed on day 35 had high T cell reconstitution in spleens but still no signs of rejection were detected. As discussed in the introduction, xenograft rejection mechanisms differ from allograft rejection. Therefore, the rejection demonstrated by Tonomura et al may be explained by the different potency of the engrafted human immune system to reject a human islet xenograft compared to a human islet allograft. A larger study comparing both xenogeneic and allogeneic islet graft rejection in BLT mice is needed to evaluate different capacity to reject allo- and xenogeneic islets by the BLT mice.

In conclusion, the BLT model was produced to improve T cell selection and development in humanized mice. This model also failed to reject human islets although these results are preliminary and may be due to limited numbers of animals used and low human cell engraftment. Regardless of the functionality of the BLT model major drawbacks preventing a widespread use of this model is the labor intensity, scarcity of human fetal tissues and overall ethical constrains associated with the use of such tissue.
5 CONCLUDING REMARKS

The incidence of T1D is increasing and at present the autoimmune process leading to T1D is poorly understood and complicate the development of intervention therapies. In this thesis we show that the β-cell response to cytokines is important for disease progression. NOD mice over-expressing SOCS-1 specifically in the β-cells have reduced incidence of diabetes and lower frequency of autoreactive T cells in the pancreas. This may be the result of inhibited CXCL10 expression in islets, which is important for the recruitment of CXCR3-expressing autoreactive T cells. Reduced MHC class I expression in SOCS-1-tg mice has also been shown by others to reduce islet recognition and accumulation of autoreactive T cells. Whether these factors could be targeted in newly-onset T1D patients as a novel therapeutical treatment remains to be elucidated.

Islet transplantation as a treatment to restore blood glucose metabolism in T1D patients is hampered by the scarcity of human islets, long-term graft failure and the severe side effects associated with immunosuppressive therapy. This calls for the rapid development of new protocols improving islet survival following transplantation. In this thesis, we show that the MSC-like MBA-1 cell line suppress T cell proliferation in vitro and enhance islet allograft mass in vivo. In recent publications increased survival of islet grafts have been demonstrated in diabetic rodent models when co-transplanted with primary MSCs. This points out to the potential use of MSCs as cell therapy in islet transplantation. MSCs provide several advantages as cell therapy; they are easily isolated and expanded and they are already approved for the use in clinical settings. One concern is the safety of these cells in terms of tumor support and formation, which is of special relevance in immunosuppressed patients. At present, the role of MSCs in cancer development has not been extensively studied and results are contradictory [176]. Therefore this is important for further investigations.

Another approach to avoid the use of immunosuppressive drugs is by encapsulating the islets inside a protective membrane such as the TheraCyte™ device. Our study indicated the safety of transplanting encapsulated islets in already sensitized recipients. This has of course to be further investigated in human subjects. One of the concerns with encapsulated islets is the altered metabolic control, which may reflect a decreased viability and quality of the islet graft inside the chamber. Further studies on glucose metabolism in animals transplanted with islets to the TheraCyte™ device may provide information allowing the development of improved protocols better preserving islet functions. Recent studies have shown that culturing islets together with MSCs improve islet quality and function in vitro and in vivo [177] and coating islets with MSCs increase vascularization [178]. These strategies would be interesting to test in our model as they could potentially improve the viability and function of the encapsulated islets. Also, in this thesis we have only studied immunoevasion strategies in allogeneic settings, however, recurrent autoimmunity pose a problem for T1D patients receiving islet transplantation. Thus, it would also be of clinical value to test these novel strategies for their potential use against islets autoimmunity in diabetic models as well.
Finally, in this thesis we show that the engrafted human immune cells in the HIS mouse model does not support rejection of human islets and similar results were demonstrated for the BLT mouse model, although these studies are somewhat preliminary. In addition, percentages and ratios of human immune cells are not always comparable to levels detected in human blood, spleen and thymus, which may complicate interpretation of results obtained in humanized mice. Our studies indicate that the HIS and BLT mice may not be optimal models to study the human immune responses leading to human islet rejection. These studies highlight the need for further improvements of the presently established humanized mouse models, as well as the development of easily accessible models. Standardized protocols limiting the high variability between different studies would also improve this field of study. All these requirements are clearly warranted since a well functional humanized mouse model would provide a valuable tool in which the events leading to islet loss after transplantation could be studied. In addition, different immunosuppressive treatments, such as the ones presented in this thesis, could be tested for their efficacies to prevent rejection and support islet survival. Islet modulations could also be evaluated in humanized mice. For example, β-cell expression of SOCS-1 as well as CXCL10/CXCR3 blockade in recipient mice prolongs survival of islets after transplantation in murine mouse model and would be interesting to test in a human setting [166, 179, 180]. Another interesting use of humanized mice would be to reconstitute them with HSCs from T1D patients to study disease progression. If such a model was feasible, prevention treatments could be evaluated.

To summarize, the presently available humanized mouse models have limited capacity to generate robust immune responses, myeloid cell lineages and long-term self-renewal [181]. Currently, efforts are being made to overcome species-species incompatibilities limiting survival and homeostasis of human HSCs. For example, delivery of exogenous human cytokines and lymphocyte growth factors, HLA class I and II transgenes as well as expression of factors suppressing phagocytosis of human cells by mouse macrophages are presently being evaluated by several groups (reviewed in [181]). It will be interesting to follow the progress in this field and the hopefully many uses of these models.
**6 POPULÄRVETENSKAPLIG SAMMANFATTNING**

Bukspottskörteln är det organ som bidrar till att producera matsmältningensymer samt reglera nivån av socker i blodet. Vävnaden som reglerar blodsockernivåerna består av s.k Langerhanska öar som är utspridda i bukspottskörteln. Öarna består av flera olika celltyper, bland annat s.k. β-celler som producerar hormonet insulin. När sockernivåerna i blodet höjs till följd av en måltid utsöndras insulin som stimulerar celler att ta upp socker. Blodsockret används sedan som bränsle till cellernas energiproduktion. Sjukdomar som karakteriseras av defekt blodsockermetabolism kallas diabetes. Ca 220 miljoner människor lider av diabetes och av dessa har 5-10% Typ 1 diabetes (T1D) som även brukar kallas barndiabetes. T1D kännetecknas av brist på insulin som leder till höga blodsockernivåer. Obehandlad T1D leder till ett livshotande tillstånd då kroppen bryter ned fett och muskler för att få tillgång till bränsle. T1D behandlas framgångsrikt med insulininjektioner men trots detta riskerar dessa patienter att på lång sikt utveckla nedsatt njurfunktion, blindhet, nervskador och hjärt-kärlsjukdomar. T1D uppstår till följd av en autoimmun process som förstör β-cellerna. Autoimmunitet innebär att en individs egna immunsystem felaktigt ser kroppsegna celler (i det här fallet β-celler) som främmande och attackerar dem. Man vet inte varför en del personer utvecklar T1D men troligen beror det på en kompleks inverkan mellan gener och miljöfaktorer.


Det finns idag inget botemedel mot T1D men blodsockermetabolismen kan återställas genom transplantation av Langerhanska öar från hjärnöda donatorer. Vid en transplantation mellan två genetiskt skilda individer stöter immunceller (framförallt T-

utvecklats i sin normala omgivning. Preliminära resultat från en annan humaniserad musmodell med mer robust T-cellsutveckling och funktion gav liknande resultat. Det är viktigt att fortsätta utveckla dessa modeller eftersom de skulle kunna svara på många frågor kring det humana immunsystemet som vi idag endast har begränsad kunskap kring.

Sammanfattningsvis, i den här avhandlingen visar vi att β-cell aktivt deltar i den autoimmuna processen i T1D. Vi visar även att MSC skulle kunna bidra till ökad överlevnad av transplanterade öar. Ett annat sätt att skydda öarna efter transplantation är att kapsla in dem. Vi visar att dessa kapslar skyddar från avstötning även i immuniserade mottagare vilket har stor klinisk relevans. Slutligen etablerade vi humaniserade möss. Dessvärre utvecklade inte dessa möss tillräckligt robust humant immunsystem för att stöta på tion och är därför inte en lämplig modell i dess nuvarande form att studera interaktioner mellan humana immunceller och transplanterade öar.
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