Studies of Innate and Adaptive Immunity in Islet Transplantation

MARIA HÅRDSTEDT
Clinical islet transplantation is today an established alternative treatment for a selected group of type 1 diabetes patients. The predominant technique for transplantation is infusion of islets in the liver via the portal vein. Obstacles to advancing islet transplantation include limited engraftment resulting from an immediate blood-mediated inflammatory reaction (IBMIR), a life-long need for immunosuppression and the shortage of organs available.

In this thesis, innate and adaptive immunity were explored in allogeneic and xenogeneic settings, with the long-term goal of preventing islet graft destruction. Methods for studying immune responses to islets in blood and engrafted islets in liver tissue (intragraft gene expression) were developed and refined. The innate response to human islets and exocrine tissue in ABO-compatible blood was characterized up to 48 h using a novel whole-blood model. Physiological changes in the blood during incubations were explored and adjusted to allow prolonged experiments. Increased production of chemokines targeting CXCR1/2, CCR2 and CXCR3 was observed, accompanied by massive intra-islet neutrophil infiltration. Notably, endocrine and exocrine tissue triggered a similarly strong innate immune response.

Two studies of adult porcine islet transplantation to non-human primates (NHPs) were performed. Expression of immune response genes induced in liver tissue of non-immunosuppressed NHPs (≤72 h) was evaluated after porcine islet transplantation. Up-regulation of CXCR3 mRNA, together with IP-10, Mig, MIP-1α, RANTES, MCP-1 and cytotoxic effector molecule transcripts, was associated with T-cell and macrophage infiltration at 48-72 h. Long-term survival (>100 days) of adult porcine islets in a NHP model was later demonstrated using T-cell-based immunosuppression, including co-stimulatory blockade (anti-CD154 mAb). Graft failure was associated with increased levels of circulating, indirectly activated T cells, non-Gal pig-specific IgG and gene transcripts of inflammatory cytokines. Microarray analysis of the response to inflammatory cytokines in cultured porcine islets identified genes involved in cell death, immune responses and oxidative stress; this gene pattern coincided with physiological changes (decrease in insulin and ATP content).

In summary, allogeneic whole-blood experiments and xenogeneic in vivo studies underscored the importance of preventing early inflammation and cell-recruitment to avoid islet graft loss in islet transplantation. Long-term survival of porcine islets in NHPs was shown to be feasible using T-cell-directed immunosuppression, including anti-CD154 mAb.

Keywords: diabetes, islet transplantation, islets of Langerhans, xenotransplantation, nonhuman primate, blood, whole blood model, innate immunity, adaptive immunity, IBMIR, chemokines

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Till Per
**Front cover photo:** Immunohistochemistry of clotted human islets incubated for 6 h in recipient whole blood. Staining for neutrophils (myeloperoxidase) shows neutrophils gathering around the islets.

**Back cover photo:** photo by Anders Krüger.
List of Papers

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

I  **Hårdstedt M**, Lindblom S, Hong J, Nilsson B, Korsgren O, Ronquist G. A novel model for studies of blood-mediated long-term responses to cellular transplants. *Accepted for publication in UJMS*


*These authors contributed equally to the manuscript

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<th>Definition</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>B cell</td>
<td>B lymphocyte</td>
</tr>
<tr>
<td>CA19-9</td>
<td>Carbohydrate antigen 19-9</td>
</tr>
<tr>
<td>CITR</td>
<td>Collaborative Islet Transplant Registry</td>
</tr>
<tr>
<td>CK-19</td>
<td>Cytokeratin-19</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>Ct</td>
<td>Comparative threshold cycle</td>
</tr>
<tr>
<td>CTLs</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunosorbent spot assay</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>Gal</td>
<td>The α-Gal (Galα1-3Galβ1-4GlcNAc-R) epitope</td>
</tr>
<tr>
<td>Gal-KO</td>
<td>α-galactosyltransferase knockout (lack the α-gal epitope)</td>
</tr>
<tr>
<td>GB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cells</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IBMIR</td>
<td>Instant blood-mediated inflammatory reaction</td>
</tr>
<tr>
<td>IEQ</td>
<td>Islet equivalents</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-inducible protein 10</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>I-TAC</td>
<td>T-cell α chemoattractant</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Mig</td>
<td>Monokine induced by gamma interferon</td>
</tr>
<tr>
<td>MIP-1α/β</td>
<td>Macrophage inhibitory protein-1α/β</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stromal/stem cells</td>
</tr>
<tr>
<td>NHP</td>
<td>Nonhuman primate</td>
</tr>
</tbody>
</table>
NK cells  Natural killer cells  
PAMPs    Pathogen-associated molecular patterns  
PBMCs   Peripheral blood mononuclear cells  
PCR     Polymerase chain reaction  
PI      Propidium iodide  
PMNs    Polymorphonuclear leukocytes  
PRRs    Pattern recognition receptors  
RANTES  Regulated on activation, normal T cell expressed and secreted  
SLA     Swine Leucocyte Antigen  
SPK     Simultaneous pancreas and kidney transplant  
STZ     Streptozotocin  
T cell  T lymphocyte  
TAT     Thrombin-antithrombin complex  
TCC     Terminal complement complex (sC5b-9)  
T1DM    Type 1 diabetes mellitus  
T2DM    Type 2 diabetes mellitus  
TF      Tissue factor  
TLR     Toll-like receptor  
TNF-α   Tumor necrosis factor-α  
Tregs   T regulatory cells  
vWf     Von Willebrand factor
Preface

This thesis work was performed during two periods of my professional life – from 2002-2005 at the Diabetes Institute of Immunology and Transplantation (DIIT) at the University of Minnesota, USA (later the Schultze Diabetes Institute) and from 2011-2014 at the Department of Immunology, Genetics and Pathology (IGP) at Uppsala University, Sweden. Before, during and after these periods I have been clinically active as a medical doctor, mainly in the field of Internal Medicine.

The molecular and cellular mechanisms behind diseases have always fascinated me; to be able to understand “the big” (the non-functional islet graft) by studying “the small” (the gene or protein expressed in the graft vicinity). A major goal of my thesis has been to develop new methods to explore the immune response after islet transplantation. The research focuses in the two laboratories I joined have colored my work. During my time in Minneapolis, I took an active part in the xenotransplantation program, working with transplantation of porcine islets into nonhuman primates (NHPs). In Uppsala, my focus has been on the development of an extended whole-blood model. Our intention with this model has been to take the work of the Korsgren/Nilsson groups on the IBMIR (instant blood mediated inflammatory reaction) a step further, exploring the initiation of an innate and adaptive immune response.
Background

Diabetes

History

Diabetes has been with humanity as far back as we have written manuscripts. The Ebers Papyrus from an ancient Egyptian grave in Thebes (1500 BC) describes a condition with “thirst” and “urine in excess” believed to be the first known description of the disease diabetes [1]. The first complete description of diabetes is thought to be the one by the Greek Aulus Cornelius Celsus (25 BC– AD 50) in his monumental 8-volume work *De Medicina*. The name diabetes (meaning “a passer through”) was first used by another Greek physician, Aretaeus of Cappadocia (AD 30-90), referring to the excessive discharge of urine. “Mellitus” (Latin for “honey”) was not added until the 18th century, referring to the sweetness of the urine.

In 1869 a German medical student, Paul Langerhans, presented his thesis *Contributions to the microscopic anatomy of the pancreas*, in which he referred to highly innervated “islands of clear cells” throughout the gland which he thought were lymph nodes [2]. The connection between the pancreas and diabetes was first established in 1889 by removing the pancreas from dogs and making them diabetic (Minkowski and Mering) [3].

The discovery of insulin is a fascinating part of medical history, demonstrating the achievements and inestimable value of a true bench-to-bedside research breakthrough. In the course of 2 years, the pancreas extract project at the University of Toronto went from testing crude extracts on diabetic dogs during the summer of 1921 to 25,000 insulin-treated diabetic patients in the US and Canada by September 1923. It has to be recognized that several successful experiments on animals using crude pancreas extracts had been performed previously. However, the Toronto team was undoubtedly the first to bring the laboratory work into clinical practice [4, 5]. Despite controversy, four men were vital to the success of the project: the surgeon Banting, the medical student Best, the physiologist professor Macleod and the biochemist Collip [6, 7]. Eli Lily reached production of commercial quantities of porcine insulin by early 1923. Banting and McLeod were honoured with the Nobel Prize in 1923 for the discovery of insulin, splitting the prize money with Best and Collip.
During the first 50 years after the discovery of insulin, its effect on metabolism was intensely investigated, first on an organism level and later on the molecular level. The development of the insulin radioimmunoassay in the 1960s by Yalow and Berson made it possible to measure and understand normal insulin physiology (Nobel Prize 1977). The complete understanding of the mechanism of insulin action remained largely unsolved until the early 1970s, when the tyrosine kinase insulin receptor was characterized [8].

Epidemiology and Etiology

Diabetes is a worldwide health problem with a global prevalence of 2.8% in 2000 and a projected prevalence of 4.4% in 2030 [9]. This estimate is based on demographic changes and made with the assumption that obesity and physical activity will remain constant.

Diabetes is characterized by hyperglycemia resulting from defective insulin secretion, insulin action or both [10]. The majority of patients fall into two categories: type 1 diabetes mellitus (T1DM; 5-10% of all cases) and type 2 diabetes mellitus (T2DM; 90-95% of all cases). T2DM is dominated by insulin resistance and a relative insulin deficiency, often associated with the metabolic syndrome. T2DM preferentially occur in the adult population and the incidence increases with age. T1DM involves a complete deficiency of insulin secretion as a result of destruction of the β cells of the pancreas. This destruction (insulitis) is believed to be autoimmune, with multiple genetic predispositions and triggered by environmental factors still poorly defined. The higher incidence in developed countries and in wealthy, uncrowded urban environments has suggested an association with wealth-related factors, e.g., differences in exposure to infectious agents [11, 12]. There is substantial evidence for a link between the onset of β cell destruction and enteroviral infections [13]. Differences in bacterial intestinal flora during upbringing have more recently been suggested as a possible explanation [14]. T1DM can occur at any age, but the time of onset peaks at five-seven years of age and near puberty. The incidence of childhood diabetes has increased worldwide over the last 30 years (in Europe, about 3-4 % yearly) [15].

Living with T1DM – complications

With the discovery of insulin in 1922, acute death from ketoacidosis and hyperglycemia was prevented. A new patient group evolved with a treatable, but not curable, chronic disease. Despite optimization of insulin treatment, diabetes leads to impaired glucose control. Hyperglycemia induces a number of alterations at a cellular level and is the major underlying mechanism of the microvascular complications of diabetes: retinopathies, neuropathies and nephropathies [16, 17]. Endothelial and smooth muscle dysfunction, together with chronic inflammation and oxidative stress, accelerate atherosclerosis.
This leads to macrovascular complications (cardiovascular disease). Impaired peripheral circulation and neuropathy result in impaired wound healing and chronic ulcers, and occasionally also in peripheral gangrene and amputation.

The diabetic population has a higher mortality rate than the reference population. Early in the course, mortality is predominantly associated with diabetic ketoacidosis and hypoglycemia [18]. The most common long-term cause of death is cardiovascular disease (45-50% of all death in T1DM), with ischemic heart disease being the most common diagnosis, followed by cerebrovascular disease [19, 20]. About 20-25% of patients with T1DM develop diabetic nephropathy, but only a minority progress to end-stage renal disease (ESRD). Development of nephropathy is a major risk factor for death within the T1DM population [21].

Living with T1DM – insulin treatment and its consequences

Insulin treatment
The β cells secrete a basal level of 0.25-1.5 units of insulin per hour and, in addition, compensate for food intake. Even after a substantial meal the blood glucose level does not normally increase above 8 mmol/L. In addition to enabling glucose uptake (primarily in skeletal muscle and adipose tissue), insulin prevents the uncontrolled hydrolysis of triglycerides and limits gluconeogenesis. It is obviously a delicate task to artificially replace the fine-tuned insulin secretion to keep the glucose level stable and prevent long-term complications.

Although they saved lives, bovine and porcine insulins were plagued by purity, variability and availability issues. The clear need for advances in insulin features soon led to impressive technological developments. The first longer-lasting insulin was developed in the 1930s by complexing insulin with a fish protein (protamine) [22]. Modifications led to the neutral protamine Hagedorn (NPH) and Lente insulins in the 1950s [23]. Insulin became the first protein to have its amino acid structure determined (by Sanger, Nobel Prize 1958), its three-dimensional structure determined (by Hodgkin, Nobel Prize 1964) and to be artificially synthesized. By the early 1990s, DNA recombinant technology had led to the production of genetically modified insulin analogs, with favorable profiles, which revolutionized diabetes care.

Today, insulin treatment with long-acting insulin once/twice daily, in combination with rapid-acting prandial insulin, is standard. Continuous subcutaneous insulin infusion (CCII) has many advantages over multiple daily injections regarding glycemic control. Insulin pump treatment, however, is costly, has a risk of incidental non-delivery and requires the patient to be attached to the system. Closed-loop devices with continuous glucose monitors and possibly bi-hormonal infusion systems are rapidly advancing, though not yet in every-day clinical practice [23].
Hypoglycemia unawareness and brittle diabetes

Tight glucose control lowers the incidence of long-term, mainly microvascular, complications [25, 26]. Iatrogenic, often asymptomatic, hypoglycemia is, however, an inevitable consequence of tight glycemic control [27]. For a well-treated T1DM patient the estimated time with hypoglycemia (p-glucose < 2.8-3.3 mmol/L) is 10 % [24]. Glucose auto- and counter-regulatory mechanisms (decreased insulin secretion, increased glucagon and epinephrine secretion) as well as neuroglycopenic symptoms become impaired over time in T1DM [24]. The autonomic response to hypoglycemia is further reduced during sleep, contributing to the feared risk of severe nightly hypoglycemia [28]. The lack of warning symptoms, resulting from attenuated autonomic, sympathetic neural responses, leads to a status of hypoglycemia unawareness. Hypoglycemia unawareness is a major risk factor for severe hypoglycemic episodes. About 20% of T1DM patients develop at least impaired awareness of hypoglycemia [29].

Hypoglycemia-associated autonomic failure (HAAF) defines the syndrome of defective counter-regulation and unawareness during hypoglycemia, leading to a vicious cycle of recurrent iatrogenic, hypoglycemic episodes [30] (Fig. 1). “Brittle diabetes” is defined as a syndrome of poor metabolic control, with severe instability of blood glucose levels, frequent and unpredictable hypoglycemic episodes and diabetic ketoacidosis. Patients with brittle diabetes are often in frequent need of emergency hospital care [31]. This somewhat vaguely defined group of T1DM patients has higher complication and mortality rates than do other T1DM patients [32]. Frequent and severe hypoglycemic episodes can lead to less intense insulin treatment and accelerated secondary complications. Social isolation and dependence are common in this group of T1DM patients, contributing to their impaired quality of life.
β cell replacement

What is an islet?

The pancreas serves two main functions: exocrine (secretion of digestive enzymes into ducts) and endocrine (secretion of hormones into the blood). The word “islet” comes from “island” and refers to the island-shaped endocrine cell clusters, 50-300 μm in diameter, dispersed within the exocrine pancreas. The endocrine pancreas, the islets of Langerhans, mainly consist of insulin- and islet amyloid polypeptide (IAPP)-secreting β cells, glucagon-secreting α cells, somatostatin-secreting δ cells and pancreatic polypeptide-secreting cells [33, 34]. The relative proportion of these different cell types in a human islet varies considerably: β cells 28% to 75%, α cells 10% to 65% and δ cells 1.2% to 22% [33]. The endocrine islets consist of a dense network of capillaries (endothelial cells) and are (partially) surrounded by a collagen capsule (Fig. 2). Comprising 1-2 % of the pancreatic volume the endocrine islets use 10-20% of the pancreatic blood supply [35]. Insulin and the other endocrine hormones are secreted directly into the blood, finally entering the portal vein.

Pancreas transplantation

In parallel with advances in insulin treatment and diabetes care, strategies for β cell replacement have evolved, i.e., pancreas and islet transplantation. The first clinical pancreas transplantation was performed in 1966 by Lillehei and Kelly at the University of Minnesota [36]. As of today’s date, > 42,000 pancreas transplantations have been performed worldwide, with a frequency of about 1,600 annually [37]. Pancreas transplantations are performed either simultaneously with a kidney (simultaneous pancreas-kidney; SPK), after a previous kidney transplantation or as pancreas alone. SPK is by far the most common procedure (90%), and has the best transplant outcome (5-year graft survival of 71%) [38]. The group of patients with diabetes and ESRD has a high mortality while on the waiting list [39]. Surgical development, centralization of the procedures and advances in immunosuppression have advanced graft (and patient) survival rates over the last decade. Pancreas transplantations have also been associated with an appreciable lowering of the risk of secondary complications of diabetes. The long-term benefit of SPK compared to kidney transplant alone is today, despite initial higher postoperative mortality/morbidity, towards favor of SPK for the diabetic patient with ESRD [40].
Islet Transplantation

History

Even though early sporadic attempts to clinically cure diabetes by replacing the lost pancreatic islets can be dated to the time before the discovery of insulin [41], the development of techniques to transplant pancreatic islets into humans did not emerge until the late 1970s [42]. The first series of clinical islet transplantations were performed by Najarian and colleagues at the University of Minnesota in 1974-77. These, however, did not lead to more than temporarly lowered glucose [43]. Isolation methodology became crucial and advances such as the Ricordi digestion chamber [44], the COBE continuous purification system [45] and controlled pancreatic distension with collagenase [46] have all contributed to higher yields and better-quality islets. The liver was found to be a feasible implantation site and the method of transplanting isolated islets by catheterization of the portal vein was developed (Fig. 3). Development of better methods for islet isolation, increased knowledge of islet cell biology, improved immunosuppression and better patient care moved islet transplantation forward during the 1990s [47]. Still, the long-term clinical outcome was disappointing. Of the 450 patients reported to receive islet transplants between 1974 and 1999, less than 10% achieved insulin independence for longer than 1 year, although 28% had still sustained C-peptide secretion [48].

A breakthrough came in the summer of 2000. Shapiro and his group from the University of Alberta, Edmonton, presented seven consecutive intraportal islet transplanted patients, on a glucocorticoid-free immunosuppression regimen, with remarkably good post-transplant function [49]. All patients had attained insulin independence after two (or, for one patient three) islet cell transplants and they were still off insulin at one year (average follow-up time) post-transplant. The major advance with the “Edmonton protocol” was the avoidance of diabetogenic immunosuppressive drugs. The donor-recipient pairs in the study were matched for blood group and cross-matched for lymphocytotoxic antibodies, however no HLA-matching was performed [49].
Figure 2. Endocrine cells and blood vessels in a human islet. Sections of human pancreas tissue stained by immunohistochemistry and visualized with fluorescent secondary antibodies. (A) Insulin-containing cells (red), glucagon-containing cells (green) and vascular smooth muscle cells/endothelial cells (blue). (B) Somatostatin-containing cells (cyan). Scale bar 50μm. Photos from Cabrera et al.; copyright © 2006 by The National Academy of Sciences of the USA [34].

Figure 3. The islet transplantation procedure. The cadaver pancreas is procured from the donor and taken to the isolation facility. Islets are isolated through enzymatic digestion of pancreas and mechanical destruction. Isolated islets are infused via a percutaneous stick into the portal vein branches in the liver of the recipient.
Immunosuppression – in organ and islet transplantation

Advances in surgical techniques preceded the understanding of immunological incompatibility in the history of kidney transplantation [50]. With a successful kidney transplantation between two identical twins in 1954, after less successful non-immunosuppressed allotransplantations, it became clear that advances in immunology were necessary for the development of allogeneic transplantation [51]. The existence of adaptive immunity and transplant tolerance was suggested in the early 1950s by Medawar, with the help of Burnet’s previous work on tolerance to encountered antigens (Nobel Prize 1960) [52-54]. In 1958, Dausset described the first human leukocyte antigen (HLA) as the results of leucoagglutination experiments (Nobel Prize 1980).

Total body irradiation was initially tried to accomplish immunosuppression, leading to prolonged graft survival in a few cases, but with high mortality. A novel drug used for cancer treatment, 6-mercaptopurine, was eventually successfully transferred to clinical transplantation [55]. The less toxic derivative of 6-mercaptopurine, azathioprine, became, together with corticosteroids, the basis for the first immunosuppressive protocols. After the discovery of heterologous anti-thymocyte globulin (ATG) in 1966 it took over a decade before the revolutionary introduction of cyclosporine in the late 1970s. Cyclosporine A (CsA; a calcineurin inhibitor) effectively inhibited T-cell proliferation and opened the way for clinical transplantations not only of cadaver kidneys but also of livers, hearts and lungs [50]. CsA was the unchallenged baseline immunosuppressant in all transplant protocols until tacrolimus (a second-generation calcineurin inhibitor) showed even better graft survival in several types of transplants in the early 1990s. Since then, novel drugs such as sirolimus (an mTOR inhibitor) and mycophenolate acid (MMF; a monophosphate dehydrogenase inhibitor) have entered the arena.

A draw-back of both corticosteroids and calcineurin inhibitors (CsA, tacrolimus), especially in islet transplantation, is their diabetogenic effect. Calcineurin inhibitors are also nephrotoxic, a substantial disadvantage for the diabetic patient. The “Edmonton protocol” for clinical allo-islet transplantation introduced a steroid-free regimen using daclizumab (IL2-receptor binding monoclonal antibody [mAb]) for induction and sirolimus plus low-dose tacrolimus for maintenance therapy. Induction therapy, given pre/peri-transplantation, is characterized by anti-inflammatory drugs and mono/polyclonal antibodies to deplete T cells or prevent T-cell activation (via IL-2 receptor inhibition) (Table 1). Maintenance therapy, given lifelong, focuses on suppression of T cells by various strategies (Table 1). Since the Edmonton protocol, there has been a shift internationally toward using T-cell-depleting antibodies with or without TNF-α inhibition (e.g. etanercept) for induction and maintenance therapy with sirolimus or MMF in combination with a calcineurin inhibitor (CsA or tacrolimus) in clinical islet transplantation [56]. Sirolimus has, however, demonstrated less favorable effects on β cell viability and engraftment, together with side effects such as poor wound healing [57, 58]. It is truly a
challenge to choose maintenance protocol in clinical islet transplantation considering the different efficacy of the various drugs and concerns regarding side effects such as diabetogenicity and nephrotoxicity. Heparin infusion is given as a standard procedure at the time of intraportal transplantation to prevent thrombosis and the IBMIR.

Table 1. Selected immunosuppressive drugs used in organ and islet transplantation. mAb=monoclonal antibody; pAb=polyclonal antibody; ATG=Anti-Thymocyte Globuline; gen=generation.

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Trade name</th>
<th>Introduced</th>
<th>Mechanism of action</th>
</tr>
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<tbody>
<tr>
<td><strong>Induction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daclizumab</td>
<td>Zenapax®</td>
<td>1999</td>
<td>anti-CD25 mAb, IL-2 rec antagonist</td>
</tr>
<tr>
<td>Basiliximab</td>
<td>Simulect®</td>
<td>1999</td>
<td>anti-CD25 mAb, IL-2 rec antagonist</td>
</tr>
<tr>
<td>ATG (antithymocyte globulin)</td>
<td>Thymoglobuline®</td>
<td>2002</td>
<td>pAb, T-cell directed antibodies</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>Campath®</td>
<td>2011/13</td>
<td>Anti-CD52 mAb, depletion of T cells</td>
</tr>
<tr>
<td>Etanercept</td>
<td>Enbrel®</td>
<td>1998</td>
<td>TNF-α inhibitor</td>
</tr>
<tr>
<td><strong>Maintenance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>Prednisone®</td>
<td>1950</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>Imurel®</td>
<td>1960</td>
<td>Purine-synthesis inhibitor, inhibits T/B cells proliferation</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>Sandimmune®</td>
<td>1978/79</td>
<td>Calcineurin inhibitor (1st gen), inhibits T cell proliferation</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>Prograf®, Advagraf®</td>
<td>1995</td>
<td>Calcineurin inhibitor (2nd gen), inhibits T cell proliferation</td>
</tr>
<tr>
<td>Mycophenolate acid</td>
<td>CellCept®</td>
<td>1995</td>
<td>Purine-synthesis inhibitor, inhibits T/B cells proliferation</td>
</tr>
<tr>
<td>Sirolimus/Rapamycin</td>
<td>Rapamune®</td>
<td>2000</td>
<td>mTOR inhibitor, inhibits T/B cell proliferation by blocking intracellular signalling.</td>
</tr>
</tbody>
</table>
**Indications for clinical islet transplantation**

In general, three groups of T1DM patients are eligible for islet transplantation today: (1) patients on immunosuppression as a consequence of a previous transplant, usually a kidney (islet after kidney; IAK); (2) patients with ESRD who are scheduled for kidney transplantation, receiving islets from the same donor (simultaneous islet kidney; SIK); (3) patients suffering from glycemic lability with frequent hypoglycemia (islets alone; IA) [59]. The vast majority of islet transplantations (85%) since 2000 has been performed as islet alone (Collaborative Islet Transplant Registry [CITR]; www.citregistry.org). Each patient eligible to receive an islet cell transplant is evaluated individually and the drawbacks with long-term immunosuppression are weighted against better glycemic control. General inclusion criteria include age (16-65 years), diabetes duration (> 5 years) and a very poor diabetes control with hypoglycemia unawareness and recurrent, severe hypoglycemic events.

**The state of islet transplantation today – “Is the glass half empty or half full”?**

Islet transplantation has to be considered a treatment in development. As present, the number of registered transplanted patients is approaching 800 worldwide (CITR; www.citregistry.org). The outcome has traditionally been measured in insulin independence and here islet transplantation has failed to produce stable long-term results: 44% insulin independence at 3 years was reported from the CITR for 2007-2010 [56]. However, graft function (C-peptide >0.3 ng/mL) was 83% in the same group of patients at 3 years. The factors affecting long-term transplant outcome are schematically summarized in Fig. 4, and show areas for future improvements. The long-term outcome, measured as insulin independence, varies between transplant centers. The most successful patient cohorts reported are approaching the results for pancreas-alone transplants at 5 years (~55% insulin independence) [60, 61]. Usually, islet recipients need islets from several donors to achieve insulin independence. Altogether, pancreas transplantation today offers superior metabolic control (a higher transplanted insulin-producing mass) at the expense of the risk of a major operation. I here further refer to a recent comprehensive review discussing the different aspects of the comparison between pancreas and islet transplantation [62]. The vast majority of islet transplanted patients benefit from better glycemic control post-transplant, with a loss of socially handicapping and life threatening episodes of hypoglycemia (Fig 5). Restoration of hypoglycemia awareness has been reported even after graft failure [63]. Reports on deceleration and even improvement of secondary diabetic complications after islet transplantation have been presented [64-67]. Maybe it is time to broaden the outcome measures in islets transplantation to include quality-of-life measures and long-term effects, together with measures of insulin independence and graft function [68, 69].
Figure 4. Factors affecting long-term transplant outcome in clinical islet transplantation. Marked with an * are areas dealt with, in one way or another, in this thesis work.

Figure 5. Nine-year data from Edmonton (2012) illustrating the disparity between insulin independence and C-peptide positivity. >70% of the patients benefit from complete loss of hypoglycemia and better glucose control post-transplant. From McCall & Shapiro; copyright © 2012, Cold Spring Harbor Laboratory Press [61].
Introduction to the scope of this thesis

General concepts of transplantation

*Autotransplantation* is the transplantation of organs, tissues or cells from one part of the body to another in the same individual. *Allotransplantation* is the transplantation between genetically non-identical individuals of the same species. *Xenotransplantation* is when the donor and the recipient belong to different species.

General concepts of transplant rejection

Organ graft rejection is divided into hyperacute, acute and chronic rejection, based on cellular/molecular mechanisms and temporal aspects of the immune response. *Hyperacute rejection* occurs within minutes or hours, is antibody-mediated, irreversible and prevents vascularization of the organ. *Acute rejection* is, in general, cell-mediated and evolves within days to months after transplantation in response to foreign antigens. The progress of acute rejection is reversible if immunosuppressive drugs are used early enough to prevent further damage. *Acute humoral (xenograft) rejection*, also termed *acute vascular rejection*, occurs predominantly in organ xenotransplantation and is basically driven by natural (preformed) xenoreactive antibodies and involves donor endothelial cells and host macrophages [70]. Even though hyperacute rejection may be avoided, acute vascular rejection can still occur days after a xenotransplantation. *Chronic rejection* occurs over months to years after transplantation. Multiple immunological mechanisms are involved, resulting in chronic inflammation and chronic, irreversible tissue damage such as tissue fibrosis and sclerosis.

Innate and adaptive immunity

The innate (natural, native) and adaptive (acquired, specific) immune system are two integrated arms of the mammalian immune system [71] (Table 2). With the main aim of defending us from infections, these systems also recognize transplanted organs and cells as foreign.
Table 2. General features of innate and adaptive immunity

<table>
<thead>
<tr>
<th>Innate Immunity</th>
<th>Adaptive Immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>■ Inherited</td>
<td>■ Aquired</td>
</tr>
<tr>
<td>■ The first line of defense</td>
<td>■ The second line of defense</td>
</tr>
<tr>
<td>■ Expresses germline encoded pattern recognition receptors</td>
<td>■ Expresses diverse and highly specific antigen receptors, somatic gene rearrangement</td>
</tr>
<tr>
<td>■ Only limited clonal expansion</td>
<td>■ Clonal expansion</td>
</tr>
<tr>
<td>■ Does not generate memory</td>
<td>■ Generates memory</td>
</tr>
<tr>
<td>■ Monocytes, macrophages, dendritic cells, PMNs, NK cells</td>
<td>■ T and B lymphocytes</td>
</tr>
</tbody>
</table>

Innate immunity in islet transplantation

The IBMIR

Allogeneic islets in contact with blood provoke a reaction called the instant blood-mediated inflammatory reaction (IBMIR) [72]. This reaction begins with an immediate thrombotic and inflammatory reaction, with activation of the complement and coagulation cascades together with platelet aggregation (Fig. 6). The IBMIR is believed to cause a deleterious and rapid loss of transplanted islets prior to engraftment [73]. Originally defined in allo- and xenoislet transplantation [72], the IBMIR has later been reported for hepatocytes and mesenchymal stromal cells (MSCs) in contact with recipient whole blood. [74, 75]. Recently, the IBMIR was also described in autologous islet transplantation [76]. The IBMIR is “a multi-component reaction that is triggered when non-blood cells come into contact with whole blood” [77].

Coagulation and complement activation

The coagulation and complement systems resemble each other in organization, both consisting of proteolytic cascades of serine proteases. The two systems interact on many different levels and display intensive crosstalk [78]. Coagulation activation occurs through both the intrinsic (contact activation) and extrinsic (tissue factor [TF]) pathways in response to islet cells in the blood [72]. The intrinsic pathway is triggered by collagen and other negatively charged molecules on the surfaces of the isolated cells [79, 80]. TF, produced by islets and exocrine tissue (shown for pancreatic ductal cells), activates coagulation through the extrinsic pathway [81-83]. The end product, thrombin, converts soluble fibrinogen into insoluble fibrin. Thrombin also catalyzes many other coagulation-related reactions and is a potent platelet activator. Platelet activation and aggregation are also activated directly by collagen or collagen-binding von Willebrand factor (vWF) [73]. The complement cascade
is triggered by various stimuli evoked when foreign cells are injected into the blood-stream and the means of complement activation is most likely multifactorial. C3 is the central protein in the complement system and it can basically be activated (via cleavage to C3a) though three different pathways: **the classical pathway**, triggered by antigen–antibody complexes that activates C1; **the lectin pathway**, by binding of mannose-binding lectin (MBL) or ficolins to carbohydrates; and **the alternative pathway**, which is antibody independent [77]. Natural antibodies in the blood directed towards extracellular components (e.g., collagen) on the isolated cell surfaces are believed to be a major trigger of complement activation through the classical pathway [84]. Coagulation and platelet activation are also proven initiators of the complement cascade, one trigger being the release of chondroitin sulfate from activated platelets [85, 86]. Inhibition of thrombin as well as TF abrogates complement activation [81, 87]. The three pathways result in the formation of the membrane attack complex (sC5b-9 in its soluble form), which inserts into the lipid layer of cell membranes and causes cell lysis.

C3a and C5a (anaphylatoxins), products of proteolytic cleavage, activate and recruit PMNs and monocytes (Fig. 6). Components of the coagulation cascades (e.g. thrombin, fibrinogen, sCD40L) have also shown to directly interact with granulocytes or macrophages and thereby enhance the inflammatory response [88-90].

**Figure 6.** The IBMIR and its main components: the coagulation system, complement system and innate immune cells. These different systems interact and produce intensive crosstalk, triggering each other during the thrombo-inflammatory reaction. TF=tissue factor, MBL=mannose-binding lectin, vWf=von Willebrand factor.
Innate immune cells

Cells of the innate immune system (e.g., monocytes, macrophages, polymorphonuclear cells [PMNs] and natural killer [NK] cells) express germline-encoded pattern recognition receptors (PRRs) that traditionally detect conserved pathogen associated molecular patterns (PAMPs) present in microbes. There are many subgroups of PRRs: transmembrane forms (e.g., the Toll-like receptor family [TLR]), cytosolic PRRs (e.g., the NOD-like receptors) and secreted PRRs (e.g., pentraxins, ficolins). In recent years, the concept of “innate alloimmunity” has been proposed, suggesting that innate immune cells have the ability to sense allogeneic non-self as well as guide the adaptive allo-response to a greater extent than was previously recognized [71, 91].

Monocytes are circulating cells that migrate into tissues and differentiate into long-living, tissue-resident macrophages or dendritic cells (DCs). They serve three main immune functions: phagocytosis, antigen presentation and cytokine production. Neutrophils comprise about two-thirds of the circulating peripheral blood leukocytes and are the dominant group of PMNs. Neutrophils are recruited to sites of injury and inflammation and play a key role in reperfusion injury following organ transplantation. They kill by phagocytosis, release of anti-microbial components and generation of neutrophil extracellular traps. NK cells are innate cytotoxic lymphocytes that express inhibitory and activating receptors. Cells that lack MHC class I antigens signal “missing self”, cannot inhibit the NK cells and are killed. Activating receptors encounter the signals induced by cellular stress. NK cells mediate cytotoxic killing through the release of perforin and granzyme B [92]. NK cells have recently been shown to have memory functions, placing them in a borderline position between innate and adaptive immunity [93].

In 2005, early innate immune cell recruitment to the allo-islet graft was investigated in a whole blood model by Moberg et al. [94]. Neutrophils were the first and predominant cells to infiltrate the islets up to 6 h and only limited infiltration of macrophages was noted (believed to be of donor origin).
Adaptive immunity in islet transplantation

Transplantation of islets between individuals of the same species (allotransplantation) demands T-cell-directed immunosuppression targeting adaptive immunity in order to prevent acute cellular rejection. The cells responsible for the adaptive immune response are T (CD4+ and CD8+) lymphocytes (T cells) and B lymphocytes (B cells).

Direct and indirect T-cell allorecognition

Allorecognition is a central concept in adaptive immunity and consists of the ability of the immune system to recognize non-self antigens. The main triggers of allorecognition are the major histocompatibility complex (MHC) molecules on the donor cells, but also other antigens, known as minor histocompatibility antigens, can provoke an allo-response [95]. T cells recognize donor MHC molecules either directly, as intact molecules on the cell surface of donor antigen presenting cells (APCs) and transplanted tissues or indirectly (mainly CD4+ T cells), as processed donor MHC peptides complexed with self MHC on the recipient APCs (Fig. 7). Direct recognition is believed to play a dominant role in acute allo-rejection, when APCs of donor origin is still available [96]. Indirect recognition may dominate later in the process of chronic rejection. To further complicate things a semi-direct pathway has subsequently been described in which recipient dendritic cells acquire intact MHC antigen from donor dendritic cells and can present antigens through direct presentation [97].

The first signal for T-cell activation is provided when the T-cell receptor (TCR) interact with the peptide-MHC complex on the APC. CD4+ T cells interact with MHC class II (expressed on APCs, presenting exogenous antigens) and CD8+ T cells with MHC class I (expressed by virtually all cell types, presenting endogenous antigens). A second signal – co-stimulation – is needed for activation. This co-stimulatory signal is received by interaction between co-stimulatory molecules and their receptors, expressed by T cells and APCs. Several co-stimulatory pathways have been recognized, with overlapping functions, active during different stages of T-cell differentiation [98]. The first co-stimulatory pathway to be defined was the CD80/CD86(APC)-CD28(T-cell) pathway; another well-known interaction is the CD40(APC)-CD154(T-cell) interaction [98]. Activation of T cells without co-stimulation may lead to T-cell anergy, T-cell deletion or the development of active immune tolerance. Also, a third signal is sometimes mentioned, referring to the cytokines needed to further stimulate T-cell activation [99].
Figure 7. Direct and indirect recognition. Direct recognition of intact donor MHC by recipient CD4+ and CD8+ T cells. Indirect recognition of processed donor MHC peptides presented by recipient MHC by CD4+ T cells. Drawn with inspiration from Game et al. [95].

Figure 8. Cytotoxic T cell killing. Cytotoxic T cells recognize target donor cells by T cell receptor (TCR)-MHC I binding. Secretory granules release perforin and granzyme B (GB). Perforin molecules polymerize in the membrane of the target cell and GB passes through the pores and induces apoptosis. Binding of the Fas antigen on target cells leads to apoptotic cell death.
**Cytotoxic T cells**

The CD8+ T cells can differentiate into cytotoxic T lymphocytes (CTLs) which are able to kill target cells. In addition to the activation signals already mentioned, alloreactive CD8+ T cells usually need concomitant help from activated CD4+ T cells to become fully differentiated effector cells. Indirectly activated CD4+ T cells can here play an important role by providing help to the CD8+ T cell [95] [100] (Fig. 7). CTLs perform their cytotoxic activity by means of two different pathways: secretion of cytolytic granules (the perforin/granzyme pathway) and direct receptor-mediated induction of apoptosis (the FasL pathway) (Fig. 8). These pathways involve three cytotoxic effector molecules: perforin, granzyme B (GB) and Fas ligand (FasL). Released perforin molecules polymerize in the membrane of the target cell, forming pores that cause membrane damage. GB can pass through the pores and induce apoptosis by direct attack on the cell nucleus. Binding of the Fas antigen on target cells leads to apoptotic cell death by a series of protein-protein interactions resulting in activation of intracellular caspases.

**B cells**

Antibodies are important mediators at different stages of allogeneic organ rejection [101]. In clinical islet transplantation the role of anti-body mediated rejection is less well known and explored. Antibodies towards the graft can be natural/preformed (exist at the time of transplantation) or induced (produced as a result of immune activation). Natural/preformed antibodies are major players in hyperacute rejection. In allotransplantation examples of preformed antibodies are antibodies towards the blood group antigens (in ABO-incompatible transplantation) or the result of pre-transplant sensitization. Thanks to routine pre-transplantation cross-matching, hyperacute rejection is rare in clinical organ and cellular allotransplantation. In the process of organ chronic rejection, antibodies are sometimes major players. Indirectly activated CD4+ T cells are believed to be important mediators of humoral alloimmunity by providing B-cell help [100].
Chemokines

Chemokines are a large family of small (8 to 11 kDa) chemoattractant proteins involved in both innate and adaptive immunity. Chemokines’ ability to recruit immune cells has made them and their receptors a growing focus of interest in transplantation immunology and in the pathogenesis of many diseases [102-105]. Chemokines are sub-classified based on the spacing of their first two cysteine residues; the CC subgroup has two adjacent first cysteines and the CXC subgroup has one amino acid in between [105]. Chemokine receptors are structurally similar, G-protein-coupled proteins classified according to their preferred ligands; CCR receptors bind CC chemokines and CXCR receptors bind CXC chemokines. Chemokines are sometimes divided into “inflammatory” and “homeostatic” based on their functions and pattern of expression [106]. Inflammatory chemokines recruit leukocytes to an inflammatory site and typically bind to more than one receptor, creating an interactive inflammatory network. Homeostatic chemokines are constitutively produced and are important for the migration of APCs and activated T cells in and out of secondary lymphoid tissue. This functional distinction is, however, not conclusive. Many chemokines can fit into both categories or neither, depending on the biological context [105, 106].

Chemokines have been thoroughly investigated in allo- and xenograft rejection in both cellular and organ transplantation [102, 103, 107, 108]. Based on predominantly small-animal studies, a spectrum of chemokines has been identified as early and late recruiters of immune cells to the graft tissue, a rough but helpful model (Table 3) [103]. The immediate ischemic/reperfusion trauma initiates a wave of chemokines that recruit mainly neutrophils (IL-8, Gro-α/β/γ), rapidly followed by macrophage chemoattractants (MCP-1, MIP-1α, MIP-1β) [103]. The critical chemokines in the later chemokine cascade (Mig, IP-10, I-TAC) are chemoattractants for CXCR3-expressing T cells.

MCP-1, which binds the CCR2 receptor, is mainly known as a monocyte attractant; however, CCR2 is also expressed by basophils, memory T cells and plasmacytoid DCs (pDCs). MCP-1 is expressed by human and animal pancreatic islets and high expression has been suggested to impair islet transplant outcomes [109-111]. When exposed to cytokines, islets have been shown to produce a wide spectrum of chemokines [112]. This observation has led to the hypothesis that chemokines produced by the islets themselves, or passenger cells, are important triggers of the donor-directed immune response in the early post-transplant period.
Table 3. Chemokines expressed “early” and “late” in response to an allograft. Modified from el-Sawy [103].

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Receptor</th>
<th>Major target leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>“Early” (3-72 h)”</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gro-α/β/γ</td>
<td>CXCL1/2/3</td>
<td>CXCR1/CXCR2</td>
</tr>
<tr>
<td>IL-8</td>
<td>CXCL8</td>
<td>CXCR1/CXCR2</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CCL2</td>
<td>CCR2</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CCL3</td>
<td>CCR1/CCR5</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>CCL4</td>
<td>CCR5</td>
</tr>
<tr>
<td>Fractalkine</td>
<td>CX3CL1</td>
<td>CX3CR1</td>
</tr>
<tr>
<td><strong>“Late” (48-72+ h)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>CCL5</td>
<td>CCR1/CCR3/CCR5</td>
</tr>
<tr>
<td>Mig</td>
<td>CXCL9</td>
<td>CXCR3</td>
</tr>
<tr>
<td>IP-10</td>
<td>CXCL10</td>
<td>CXCR3</td>
</tr>
<tr>
<td>I-TAC</td>
<td>CXCL11</td>
<td>CXCR3</td>
</tr>
</tbody>
</table>

The receptors CXCR3 (ligands: IP-10, Mig and I-TAC) and CCR5 (ligands: MIP-1α, MIP-1β and RANTES) are, at an inflammation site, preferentially expressed by activated CD4+ Th1 cells and have been shown to be important mediators of cellular rejection [107]. Both these receptors are also expressed by NK cells, a small portion of monocytes (CCR5), B cells (CXCR3) and subgroups of dendritic cells (CXCR3 and CCR5). Both the expression and beneficial blockage of CCR5 and CXCR3 have been demonstrated in animal models of allo- and xeno-islet transplantation, leading to a discussion of their relative contribution in islet graft rejection [113-116]. IP-10 antibody treatment has been shown to prolong graft survival in one of these murine models of islet allotransplantation [115]. Interestingly, CXCR3 and IP-10 have been suggested to take part in the recruitment of lymphocytes to the insulitis lesions of recent-onset type 1 diabetes [117].

CCR7 is a major homing receptor for the immune system that is expressed by T cells in different stages of maturation as well as by mature, antigen-loaded, dendritic cells. Maturation of dendritic cells after antigen loading is accompanied by a preferential switch in receptor expression to CXCR4 and CCR7 [118].
Islet xenotransplantation

In 2009, a comprehensive consensus statement was published by the International Xenotransplantation Association (IXA) regarding conditions for clinical trials of porcine islet products in T1DM [119]. Promising results with transplantation of porcine islets into nonhuman primates (NHPs), reporting normoglycemia >6 months, have been reported from at least six independent research groups [120-123]. These studies have included adult or neonatal porcine islets transplanted either directly intraportal or encapsulated subcutaneous/intraperitoneal [124]. The first scientific attempt to transplant porcine islets to diabetes patients was made by Groth et al. in 1994 and resulted in no advantage regarding glucose control but detectable porcine C peptide in urine >300 days [125]. From Mexico has come a report of pediatric patients transplanted with pig islets together with Sertoli cells in a subcutaneous chamber, which resulted in highly reduced insulin doses [126]. In China a clinical xenoislet transplant study including 22 patients was performed in 1999-2005 [127]. The first report from a clinical trial in New Zealand with alginate-encapsulated porcine islets has just recently been published, demonstrating a decrease in hypoglycemic events but no reduction in insulin dosage [128]. There is an ongoing ethical discussion concerning clinical trials, in which the xenotransplantation community has stressed the importance of a beneficial justification based on relevant preclinical models [119, 129].

The rationale behind islet xenotransplantation is organ shortage. Pigs have been suggested as good candidates for islet donation. Pig insulin differs from human insulin in only one amino acid and it has been successfully used in humans to treat diabetes. Pigs are easy to breed and an established domestic animal. Pig donors can be genetically modified (see Future perspectives below) [130]. Fetal, neonatal and adult (>6 months) porcine islets all have particular advantages and disadvantages related to isolation and transplantation [130]. The advantage of transplanting adult islets is that they start to function immediately after isolation/transplantation and express low quantities of the α-gal (Galα1-3Galβ1-4GlcNAc-R) epitope [131, 132]. Disadvantages with adult islets include a variable outcome of isolation and their vulnerability because of poorly developed peri-insular matrix (“islet capsule”) [133].

Transmission of infectious agents from the recipient pig to the donor patient and the human population has for long been debated. Avoidance of many bacteria, viruses, protozoa and fungi is possible through breeding in bio-secure facilities; however, the major concern has been transmission of porcine endogenous retrovirus (PERV). To date, no active replication has been detected when humans and NHPs receiving porcine cells or tissues have been monitored, suggesting that this potential event is less of a problem than feared. Techniques for preventing replication of the virus, if transmitted, have also evolved [134].
Immunity in islet xenotransplantation

Hyperacute rejection and xenoreactive antibodies

In xenogeneic organ transplantation, hyperacute and acute vascular antibody-mediated rejection are of major concern (Fig. 9). The predominant antigen triggering hyperacute rejection is the porcine-specific carbohydrate antigen called the α-gal epitope [135]. Humans, apes and Old World NHPs do not have this epitope but are exposed to the antigen through the gut and therefore produce natural anti-Gal antibodies [136]. In hyperacute rejection preformed donor specific antibodies bind to donor cells (in organ transplantation predominantly endothelial cells) and initiate complement activation. Elimination of the α-gal epitope prevented hyperacute rejection in a study of pig-to-NHP solid-organ transplantation [137]. Besides from the anti-Gal antibodies there are also antibodies towards non-α-gal epitopes expressed in the pig (non-Gal antibodies). These epitopes consist of other carbohydrate structures and peptides [138].

The xeno-IBMIR

Avascular adult and neonatal porcine islets have repeatedly been shown, in rodent and NHP models, to be able to escape fulminant hyperacute rejection [139, 140]. At least a sufficient part of the xeno-islets survive to engraft and retrieve metabolic function [121]. In addition to the lack of an immediate donor endothelial cell interactions (compared to vascular anastomosis in organ transplantation), adult porcine endocrine and exocrine pancreatic tissue have a markedly lower expression of the α-gal antigen [131, 141]. The xenogeneic islets, however, launch a reaction that resembles an allo-IBMIR, with immediate coagulation/complement activation and with TF as a key mediator (Fig. 6) [142]. Early antibody and complement binding to the pig-islet surface indicate the involvement of natural xenoreactive antibodies as well [143]. Complement activation though the alternative pathway has also been demonstrated in the adult porcine islet-to-NHP model [144]. Species differences in complement-regulatory proteins make porcine islets more susceptible to complement destruction [145]. Transgenic expression of human complement regulators, CD55 (decay-accelerating factor [DAF]) and CD59, protect pig islet xenografts from destruction in vitro and in vivo [146] [147]. The difference between autologous, allogeneic and xenogeneic (adult porcine islets) IBMIR was recently explored in pig and human blood demonstrating a more devastating damage of xeno-islets in vitro, associated with antibody deposition and complement activation [148]. The degree of antibody-mediated destruction in the xenogeneic setting seems to vary between different models. Most likely factors as differences in isolation and culturing techniques, porcine age and strain can affect the level of this early devastating complement attack.
Figure 9. Innate and adaptive immune responses triggered by organ transplantation and intraportally transplanted islets, in the allogeneic and xenogeneic setting, respectively. The figure presents the predominate mechanisms of rejection in the different settings. Allotransplantation is here referred to as clinical ABO-compatible transplantation after cross-matching. Question marks (?) indicate that the initial xenogeneic IBMIR reaction sometimes resembles the hyperacute rejection in xeno-organ transplantation. Chronic rejection is not described for transplanted xeno-organs or cellular grafts.

**Cellular rejection**

Following the IBMIR, acute cellular rejection threatens the transplanted xenogeneic islets. Extensively studied in rodent models, xeno-islet rejection was suggested to be a predominantly CD4+ T-cell-driven process, with macrophages as the main mediators [149-154]. Originally, indirect recognition of processed swine leukocyte antigens (SLA) presented by host APCs was thought to provide the main initiating event in xenograft rejection, with a reaction resembling delayed hypersensitivity (DTH) [155]. However, using a mixed lymphocyte reaction (MLR) technique, researchers have demonstrated direct recognition of porcine MHC by human T cells, suggesting involvement also of direct recognition, despite the species barrier [156]. Supposedly the resemblance in MHC-structures over the species barrier will determine the degree of direct recognition [157]. Involvement of xenoreactive CD8+ T cells has subsequently also been demonstrated [158, 159]. Most likely, the T-cell dependent islet xenograft destruction includes all arms of the adaptive immune response (i.e., cytokine production, recruitment of macrophages and NK-cells, help to xenoreactive B cells as well as direct T-cell cytotoxicity), even though the CD4+ T cells are the key players in the drama [160, 161].
Cytokine and glucose responses in cultured islets

The inflammatory cytokines IL-1β, TNF-α (mainly macrophage-generated) and IFN-γ (mainly T-cell-generated) are important mediators of insulitis in T1DM [162, 163]. Combinations of these cytokines have strong synergistic effects regarding β cell stress and death. Exposing cultured islets to different combinations of IL-1β, TNFα and IFN-γ is an established model for β cell destruction in T1DM. Eizirik and coworkers are noteworthy for their continuous work on mapping the gene network behind cytokine-mediated β cell stress [164]. Many of these studies have used FACS-isolated rodent islets or INS-1 cells [165-167], but there are also studies on human islets [112, 168].

In T2DM, chronic exposure to hyperglycemia and/or free fatty acids (FFA) have been suggested as mediators of disease progress [169]. Hyperglycemia cannot be the primary mover in the pathophysiology of T2DM, but it contributes to the progression of the disease. Culture studies of prolonged hyperglycemia in rodent and human islets have demonstrated changes in glucose-stimulated insulin secretion [170-172]; other studies have shown alterations in β cell function and even pro-apoptotic events as the result of “glucotoxicity” [173].

Models for studying islet cell transplantation

Whole-blood models

In vitro whole-blood models have been crucial for describing the thrombo-inflammatory reaction, the IBMIR, following infusion of donor islets into the portal vein of the recipient [72, 81, 82]. Most experiments with cells or cell clusters have been performed using rocking tubing loops with closed or open ends [72, 81, 82, 174]; a few experiments have also been conducted with rocking test tubes [76, 94]. In biomaterials research, the rotating loop model is often used, as well as the slide chamber model [175-177]. Crucial to experiments with islets is reducing the shear forces in order to prevent fragmentation of the cell clusters. Therefore, it is advantageous to choose models with less vigorous circulation of the blood. All of these whole-blood experiments have been performed over a maximum of 60 min, with a few studies extending over 6 h [76, 94, 178]. To my knowledge, no whole-blood model, running beyond this time frame, has previously been described.

Animal models

Animal models have a long history in diabetes research. Originally exclusively using pancreatectomized animals (preferentially dogs and rabbits), experimenters started to use drugs to induce diabetes in the 1940s (alloxan) and 1960s (streptozotocin [STZ]) [179]. STZ was used as chemotherapeutic agent
for cancer because of its inhibition of DNA synthesis. However, the drug also activates poly-ADP ribosylation and is transported by GLUT2 into the β cells. The last two properties are important for the drug’s β cell toxicity [180]. In addition to induced animal models, there are spontaneous diabetic models: the nonobese diabetic (NOD) mouse and the biobreeding (BB) rat. The animals in both these models suffer from an autoimmune attack of the islets that induces insulitis [179].

NHPs have been used in islet cell research over the last 15 years: e.g., Rhesus macaques (*Macaca mulatta*), cynomologus macaques (*Macaca fascicularis*) and baboons (*Papio*). The animals have been rendered diabetic by pancreatectomy or through STZ infusion [181]. Limitations of the diabetic macaque model as a model for xenotransplantation of porcine islets to humans have been highlighted recently [182]. One important aspect that was mentioned is the metabolic differences between pigs and macaques which might result in mild hyperglycemia in the transplanted NHPs [183, 184]. Metabolic factors such as the levels of fasting and stimulated blood glucose are more similar between human and pigs [183]. In another study, however, the glucose-stimulated secretion from pig islets was lower than that from either human or NHP islets [184].
Aims

General aims

1) *To develop new methods* to study immune responses in intraportal allo- and xeno-islet transplantation.

2) To *increase knowledge* about innate and adaptive immunity in allo- and xeno-islet transplantation. By gaining insight into the immune response to transplanted islets, improve immunomodulation to *inhibit the destruction and rejection* of islet grafts.

Specific aims

*Paper I*: To develop a novel whole-blood model for time-wise extended analysis (48 h) of the immune response to a cellular islet graft.

*Paper II*: To explore the IBMIR and initiation of innate immunity up to 48 h post-transplant in a novel whole-blood model of human islet allotransplantation.

*Paper III*: To explore critical events in leukocyte recruitment and graft destruction within 72 h post-transplant by analyzing immune response genes.

*Paper IV*: To evaluate long-term xenograft survival in cynomolgus macaques on a T-cell targeting immunosuppression regimen, including co-stimulatory blockade. To explore the adaptive immune mechanisms of xenograft rejection.

*Paper V*: To evaluate the physiological and molecular response of isolated porcine islets to inflammatory cytokines and hyperglycemia.

Papers listed in order, based on the biological events studied (Fig. 10).
Figure 10. Papers I-IV included in this thesis describe temporally the different parts of the immune response to intraportally transplanted islets. Paper V describes the stress response of cultured porcine islets to inflammatory cytokines and hyperglycemia.
Considerations on methods and design

A detailed description of the materials and methods used is given in the attached manuscripts (papers I-V).

A novel whole-blood model (papers I and II)

Prolonged incubations and smaller volumes (paper I)

As previously stated, there is a lack of in vitro whole-blood models extending beyond 6 h. To study the innate response to intraportally transplanted islets over longer periods, we aimed at developing a whole-blood model for extended incubations. The loop models used so far often require volumes of approximately 4-7 mL blood to attain appropriate blood flow in the system. This requirement demands the availability of high volumes of valuable cellular material and drugs to be tested, and can make it impossible to run multiple treatment groups and replicates during the same experiment.

In paper I we present a novel whole-blood model running for up to 48-72 h with blood volumes of only 1 mL per treatment. Heparinized tubing was cut into suitable lengths (6 cm for 1 mL of blood) and sealed at one end. One mL of blood was added and the tube was sealed or clipped at the other end to form a small bag (Fig. 11). Multiple tubing bags were attached to a rotating wheel (10 rpm) placed in a 37°C cabinet. To avoid clotting and facilitate gas exchange, an air bubble was left in the bag for the blood to move freely.

Human islets (13-15 μL, corresponding to ~4300 IEQ), with a purity of ~70%, or an equal volume of exocrine tissue were added to the blood in the treatment groups (paper II). The negative control was whole blood with the addition of 13-15 μL PBS. The positive coagulation control consisted of whole blood with addition of thromboplastin.

Blood handling (paper I)

To maintain a low coagulation/complement background activation, every step in the experimental design is important. Free flow of blood from the vessel during blood drawing, working gently with the blood when collecting and pipetting, making sure all equipment in contact with blood is well heparinized [185], keeping blood flow at a gentle speed during incubation [185], designing
the model with smooth surfaces and avoiding kinks that can create unnecessary shear forces [186, 187] are all necessary. Even the diameter of the tubing used during incubations matters for complement and coagulation activation, with preference for a tubing with an inner diameter of least 6 mm [188].

**Physiologic parameters and final additives (paper I)**

Several parameters of blood physiology were measured during method development: blood gas analysis with pH, lactate, cation (Na⁺, K⁺, Ca²⁺) and Cl⁻ concentrations, osmolality, leukocyte viability (7AAD/annexinV staining), hemolysis (LD activity) and coagulation/complement activation (TAT, C3a, sC5b-9). To maintain a balanced physiological environment beyond 6 h, concentrated glucose (833 mmol/L) and sodium hydrogen carbonate (NaHCO₃; 1 mol/L) were added at regular intervals based on analyses of glucose, pH, ions and osmotic pressure. For this purpose, a small hole was left open at one end of the bags (Fig. 11c). The additives were injected, using a Hamilton needle, toward the plastic inner surface of the tubing right above the blood surface.

**Protein detection (paper II)**

At the end of the incubations, EDTA was added, and the blood samples were centrifuged for plasma collection. Three different methods for plasma protein detection were used in paper II. Thrombin-antithrombin (TAT), C3a, and sC5b-9 were measured using enzyme-linked immunosorbent assays (ELISA). Multiplex protein analyses were performed for 18 cytokines/chemokines and soluble CD40L (sCD40L) using multiplex Luminex xMAP Technology. Immunoassays for protein analysis using a GyroLab workstation (Gyros, Uppsala, Sweden) were performed for detection of TF, IL-6, IL-8, and MCP-1.

**Immunohistochemistry (Paper II)**

EDTA-treated blood samples (EDTA blood) were centrifuged, and plasma was collected. The remaining pellet was rinsed with PBS and clots, with embedded pancreatic tissue, were fixated in formalin for 24 h; thereafter paraffin-embedded. The paraffin-embedded clots were cut in serial sections and stained manually using standard immunoperoxidase techniques. Primary antibodies were directed against endocrine tissue (chromogranin A), neutrophils (myeloperoxidase [MPO]), monocytes/macrophages (CD68, CD14, CD163) and T cells (CD3). Counterstaining was performed with hematoxylin & eosin (H&E).
**Figure 11.** The rotating tubing bag whole-blood model. (a) Multiple tubing bags attached to a rotating wheel in a 37°C cabinet using rubber bands. (b) Tubing bags containing 1 mL of blood closed by sealing or with a clip. (c) A small hole was left open for injections of additives (marked by an arrow).

**Figure 12.** The porcine islet-to-NHP transplant model. Islets are immediately isolated from the donor pig pancreas. After 48 h of culture the islets are transplanted to the nonhuman primate recipient through intraportal infusion. The primate has previously been rendered diabetic by a streptozotocin infusion.
Islet xenotransplantation in nonhuman primates (papers III and IV)

Working with nonhuman primates

Primate studies are labour-intensive, involving surgery, sacrifice and necropsy procedures, induction of diabetes, blood glucose measurements, insulin injections twice daily, daily dosing of immunosuppressive drugs and blood sampling. Prior to islet transplantation, all animals received an intravenous port used for venous blood sampling [189]. After rendering animals diabetic by high-dose intravenous STZ, diabetes was defined as blood glucose >300 mg/dl, K values (intravenous glucose tolerance test) <1.0, macaque C-peptide <0.2 ng/ml and negative C-peptide response to intravenous arginine challenge. Intraportal islet transplantation was performed through a microlaparotomy, with injection of islets into a mesenteric tributary to the portal vein (Fig. 12). During procedures including surgery (port placements, islet transplantation, sacrifice) the animals were put to sleep using general anaesthesia.

Blood sampling, immunoassays and histopathology

Sampling intensity differed between the two NHP studies referred to in this thesis and for complete methods description I refer to Kirchhof et al. [140] and paper IV (supplementary methods). Metabolic status was evaluated by blood glucose measurement twice daily, weekly porcine C-peptide, and intravenous glucose tolerance and arginine stimulation tests. Standard laboratory testing (hematology and chemistry, including liver panel) was done repeatedly.

The concentrations of IgG and IgM Gal-specific antibodies in serum from NHP recipients were determined by an ELISA assay. IgG and IgM antibodies to non-Gal epitopes were determined by flow cytometry. Enzyme-linked immunosorbent spot (ELISPOT) assays were performed to detect IFN-γ secreting donor-reactive T cells in the peripheral blood [190]. By stimulating recipient responder cells with both lysated and non-lysated donor stimulator cells, indirectly and directly activated donor-reactive T cells could be quantified.

Histopathological analysis, including immunohistochemistry, was performed for liver tissue (bearing xeno-islets) after sacrifice. Islet graft status was evaluated and classified based on insulin staining and cellular infiltration.
**Paper III – Natural history study**

In 1998 a natural history study of porcine islet transplantation to NHPs was performed at The Diabetes Institute in Minneapolis (Kirchhof et al.) [140]. This study included eight non-immunosuppressed Rhesus macaques, of which six animals were rendered diabetic prior to transplantation. The animals received 20,000 IEQ/kg of adult porcine islets, together with heparin, intraportally. Two animals were sacrificed at 12, 24, 48 hours and 72 hours respectively. After sacrifice each NHP underwent a necropsy.

**Paper IV – T-cell-directed immunosuppression**

In 2002-2004, our group performed an extensive study with NHPs, aiming for long-term survival of adult porcine islets using a T-cell-directed immunosuppression regimen. The rationale behind this study was based on the observation that adult porcine islets escape hyperacute rejection and primarily undergo cellular rejection mediated by T cells and macrophages [140]. The definite endpoint was 6 months follow-up with a functional islet graft. Twelve diabetic, immunosuppressed, fully heparinized cynomolgus macaques received 25,000 IEQ/kg of adult porcine islets, intraportally. The animals were divided into three different treatment groups (see Results, Table 6). The NHPs in group A (N=3) were given basiliximab for induction and FTY720 plus everolimus for maintenance immunosuppression. Group B (N=4) received, in addition to the treatment in group A, the human anti-CD154 mAb (ABI793) and group C (N=5) received both ABI793 and leflunomide. The immunosuppressive drugs used are summarized in Table 4.

The logic behind the immunosuppression regimen was to attack T-cell activation and proliferation through different mechanisms. Induction with basiliximab (an IL-2 receptor antagonist) on days 0 and 4 post-transplant is intended to shrink the pool of activated T cells. FTY720 attracts T cells to lymphoid tissue and prevents the cells from homing to the graft. Everolimus, a derivate of sirolimus, blocks intracellular downstream signaling of IL2/IL15, prohibiting T-cell proliferation. Anti-CD154 mAb (ABI793) blocks CD40-CD40L co-stimulatory interactions that are essential for T-cell activation. Leflunomide has a wide range of anti-inflammatory traits, supressing TNF-α-induced tissue injury and xenoreactive antibody responses [191, 192]. FTY720, everolimus, and leflunomide were all given from 7-10 days pre-transplant and thereafter daily. ABI793 was given from day 5 pre-transplant, with more intense administration up to 11 days post-transplant; thereafter, it was given weekly. Addition of Anakinra (IL-1 receptor antagonist) to the protocol (given to three animals in group B) was partially based on a previous pilot study with monkey and pig IL-1β mRNA expression in livers bearing rejected or nonfunctional xenografts [193].
Table 4. Immunosuppressive drugs used during the long-term xenotransplant study (paper IV). Adm. = Administration; i.v.=intravenous; s.c.=subcutaneous; i.m.=intra-muscular, rec=receptor

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Brand name</th>
<th>Adm.</th>
<th>Use in the study</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Induction</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Basiliximab</td>
<td>Simulect®</td>
<td>i.v.</td>
<td>Group A, B, C</td>
<td>anti-CD25 mAb, IL-2 rec. antagonist</td>
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<tr>
<td><strong>Maintenance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>FTY720</td>
<td>oral</td>
<td>Group A, B, C</td>
<td>Targets the S1PR1 rec on lymphocytes, inhibits emigration from lymphoid organs</td>
<td></td>
</tr>
<tr>
<td>Everolimus</td>
<td>RAD®</td>
<td>s.c.</td>
<td>Group A, B, C</td>
<td>Sirolimus derivate, mTOR inhibitor, inhibits T/B cell proliferation</td>
</tr>
<tr>
<td>ABI93</td>
<td>i.v.</td>
<td>Group B, C</td>
<td>anti-CD154 mAb; costimulatory blockade</td>
<td></td>
</tr>
<tr>
<td>Leflunomide</td>
<td>Arava®</td>
<td>oral</td>
<td>Group C</td>
<td>Pyrimidine synthesis inhibitor; has a wide range of anti-inflammatory properties</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>Prograf®</td>
<td>oral</td>
<td>Group C (animals not on FTY720)</td>
<td>Calcineurin inhibitor (2nd gen), Inhibits T cell proliferation</td>
</tr>
<tr>
<td>Anakinra</td>
<td>Kineret®</td>
<td>s.c.</td>
<td>Group B (3 animals)</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>RATG</td>
<td>Thymoglobulin®</td>
<td>i.v. and i.m.</td>
<td>Occasionally to prevent rejection in Group A and B (3 animals)</td>
<td>Rabbit anti-thymocyte globulin</td>
</tr>
</tbody>
</table>
Intragraft gene expression (papers III and IV)

Liver tissue bearing islet xenografts was collected at sacrifice. In the second study (paper II), lobectomy during the transplant period was performed in four animals and liver tissue collected. A method for intragraft gene expression analysis in liver tissue using real-time PCR was developed. Here a few methodological considerations are discussed:

Collecting and processing liver tissue for mRNA isolation

After being weighed and macroscopically evaluated, the liver was cut into small pieces for further analysis and storage. For gene expression analysis liver tissue was initially snap frozen, later first incubated over night at 4°C in the preserving solution RNA later® (Qiagen), previous to storage in a -80°C freezer. Initially, liver samples were collected randomly (paper III) for RNA isolation, but during method development we started to sample tissue from selected liver lobes (paper IV). This decision was made based on the indication that islet distribution within the liver of transplanted NHPs might not be exclusively random (unpublished data).

We considered how much liver tissue was needed to get a representative piece of the islet graft and account for the variation related to dispersion of the islets within the liver tissue. In a first gene expression study of allografts in intraportally transplanted NHPs, we isolated mRNA from multiple 30 mg (4-6) samples of liver tissue using the RNeasy mini kit (Qiagen) [194]. Through these experiments we obtained an understanding of the variation in mRNA expression of cytotoxic effector molecules within the liver of the same recipient, using 120-180 mg of liver tissue. Based on these data and practical considerations, we finally extracted mRNA from 600 mg pieces of liver tissue. The quality of the isolated total RNA was validated by RNA 6000 Nano LabChip technology.

Species-specific primers

To separately study the genes expressed by the pig (donor) and monkey (recipient), we designed species-specific primers. The gene sequences for pig and monkey, respectively, were aligned and areas of species differences were identified for primer design. By designing intron-spanning primers, we avoided amplifying contaminating genomic DNA. Primer concentration was optimized and specificity was confirmed by crosswise negativity using cDNA from activated peripheral blood mononuclear cells (PBMCs) isolated from human, Rhesus monkey and pig (Fig. 13). Amplification of the desired PCR product was verified by agarose gel electrophoresis and specific melting temperature for varying lengths of oligonucleotides given by Dissociation Curve
1.0 software [195]. For every PCR run the dissociation temperature was given for each amplified product, to verify the correct gene amplification.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>P</th>
<th>R</th>
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<tbody>
<tr>
<td>β₂M</td>
<td>Rhesus</td>
<td></td>
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<tr>
<td></td>
<td>Pig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Rhesus</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Pig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>Rhesus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pig</td>
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</table>

*Figure 13. Validation of real-time PCR assays. Species-specific amplification of porcine (P) and Rhesus monkey (R) cyclophilin, IL-1β and TNF-α. Real-time PCR was run on reactions containing activated PBMC cDNA from pigs and monkeys, together with species-specific primers. The reaction product were electrophoresed on an agarose gel and stained with ethidium bromide. Results were confirmed by specific melting temperature analysis. From Hårdstedt et al. (paper III); copyright © 2005, John Wiley and Sons.*

**Real-time PCR**

Reverse transcription to cDNA was performed prior to real-time PCR. The SYBR green fluorescent dye, binding to dsDNA, was used for detection of amplified gene transcripts [196]. The cycle at which the fluorescence signal reaches a set threshold value (Ct value) corresponds to the concentration of transcripts in the starting sample. Data were analyzed by the comparative threshold cycle (Ct) method [197, 198]. Adjustments were made for average amplification efficiencies for each primer pair, as determined with LinReg software (outliers excluded using the KOD method) [199, 200]. By adjusting for different amplification efficiencies, we could more accurately compare the relative expression of different immune response genes within the tissue (paper III). Data are presented as the relative fold change differences between individuals/groups after normalization to the expression of a housekeeping gene (cyclophilin A or β-actin).
Microarray data analysis (paper V)

Incubation of islets for 48 h under four different conditions preceded RNA isolation and pairwise hybridization to a new first-generation oligo set for a pig microarray (Qiagen Array-Ready Oligo Sets for pig, v 1.0 and extension 1.0) [201]. The following culture conditions were compared: (1) 5.6 mM glucose, (2) 16.7 mM glucose, (3) 5.6 mM glucose + cytokines, (4) 16.7 mM glucose + cytokines (Fig. 14). A mixture of three different porcine recombinant cytokines (IL-1β, TNF-α and IFN-γ) were used in concentrations based on a previous study of porcine islets [202].

cDNA labeled with Cy3 or Cy5 was hybridized in four different contrasts (treatment pairs) based on culture conditions. This made up a loop design model (Fig. 15A). For each contrast dye-swap replicates were performed. We used a mixed analysis of variance (ANOVA) model (R/Maanova software) for analysis of the data which allowed us to explore the effect of one treatment regardless of the other (main effects; Fig. 15B). Our mixed model was:

\[ y_{gijk} = \mu + D_i + A_j + S_k + C_g + G_g + (CG)_{g} + \epsilon_{gijk} \]

where \( \mu \) represents an overall mean value, \( D \) represents the dye effect, \( A \) is the main effect of the array, \( S \) is the main effect of the sample replicates, \( C \) is the main effect of cytokine exposure, \( G \) is the main effect of glucose exposure, \( CG \) is the interaction effect of glucose and cytokine exposure, and \( \epsilon \) is the stochastic error.

**Figure 14.** Porcine islets were incubated for 48 hours under four different conditions.

Classical analysis of microarray data consists of pairwise comparisons within a contrast using t-tests (Fig. 15a). A development in the analysis of microarray data with more than one contrast was the use of ANOVA models, including mixed ANOVA models, beginning in the early 2000s [203-205]. In a mixed ANOVA model both fixed (e.g., treatment, dye effects) and random (e.g., array effects) effects can be integrated and evaluated.
Figure 15. Illustration of the loop design model used for the experiments in paper III: (A) Data analysis by pairwise comparisons. (B) Data analysis by using an ANOVA model.

Mixed-model ANOVA analysis made it possible to evaluate the interaction between the cytokine and glucose effect on the gene expression. Since we had cultured the islets with cytokines and elevated glucose in all four combinations the interactive effect between the two conditions could be evaluated statistically (Fig 14). The fold change differences in these interactive effects were marginal, and the biological significance was therefore unclear. Therefore these data were not included in the final paper. However, the statistical method for analyzing the interactive effects of two treatments remains attractive.

**Measurements of islet quality (paper V)**

Paper V presents data for islet quality and function, in relation to gene expression findings. Viability, measured as membrane integrity, total intracellular insulin content and ATP content were assessed in porcine islets exposed to cytokines and elevated glucose for 2, 4 and 8 days (Fig. 14). Membrane integrity was measured as FDA/PI staining (fluorescein diacetate/propidium iodide), insulin content by an ELISA assay and ATP content using a bioluminescence assay. Insulin and ATP content was normalized to DNA content.

**Ethical considerations**

There are numerous of practical and ethical considerations when performing preclinical research using NHPs. Respecting animal welfare is crucial, not only for ethical reasons, but also to ensure the internal validity of study results. The guiding principles for ethical use of animals in research can be summa-
rized in the three Rs: Replacement, Reduction and Refinement. These principles are important to use as a guide in daily work with research animals. The research group in Minneapolis has tremendously advanced their work with NHPs before, during, and since the time I spent in the laboratory. Examples of this advancement are the development of the technique for subcutaneous port placement [189, 206], STZ induction of diabetes [207, 208] and animal handling [209].

The ethical review board in Uppsala approved the drawing of blood from healthy donors and the use of endocrine and exocrine cells obtained during islet isolation from human pancreata (papers I-II). Studies involving NHPs and porcine islets were conducted as approved by University of Minnesota Institutional Animal Care and Use Committee (IACUC) and according to National Institutes of Health guidelines (papers III-IV).

**Statistical analysis**

*Working with a small number of N*

Using isolated human and porcine islets as well as large animals in studies demands keeping the N number small for practical, economic and ethical reasons. However, this is a delicate balance since too small numbers can result in meaningless research. In all my studies I have been fortunate to work with relatively large differences between treatment groups and despite limitations in the number of N I have been able to present significant statistical analyses. Presenting patterns noticed for single animals in a study (i.e., such as a case study) can add valuable information that disappears when one tries to present the results in the context of a group (paper III).

*Skewness and dependence*

All data were evaluated for normality prior to using parametric statistical analysis. Gene expression data from real-time PCR are presented as ratios of expression levels between different genes and different treatments. The nature of ratio data is right skewness. Also, protein expression data for cytokines and chemokinones turned out to be highly right-skewed. Log transformation helped to achieve normal distributions and allow me to use parametric statistics in my papers. Nonparametric statistics were also sometimes run in parallel (paper II) to ensure that the trends were stable. Some other biological data, such as glucose and electrolyte concentrations, exhibited a normal distribution without transformation.

Dependence was a major issue with my blood experiments, which involved multiple sampling over time from one donor-recipient pair and different treatments within one donor-recipient pair. Paired t-tests and repeated measures
ANOVA were used to account for these dependences. For repeated measures ANOVA analysis, the assumption of sphericity was considered using Mauchly's sphericity test. Repeated measures ANOVA analysis was verified with statistical comparisons of the area under the curve (AUC) to confirm the main effects reported (papers I and II).

**Cluster analysis and heat maps**

Statistical analyses of microarray data with comparisons between groups (differential expression analysis) have been discussed above. A second objective in data mining of large data sets can be to cluster data in groups based on similarities in expression profiles [210]. These analyses have classically been performed for gene expression microarray data by using hierarchical cluster analysis and visualization with heat maps [211]. Advances in proteomics have also made use of these methods [210]. Cluster analysis and illustration of gene and protein data using heat maps can also be useful when presenting smaller data sets with expression of multiple genes and proteins (papers II and III). One has to be careful with data processing prior to analysis, since normalization and mean centering of data affect the clustering and appearance of the data.
Results

Blood physiology during long-term incubations (paper I)

To my knowledge, there is no literature to date on blood physiology during long-term incubations in 37°C. To study the immune response over longer periods in a whole-blood model, it is crucial to evaluate the physiological conditions for the experiments. Paper I is a comprehensive report on the physiological changes in human whole blood during 72 h incubation at 37°C.

Basic glucose consumption in the whole blood was high, even higher when pancreatic cells were added. Energy depletion resulted in ion disturbances within 24-48 h, with dramatically increased K⁺ and decreased Na⁺ and Ca²⁺ concentrations (Fig. 16). The addition of glucose, kept at 3-10 mmol/L, improved the ion balance but led to metabolic acidosis. Addition of concentrated NaHCO₃ every 12 h finally balanced the severe acidotic environment, maintaining the pH at >6.8 over 48 h (Fig. 17).

Figure 16. Changes in Na⁺, K⁺ and Ca²⁺ concentrations in whole blood during incubation for 72 h (37°C) in our rotating tubing bag model.
The glucose concentration was adjusted to 10 mmol/L every 12 h based on glucose measurements, adding volumes of 5-10 µL of concentrated glucose (833 mmol/L) as needed (Fig. 17). During method development, blood gas analysis was performed every 12 h to guide the addition of concentrated NaHCO₃ (1 mol/L) based on pH and standard bicarbonate. In the final experiments, a protocol with set volumes was followed (10 µL at 12 h, 15 µL at 24 h, and 7 µL at 36 h). With the fine-tuned addition of glucose and NaHCO₃, hemolysis (LD activity) was prevented for up to 72 h of incubation and leukocyte viability (based on 7AAD/annexinV staining) was better preserved.

Paper I discusses the relative importance of the physiological changes during long-term experiments and how these changes potentially affect immune cells and coagulation/complement activation. In a closed system, it became a delicate balance between preventing energy depletion/acidosis and the increased osmotic pressure, measured as osmolality. In conclusion, a pH above 6.8 and an osmolality below 400 were considered acceptable for immune and coagulation studies. Since these limits, together with cation disturbances, were compromised beyond 48 h (especially with addition of exocrine cells) we believe that precautions must be taken in future in vitro studies beyond this time limit.

![Figure 17. Schematic figure showing the addition of glucose and NaHCO₃ during long-term incubations of whole blood. The aim was to keep p-glucose at 3-10 mmol/L and pH >6.8 throughout the experiment.](image-url)
Immune response to human islets in blood (≤48 h)  
(paper II)

Paper II describes the blood-mediated response to human islets, exocrine tissue and thromboplastin up to 48 h.

The IBMIR

The addition of human islets, exocrine cells or thromboplastin resulted in coagulation and platelet activation, as demonstrated by clotting, platelet consumption, TAT, and sCD40L production. TAT and sCD40L exhibited early high concentrations (peaks at 6-12 h). TF gradually increased in response to islets and exocrine tissue. Noticeable, clots surrounding human islets dissolved from 12 h onward; this was not seen for clots surrounding exocrine tissue. C3a already exhibited high concentrations in response to pancreatic cells and thromboplastin at 6 h, and the levels remained high for 48 h. sC5b-9 production responded strongly to the human islet, exocrine tissue and thromboplastin with increasing concentrations from 6 h on.

Chemokines and cell recruitment

Immunohistochemistry revealed cellular infiltration. Neutrophils (MPO+ cells) and monocytes/macrophages (CD14+, CD163+ cells) had gathered around the islets and exocrine tissue at 6 h. Over the 48 h observation time there was a massive neutrophil infiltration (Fig. 18), whereas monocytes/macrophages were found infiltrating the islet and exocrine tissue in only few to moderate numbers. Toward the end of the 48 h incubation, T cells (CD3+ cells) were seen gathering around the exocrine tissue in some samples; this was not noticed in the endocrine preparations.

Several chemo/cytokines were produced in increasing levels in response to human islets and exocrine tissue. Some of these (MCP-1, IL-8 and IL-6) were already expressed at high concentrations at 6 h, thereafter further increasing over 48 h. Others were building up from 6-12 h (Groα/β/γ and I-TAC) or later (IP-10 and Mig). Exocrine tissue tended to trigger a higher level of innate immune response than did an equal volume of islets; this difference was particularly evident for the production of MCP-1 and the CXCR3-ligands. Thromboplastin provoked similar levels of platelet consumption and TAT/sC5b-9 formation (coagulation/complement activation). However, in most samples with thromboplastin added, the levels of CXCR1/2 ligands, MCP-1, IL-6, and CXCR3 ligands were equal or closer to those seen for incubation of whole blood alone.
Figure 18. Immunohistochemistry staining of human islets incubated in ABO-compatible blood for 6 and 24 h. Photos show formalin-fixed, paraffin-embedded sections of clotted islets stained with hematoxylin & eosin (H&E) and myeloperoxidase (MPO; staining for neutrophils). Photos show increasing infiltration of neutrophils over time.

Figure 19. Cluster analysis illustrating the different expression levels of immune response genes in liver tissue between animals sacrificed early (12-24 h) and late (48-72 h). Elevated expression levels, compared with average, are shown in increasing intensities of red and reduced levels are shown in green. No change is black. From Hårdstedt et al. (paper III); copyright © 2005, John Wiley and Sons.
Immune response to porcine islets in the liver (≤72 h) (paper III)

The gene expression analyses in paper III were based on a natural history study of porcine islets transplanted to NHPs. The essential conclusion of the original paper (Kirchhof et al.) was that transplanted xeno-islets, despite complement binding, do not vanish as a result of hyperacute rejection. The islets instead transiently reverse diabetes before being attacked by acute cellular rejection [140]. Immunohistological evaluation revealed infiltration of CD4+ and CD8+ T cells and macrophages over the first 72 h, with a higher abundance at 48-72 h. Neutrophils were abundant at 12-24 h, but not thereafter.

Table 5 summarizes the genes studied in paper III. By comparing the transcript levels for animals sacrificed early (12-24 h) and late (48-72 h) and correlating the transcript levels with immunohistological cell infiltration (%D islets), we were able to identify genes that appear to be important for cell recruitment and cellular rejection (Fig. 19).

<table>
<thead>
<tr>
<th>Table 5. Selected genes studied in Paper III. All primers were specific for NHP unless otherwise indicated.</th>
</tr>
</thead>
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<tr>
<td><strong>Identification of porcine tissue</strong></td>
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<tr>
<td><strong>Early cell recruitment</strong></td>
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<td>Chemokines</td>
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<td>Chemokine receptors</td>
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<td>T cell markers</td>
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<td><strong>Early graft destruction</strong></td>
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<td>Inflammatory cytokines</td>
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<td>Cytotoxic effector molecules</td>
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CXCR3 mRNA and transcripts for the ligands IP-10 and Mig were identified as being induced and correlated with cellular infiltration. Also, MIP-1α and RANTES transcripts increased over time, but the receptor CCR5 mRNA did not. MCP-1 mRNA was highly expressed, with increasing levels over time. Perforin, GB and FasL transcripts were more abundant at 48-72 h. The CD4/CD8 mRNA ratio suggested a relative increase in CD8+ T cells among the total T cells over the study period. Interestingly, we noticed a correlation between cell infiltration and the expression of the homing receptor CCR7. Antigen-loaded, mature dendritic cells, and activated T cells switch to expressing the receptor CCR7 in order to guide migration to secondary lymphoid tissue.
Reflections concerning gene expression data:

- In Fig. 5 in paper III, the relative abundance of mRNA in the liver tissue of rejecting animals is shown. Perforin, GB and FasL mRNA (cytotoxic effector molecules) were studied in liver tissue from several groups of transplanted NHPs (papers III, IV and [194]). Among the cytotoxic effector molecules, perforin was always the most highly expressed, followed by FasL. GB had a very low baseline expression in all studies. In paper III, GB was undetectable in early rejecting animals at 12-24 h.

Long-term survival of porcine islets in a nonhuman primate model (>100 days) (paper IV)

Immunosuppression and graft survival

Graft survival of porcine islets in NHPs for more than 100 days was presented in a Brief Communication in *Nature Medicine* 2006 (Paper IV). In the same issue, a group from Emory presented equally long-term survival of neonatal porcine islets in NHPs [121]. In both these studies, similar immunosuppressive protocols were used, including co-stimulatory blockade with anti-CD154 mAb [120, 121] (Table 6).

Table 6. Intervention groups in paper IV.

<table>
<thead>
<tr>
<th>Group A (N=3)</th>
<th>Group B (N=4)</th>
<th>Group C (N=5)</th>
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<tr>
<td>Imunosuppression</td>
<td>Basiliximab</td>
<td>Basiliximab</td>
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<td></td>
<td>FTY720</td>
<td>FTY720</td>
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<tr>
<td></td>
<td>Everolimus</td>
<td>Everolimus</td>
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<tr>
<td></td>
<td>ABI793</td>
<td>ABI793</td>
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<tr>
<td>Islet graft survival</td>
<td>24, ≥39, 45</td>
<td>47, 54, ≥73, ≥187</td>
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Reversal of diabetes and long-term graft survival of adult porcine islets was achieved without elimination of the gal-epitope. Graft survival improved substantially from group A (median, 30 d) to group B (median, 63 d) and group C (median, 140 d) (Fig. 20). The animal with the longest graft survival (group B) was sacrificed at day 187 with a functional graft (Fig. 21). Our study demonstrated long-term xenograft survival, but the graft survival was associated with high morbidity (i.e. CMV infections, anaemia) as a result of heavy immunosuppression. In particular thromboembolic events were induced in eight of nine animals treated with the anti-CD154 mAb. For histopathologic results I refer to supplementary table 2 in paper IV.
Figure 20. Outcome of the long-term survival study of porcine islets transplanted to NHPs. Kaplan-Meier estimates of islet xenograft survival in groups A, B and C. Symbols indicate seven euthanasia censors and one death censor. From Hering et al. (paper IV); copyright © 2006, Nature Publishing Group.

Figure 21. (A) Blood glucose measurements (black lines=morning glucose; dotted lines=evening glucose) and exogenous insulin given (gray bars). (B) Results of intravenous glucose stimulation tests pre-STZ (dotted line), post-STZ (black line) and after transplantation (gray lines). From Hering et al. (paper IV); copyright © 2006, Nature Publishing Group.
**Xenoreactive antibodies and xenogeneic T-cell response**

There was no increase in serum IgM or IgG anti-Gal antibodies in rejecting animals and graft tissue did not stain with Gal-specific (isolectin B4) staining. However three of four NHPs with ongoing rejection exhibited elevated levels of non-Gal pig-specific IgG in serum.

ELISPOT assays detecting IFN-γ-secreting donor-reactive T cells revealed increased number of circulating, indirectly activated donor-reactive T cells in NHPs with ongoing xenograft rejection.

**Intragraft gene expression (Paper IV)**

Pig-specific insulin mRNA in the livers at sacrifice was positively correlated to a better graft status, as classified by histopathological scores (Fig. 22, unpublished data). A higher histopathological score reflects a higher total number of well-preserved islets found in the xenograft. Pig-specific mRNA for CD4, IL-1β and TNF-α was not reliably detected in the liver tissue.

Inflammatory cytokine mRNA was down-regulated in Group B and C animals. IL-1β mRNA expression was significantly lower for Group B and C animals (receiving co-stimulatory blockade). TNF-α mRNA expression tended to be lower in Group C animals (addition of leflunomide treatment). The expression of *cytotoxic effector molecule* transcripts (Perforin, GB and FasL) did not show any differences between the treatment groups. However, GB, and to a lesser extent perforin and FasL, were suppressed in association with high trough levels of anti-CD154 in blood (paper IV).

**Reflections concerning gene expression data:**

- Gene expression data was reported relative to the expression in liver tissue from nine untreated, control, monkeys (paper IV). The rationale behind comparing levels of immune response genes between heavily immunosuppressed, previously STZ-treated and transplanted animals with untreated controls is vague. In a previous study on cytotoxic effector molecule mRNA expression in peripheral blood pre- and post-transplant, I concluded that T-cell-directed immunosuppression substantially decreases the levels of lymphocytes, and therefore also cytotoxic effector molecule transcripts (unpublished data).

- In addition to the effects of drug exposure, several factors seemed to affect the expression of immune response genes: the stage of rejection, the time from the onset of rejection, and inflammation and infection (e.g., local inflammation due to surgery, or CMV infection). Since gene expression was time-related, the status of the animal close to the time of tissue collection was crucial for the interpretation of the data.
Figure 22. Correlation between porcine mRNA transcripts levels and graft status. Real-time assessment of porcine mRNA within liver tissue using species-specific primers; low Ct value reflects a higher level of mRNA. Histopathological scores reflecting graft status where a high score equals a more well-preserved, less rejected graft (%A islets (well-preserved islets) x total number of counted islets in the tissue). Unpublished data, Hårdstedt 2004.

Gene expression in response to glucose and cytokines in cultured porcine islets (paper V)

Physiological (membrane integrity, insulin and ATP content) and molecular changes in response to two well-documented conditions in islet cell biology were evaluated: inflammatory cytokine stress and hyperglycemia (see further in the Introduction). In paper V, the top 25 differentially expressed genes in response to 48 h exposure to cytokines and glucose were presented (cutoff p<0.05), together with main functional groups of genes based on gene ontology categorization (EASE software) [212].

During our data analysis, a more stringent list of genes were also selected (p<0.01), in an attempt to explore pathways and functional groups of genes differentially expressed. This selection was not based on the relative fold-change increase/decrease and included genes with both high and low relative expression. The data are therefore less trustworthy concerning up/down-regulation of single genes but are of more interest for studying functional groups of genes. These analyses were not included in the final manuscript (paper III). In the following sections, I present some reflections based on this data set (cutoff, p<0.01). Naturally, this discussion highly overlaps with the results presented in paper III (cutoff, p<0.05).
Cytokine response

The effect of exposure to IL-1β, TNF-α and IFN-γ was dominated by increased expression of genes involved in immune response, stress response and death/apoptosis, as well as genes involved in cellular and oxidative defense strategies (paper V and Fig. 23). Transcription factors and genes involved in signal transduction were two big groups that were up-regulated. Genes involved in antigen presentation (MHC-related genes, proteasome components, genes involved in antigen processing and presentation), complement activation (i.e., C3, C1s) and chemokines (MCP-1, CXCL16) were increased.

Up-regulation of previously defined IFN-γ-induced genes was noticed [165, 167]. The JAK-STAT pathway was induced, together with SOCS-3, a negative feedback regulator of IFN-γ signaling [213]. A key regulator of cytokine (IL-1β/TNF-α)-mediated stress signaling in β cells is the transcription factor NFκB [165]. NFκB as well as the NFκB-inhibitors, NFκB inhibitor alpha and RelA-associated inhibitor, were induced in response to cytokines. Cytokines are known to trigger ER stress in β cells, leading to impaired cellular function and eventually to cell death [214-216]. Genes known to contribute to, or be the result of, ER stress were differentially expressed under cytokines (Fig. 23) [217]. Caspase-3, an “end-stage” effector involved in apoptosis, was increased, together with inhibitors of apoptotic events: Bax inhibitor-1 and nuclear protein 1. Genes involved in vesicle-mediated transport and secretory pathways were down-regulated in response to cytokines, suggesting impaired functioning of the ER/Golgi system. The following genes involved in oxidative defense was up-regulated in response to cytokines (previously shown in oxidative stress and/or expressed in islets): enzymes from the thiol antioxidant system (thioredoxin, peroxiredoxin I) [218], metallothionin-IA (MT-1A) [219], glutathione transferase [220], glutaredoxin 2 and calcipressin [221].

Glucose response

Elevated glucose over 48 h was associated with an elevated metabolic activity with increased expression of genes involved in metabolism, transportation, and energy production (Fig. 24). Important enzymes in glycolysis were up-regulated together with pyruvate dehydrogenase. The expression of several genes involved in lipid metabolism was increased e.g., apolipoprotein II and A-IV precursors; also IDII protein, which is involved in cholesterol synthesis. Many genes active in protein synthesis and intracellular transport were up-regulated, as well as genes involved in protein destruction and processing (genes involved in the ubiquitin system). Interestingly, genes involved in oxidative defense were both up- and down-regulated by glucose. Thioredoxin
was up-regulated under these conditions, as was thioredoxin-interacting protein (TXNIP), an inhibitor of thioredoxin. The expression of both metallothionin IA and III was decreased in response to glucose.

**Islet quality and energy utilization**

Exposure to inflammatory cytokines or hyperglycemia was associated with a reduction in the intracellular ATP and insulin content over time (paper V). These changes were more evident for cytokine then glucose stress. Insulin content showed a significant decrease by day 4, however the decline in ATP content failed to reach a statistical significance due to high variation in measurements and small number of N. Islet cell viability assessed by membrane integrity (FDA/PI) was virtually unaffected by the treatments up to 8 days.

Figure 25 is an attempt to visualize the gene expression changes (at 48 h) supposedly leading to the decline in intracellular ATP in response to cytokines and glucose. The drop in ATP under cytokine stress can be explained by both elevated energy utilization and impaired ATP production. Genes involved in mitochondrial processes such as β-oxidation and ATP production were found to be down-regulated (e.g., ATP synthase delta chain). Mitochondrial oxidative metabolism generates an estimated 98% of β-cell ATP [222]. The expression of genes encoding enzymes involved in glycolysis, as well as the metabolism of other carbohydrates, was depressed. One exception was glucokinase, whose expression was increased.

Exposure to hyperglycemia also tended to decrease ATP content over time. Elevated glucose increased the expression of genes involved in cell proliferation and protein synthesis, and also anti-proliferation and proteolysis. For changes in the protein profile and induction of metabolic processes, degradation of proteins that are no longer needed is also important. Hyperglycemia induced metabolic activities leading to a higher demand for energy. Glucose, however, also up-regulated genes involved in energy production (such as genes in the glycolysis and electron transport chain), somewhat balancing the intracellular energy utilization (Fig. 25).
**Figure 23.** Cytokine response. An attempt to visualize selected microarray findings in response to cytokines. Genes were selected by mixed-ANOVA model analysis (R/Maanova) using a significance cutoff of 0.01. Small arrows in front of the gene name indicate up-regulation or down-regulation in response to cytokines; the symbol "\( \uparrow \)" indicates inhibition. Unpublished analysis, Hårdstedt 2005.

**Abbreviations:** C1NH (plasma protease C1 inhibitor precursor), CEBPB (CCAAT/enhancer binding protein β), CRAC1A (GTPase cRac1A), CTF1 (cardiotrophin 1), CYP2C18 (cytochrome P450 2C18), G3BP-1 (Ras-GTPase binding protein 1), GADD45 (growth arrest and DNA damage-inducible), GST (glutathione transferase), Grx (glutaredoxin 2), HSP-70 (heat shock protein (70.4 kD)), HSP-33 (heat shock protein 33), IDO (indoleamine 2,3-dioxygenase), IRF1 (interferon regulatory factor 1), IRF2 (interferon regulatory factor 2), IRF7A (interferon regulatory factor 7A), I-TRAF (TRAF family member-associated NFKB activator), MnSOD (manganese superoxide dismutase), MT-IA (metallothionein-IA), NFKBIA (NF-kappaB inhibitor alpha), NFKB2 (NFkappa B2), Prx (peroxiredoxin 1; thioredoxin peroxidase 2), R-Ras (p23) (Ras-related protein R-Ras (p23)), RAN (Ras-related nuclear protein), SOCS3 (suppressor of cytokine signaling 3), STAT1 and 2 (Signal transducer and activator of transcription 1 and 2), TAP1 and 2 (antigen peptide transporter 1 and 2), TAPBP (Tap-binding protein), TLOC1 (translocation protein 1), TRAPPC4 (trafficking protein particle complex subunit 4), Trx (thioredoxin), VPS35 (vacuolar sorting protein 35), WARS (tryptophanyl-tRNA synthetase), XBP-1 (X-box-binding protein-1).
Figure 24. Glucose response. An attempt to visualize selected microarray findings in response to hyperglycemia. Genes were selected by mixed-ANOVA model analysis (R/Maanova) using a significance cutoff of 0.01. Small arrows in front of the gene name indicate up-regulation or down-regulation in response to glucose. Unpublished analysis, Hårdstedt 2005.

Abbreviations: Apo-AIV (apolipoprotein A-IV precursor), Apo-CII (apolipoprotein C-III precursor), ATF4 (activating transcription factor 4), BTG1 (B-cell translocation gene 1, anti-proliferative), BTG2 (B-cell translocation gene 2, anti-proliferative), C14orf130 (chromosome 14 open reading frame 130), CCNA2 (cyclin A2), CREG1 (cellular repressor of E1A-stimulated genes 1), DDC (dopa decarboxylase), Dyrk1b (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1b), EEF2 (eukaryotic translation elongation factor 2), EIF4G2 (eukaryotic translation initiation factor 4 gamma), FACE-2 (farnesylated-proteins converting enzyme 2), FBXO2 (F-box protein 2), GPI (glucose phosphate isomerase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), GBE (glycogen branching enzyme), HNRPA1 (heterogeneous nuclear ribonucleoprotein A1), hNRP (NAP-1 related protein), ID1 (isopentenyl-diphosphate delta isomerase), KDELR2 (KDEL endoplasmic reticulum protein retention receptor), MMP7 (matrix metalloproteinase 7), MRPS16 (mitochondrial ribosomal protein S16), MRPL11 (mitochondrial ribosomal protein L11), NCBP2 (nuclear cap binding protein subunit 2), ODC (ornithine decarboxylase), OXA1L (oxidase assembly 1-like), PSMB3 (proteasome subunit β 3), PSMB10 (proteasome subunit β 10), 6-phosphogluconolactonase (6PGL), PKM2 (pyruvate kinase), RAMP4 (ribosome associated membrane protein 4), RNF10 (ring finger protein 10), RNF146 (ring finger protein 146), RNP21.4 (Cop-coated vesicle membrane protein p24 precursor), RPS14 (ribosomal protein S14), Syx5 (syntxin 5), TICAM2 (TLR-like receptor adaptor molecule 2), UBE3B (ubiquitin protein ligase E3B), USF1 (upstream transcription factor 1)
Figure 25. An attempt to visualize energy utilization in response to glucose (left panel) and cytokines (right panel), with selected genes presented. In the center are the mitochondria of the β cell. Small arrows in front of gene name indicate up-regulation or down-regulation in response to cytokines and glucose. Unpublished analysis, Hårdstedt 2005.

Abbreviations: ALDOC (aldolase C, fructose-bisphosphate), GAPDH (glyceraldehyde phosphate dehydrogenase), GPI (glucose-6-phosphate isomerase), TIM (triose-phosphate isomerase), OXA1L protein (oxidase [cytochrome c] assembly 1-like), PK (pyruvate kinase),
Long-term blood model – pros and cons (paper I)

A major advantage of a human model is avoiding data from animal models with unknown clinical applications. Initial testing of drugs, biocompatibility of materials and toxicity in a human whole-blood model prior to animal experiments is an attractive option. There is an ethical requirement to find alternatives to animal models and I have seen our work with this long-term human blood model as a step in this direction. A disadvantage with a closed whole-blood system is obviously the lack of blood exchange. The blood is re-circulating within the tubing bag during the observation time, which results in accumulation of coagulation/complement products as well as cytokine/chemokines. When studying immune responses, the lack of interactions with the recipient’s endothelial cells and lymphoid tissue is another consideration compared to the in vivo situation. The artificial inner surface of the tubing, covered with heparin, diminishes coagulation activation but does not by other means behave like vascular endothelium.

When a blood model is being designed, it is a fundamental necessity to define what conditions the model should mimic. Here, our aim was to mimic the environment in the portal vein liver branches, where islets are trapped early after intraportal islet transplantation [223]. Blood circulation around the trapped, thrombotic islets in the portal vein branches is most likely limited, as in our model. Rapid coagulation and complement activation occur in vivo together with a high metabolic consumption. This causes lowering of the pH in the microenvironment surrounding the islets, trapped within the clots. We chose not to add anticoagulants, since these agents will influence innate immunity, which meant that we had to allow for a higher intrinsic background. Depending on future applications of the model, evaluation of background activation and possibly the addition of low doses of anticoagulants can be beneficial.

Energy depletion leading to inhibition of energy-requiring ion pumps and severe ion changes needed to be prevented. Intracellular signalling during immune cell activation as well as coagulation and complement activation are dependent on calcium ion homeostasis [224, 225]. β cells are also dependent on ion (K⁺ and Ca²⁺) homeostasis and ATP/ADP ratios for insulin release and normal cell function [226]. A literature review on how pH and osmolality affect immune function was performed (paper I). There was a balance between
compensating for acidosis with addition of NaHCO₃ and creating a too high osmotic pressure within a closed system. This challenge, together with ion changes, finally made us decide to end the experiments after 48 h incubation.

The important first hours and days (papers II and III)

Intraportally transplanted islets go through an exceptionally traumatic event at the moment of transplantation. Only about 50% of the islets, at the most, will engraft into the liver and produce insulin (Fig. 26) [227]. Thrombi around the islets formed within the first 6 h in our model. The initial coagulation activation was supposedly driven by platelet activation and the intrinsic coagulation pathway, triggered by exposure of extracellular matrix components (e.g., collagen) and endothelial cells on the islet surface [79, 80, 228]. This event was followed by increased concentrations of TF, produced by the pancreatic tissue and leukocytes, including neutrophils [81, 229]. An equilibrium was reached between thrombosis and fibrinolysis; clots surrounding the islets partly resolved over the study period. Complement cascades accumulated terminal complexes (sC5b-9) simultaneously with increased chemokine production and attraction of immune cells to the graft site. In the clinical setting, the clotted islets adhere to the vessel wall or are trapped in portal vein branches because of the size limits of their lumens [223]. Revascularization of the islets is believed to happen within about 7-10 days after transplantation.

Cells initially recruited to the graft vicinity (neutrophils and monocytes) are critical in shaping the innate immune response. Platelets, which instantly surround the islets and contribute greatly to the “innate” environment, should also be mentioned here. The activated platelets form complexes with leukocytes (granulocytes and monocytes) and trigger coagulation/complement activation by various means, providing a surface for coagulation factors and releasing chondroitin sulfate [230] (see further in the Discussion, CD40L – a partner in multiple crimes). Activated platelets seem to balance complement activation by binding complement inhibitors and may also promote vascularization through the release of vascular growth factors [231]. Accompanying the early cell recruitment, IL-8, MCP-1 and IL-6 were already produced at high levels by 6 h in our model, with increasing concentrations over the 48 h. These chemokines have previously been shown to be secreted by human islets as well as by several types of innate immune cells [109, 232, 233]. Concentration of other chemokines were built up from 6-12 h (Groα/β/γ and I-TAC) or later (IP-10 and Mig). Interestingly, the pattern of chemokines induced in our whole-blood model was nearly concordant with previous measurements in peripheral blood up to 7 days after clinical islet transplantation [234]; however, we could not confirm the up-regulation of MIP-1α and MIP-1β.
Ten years after the discovery of the IBMIR, the initial destruction of islets post-transplant has again become a focus of attention [235, 236]. Also in organ transplantation, innate immunity has been suggested to play a more prominent role than previously recognized [71, 237]. It is obvious that what happens within hours and days after transplantation is crucial not only for the short term, but also for long-term transplant outcome. In addition to the initial destruction of islets, the acute inflammatory response (IBMIR) shapes and accelerates the later adaptive immune response [143]. In the post-glucocorticoid era of clinical islet transplantation, induction protocols including TNF-α inhibition (etanercept), have recently been suggested to be beneficial for long-term transplant outcome [60, 238]. Further long-term studies are needed to clarify the relative effect of TNF-α inhibition and the novel T-cell depressants used in these protocols.

To “hide” the islets from IBMIR, and thereby preventing early destruction and decreasing immune activation, seems crucial (see Future perspectives). Potential targets include platelet activation, complement and coagulation activation as well as blockade of innate cellular recruitment. Targets for chemokine blockade, based on my studies, would be CXCR1/CXCR2 (with ligands IL-8 and Gro-α/β/γ), CCR2 (with ligand MCP-1) and CXCR3 (with ligands IP-10, Mig, I-TAC) (Fig. 27). The reasoning behind a chemokine blockade is to reduce/withhold immune cell infiltration and activation during the vulnerable phase of engraftment and attenuate the immunogenicity of the graft tissue.
by diminishing cellular stress. *Reparaxin*, a dual CXCR1/2-antagonistic drug, recently improved graft function one month post-transplant when added to the induction protocol (day -1 to day 7 post-transplant) in a small clinical trial of islet transplantation [234]. CCR2-deficient recipients and CCR2 blockade have had beneficial effects on islet graft survival in murine models of islet allo- and xenograft transplantation [113, 239-241]. Murine models of islet allotransplantation have demonstrated diminished immune cell recruitment and/or prolonged islet graft survival in CXCR3 deficient recipients or with the use of CXCR3- and IP-10 directed antibodies [114-116].

![Diagram](image)

**Figure 27.** Three chemoattractant chemokine-chemokine receptor axes were identified in my studies: CXCR1/2 (ligands IL-8 and Groα/β/γ), CCR2 (ligand MCP-1) and CXCR3 (ligands IP-10, Mig and I-TAC).

**Transplantation of non-endocrine tissue – for better or for worse? (paper II)**

A close-to-the-clinic question as a consequence of comparing the innate immune response to human islets and exocrine tissue was: Is transplantation of non-endocrine tissue deleterious, positively helpful, or insignificant with relation to transplant outcomes? Today, the predominant approach in clinical practice is to prioritize a high total β-cell mass, also including medium-density fractions with a lower purity at transplantation. The Edmonton and Miami groups presented the composition of their islet grafts, as assessed by different cell identification methods (immunostaining [242] and FACS [243]). In the Edmonton material, the endocrine fraction represented, on average, 41% (23%
β cells), the ductal fraction 24%, and the acinar fraction 35% at transplantation, corresponding to an average purity of 67% as assessed by dithiazone staining [242]. When the different cell components were correlated with long-term patient outcomes, a higher percentage of ductal cells (anti-cytokeratin [CK]-19 positive cells) was associated with a superior transplant outcome. The Miami group identified ductal cells by using anti-CK-19 and anti-carbohydrate antigen 19-9 (CA19-9) (in FACS analysis) [243]. They stressed the difficulty inherent in identifying the cell types by showing that anti-CK-19 antibodies also stained endocrine cells (α and δ cells), whereas CA19-9 also stained some amylase-positive cells.

Ductal cells have been suggested to be progenitor cells for β cells and to be involved in supporting engraftment [244]. They, however, also produce TF and promote coagulation and the IBMIR [83]. Acinar cells are not believed to contribute to the regeneration of endocrine cells [245]. Both isolated ductal and acinar cells have shown to express cytokines/chemokines (e.g., MCP-1, IL-6 and TNF-α) [243, 246]. Interestingly, high-density layers of ductal cells produced higher concentrations of inflammatory cytokines (IL-1β, TNF-α and IFN-γ) and chemokines (e.g., MCP-1 and Mip-1β) compared to low-density layers [243]. Our whole-blood study demonstrates that exocrine tissue trigger an innate immune response of similar magnitude as an equal volume of human islets. The production of TF and some of the early immune cell chemotactants (MCP-1 and CXCR3-ligands) were higher in response to exocrine tissue than human islets. Our exocrine tissue was assessed from impure, high-density fractions from the isolation procedure. Histological evaluation indicated that these fractions contained predominantly acinar tissue. Ductal structures were occasionally seen in the endocrine fractions.

In summary ductal tissue may be of benefit to islet regeneration post-transplant, but equally beneficial qualities for acinar tissue has not been described. Recent data from our group showed that graft function at one month (the ratio of C-peptide to glucose and creatinine) was negatively correlated with an increased exocrine volume at transplantation [247]. Based on this observation, one can speculate that a limited mass of co-transplanted ductal cells is beneficial, whereas acinar tissue may be deleterious for engraftment and graft survival.

Allo versus xeno – differences and similarities (papers II, III and IV)

None of the studies included in this thesis was explicitly designed to compare the immune response to islet allo- and xenografts. The response to a human islet graft was evaluated in ABO-compatible human blood (≤48 h). Xenograft immunity was evaluated in a natural history study of porcine islets to non-
immunosuppressed NHPs (≤72 h) and an interventional study (>100 days) with immunosuppressed NHPs. After working for several years with both allo- and xeno-models, I am taking the opportunity here to reflect on the differences and similarities between the two, based on my experiences and others.

As mentioned, many features of the IBMIR response is similar between the allogeneic and xenogeneic settings (see Introduction). A recent in vitro study showed IgM/IgG binding, complement activation and islet damage in response to xeno-islets (pig-to-human), but much less so in response to allo-islets (human-to-human) within 60 min in vitro [148]. Two cooperative, but independent, mediators of the acute xeno-IBMIR response are natural xenoreactive antibodies and the complement system [248]. The α-gal epitope (to a lesser extent expressed on adult porcine islets), together with other xenogeneic epitopes, binds preformed/natural antibodies and also triggers the production of xenoreactive antibodies post-transplant [132, 138, 249]. As mentioned, species-differences in complement-regulatory proteins make porcine islets more susceptible to complement destruction. In the unique natural history study of porcine islets in non-immunosuppressed NHPs, IgM/IgG antibodies and complement deposits were noticed on the surface of the islets at 12-24 h (Kirchhof et al.) [140]. However, a substantial amount of the xenogeneic islets did not vanish due to hyperacute rejection. IgM/IgG and complement attached to the porcine islets decreased over the 72 h observation period. Serum complement activation products (C3a/sC5b-9) increased at 12 h, but thereafter went back to pretransplant levels. In contrast to van der Windt et al.’s findings, referred to above [148], other in vitro studies in the allogeneic setting have demonstrated binding of natural antibodies and complement products also to allogeneic islets [84]. However, the concentration of natural antibodies towards the xenogeneic epitopes (predominantly carbohydrates) must be of much larger magnitude [132]. In clinical allotransplantation, the ABO-blood group antigens resemble the α-gal epitope and ABO-incompatible transplantations exhibit certain similarities to xenotransplantation [250].

These xenotransplant data (paper III and Kirchhof et al.) can be compared with the kinetics in our allotransplant whole-blood model, showing gradually increasing concentrations of sC5b-9 up to at least 72 h. In both settings, the neutrophils were the first infiltrating immune cells. In pig islet xenografts, neutrophils (identified by elastase staining) were the predominant infiltrating cells at 12-24 h but disappeared when T cells and macrophages took over. Cytotoxic effector molecule transcripts (perforin, GB and FasL) and CD8 mRNA increased at later time points, indicating that CD8+ T-cell activation and cytotoxic killing had already begun by 48 h in non-immunosuppressed monkeys (paper III). Important to remember is that also NK cells can possess cytolytic killing by the perforin/granzyme pathway [92]. However, in our rejecting NHPs, immunohistological staining for NK cells (CD56) revealed no evidence of NK cells in association with the xenografts [140]. In the whole-
blood model, there was an ongoing infiltration of neutrophils over the 72 h study period, but T cells were not seen infiltrating the islet allografts. These findings could simply describe the differences between working in two very different models. However, they are in line with our knowledge of a faster and more vigorous xenogeneic immune response [251, 252]. Natural anti-pig antibodies, immediately binding to the pig islet xenografts, may take active part in the rapid launching of cellular rejection [140].

If the porcine islets survive the initial innate “knockout,” a subsequent cellular (mainly T-cell-mediated) response soon takes over. Long-term islet allograft survival in NHPs had previously been demonstrated with immunosuppression similar to that given to the group A animals in paper IV (basiliximab, everolimus and FTY720) [253]. In the xeno-study, twice as many islets (IEQ) were transplanted (25,000 IEQ/kg compared to 10,000 IEQ/kg) and higher doses of everolimus/FTY720 were given. Allograft survival on this regimen was >6 months (termination of the study), whereas xenograft survival was 24-45 days. This illustrates the fundamental differences between an allo- and xeno-response in larger animals. However, the mechanisms behind this difference are not fully mapped. To say that a xenogeneic immune response is merely more vigorous is probably an oversimplification. Most likely, different immunological mechanisms are involved or emphasized.

Elevated levels of pig-specific non-Gal IgG were identified in the rejecting primates in our study, suggesting a humoral component. Numerous foreign proteins in the xenografts give rise to a large repertoire of immunogenic peptides, stimulating a diverse set of T cells (and B cells) [249]. As previously mentioned, indirect but also direct recognition of xenogeneic MHC-derived peptides or MHC-molecules, respectively, have been described in pig-to-human xeno-responses [254]. In our long-term porcine islet-to-NHP study, there were high levels of indirectly activated IFN-γ-secreting donor-reactive T cells in rejecting animals, indicating incomplete suppression of indirect immune recognition. The results concerning cytotoxic T cells were less conclusive. However, there was a trend toward lower cytotoxic effector molecule mRNA expression in association with high trough levels of co-stimulatory blockade at sacrifice. Whether the blockade of CD154 has a crucial direct effect on CD8+ T-cell activation or mainly act by impairment of CD4+ T-cell help is hard to conclude from the literature. Memory CD8+ T cells have been shown to be resistant to CD154 inhibition [255]. However, CD4+ T-cells interaction with dendritic cells (involving CD40-CD154) seems to be essential for the priming of CD8+ T cells [256]. In conclusion, our long-term NHP study suggested two mechanisms of adaptive xenograft rejection that might be of exclusive importance: the humoral component (pig-specific non-Gal antibodies) and indirect T-cell activation.
CD40L – a partner in multiple crimes? (papers II and IV)

Today, our long-term survival study of porcine islets from 2006 is a part of xenotransplantation history. A handful of research teams have later shown similar graft survival (>6 months) in the porcine-islet-to-NHP model [257-259]. In virtually all of these protocols, anti-CD154 mAb was included. The rationale behind anti-CD154 mAb treatment is blocking the co-stimulatory signal between T cells (expressing CD154=CD40L) and APCs/B cells (expressing CD40). Through this treatment, T-cell activation and B-cell proliferation, as well as antibody switching from IgM to IgG, are prevented [260]. Interestingly, co-stimulatory blockade have been suggested to efficiently block the indirect pathway of T-cell activation [261]. Unfortunately, the high frequency of thromboembolic events associated with anti-CD154 treatment brought this treatment to a halt. Interesting to mention is the fact that the Emory team did not experience thrombotic events when giving anti-CD154 mAb, restricted to only six doses for induction [121].

The pro-thrombotic activity of CD154 mAb is most likely caused by the expression of CD40L on activated platelets [262]. Once activated, platelets release pro-inflammatory soluble CD40L (sCD40L) within minutes to hours [263]. This phenomenon was also demonstrated by us in vitro in response to a human islet transplant (paper II). It has been estimated that >95% of the circulating CD40L is derived from platelets [263]. Through CD40L, platelets interact with and activate CD40-expressing endothelial cells [260]. To complicate the matter even further, platelets also express CD40 and have been shown to interact with CD154-expressing T cells, generating a potential circle of amplified immune responses [264, 265]. Also pancreatic islets have shown to express CD40 [266]. CD40-CD40L interaction lead to islet secretion of IL-6, IL-8, MCP-1 and MIP-1β [267]. The CD40-C40L signaling is important for T/B-cell activation and proliferation. However, it is obviously involved in various aspects of inflammation and immune response and notably tightly linked to activated platelets [260]. Did we, in addition to dampening the adaptive cellular immune response, also affect a complicated network of inflammatory signaling with our CD154 mAb treatment?

Blockade of the CD40-CD154 interaction between T cells and APCs has been shown not only to inhibit activation of graft-reactive T cells but also to promote tolerance by enriching T-regulatory cells (Tregs) [268]. Interestingly, the induction of tolerance might be dependent on the “immunosuppressive environment” and the inflammatory state of the graft [269]. This further stresses the importance of preventing the IBMIR (i.e., inhibiting inflammatory stress). Based on studies in mice, the key is to combine co-stimulatory blockade with a drug that induces apoptosis in the alloreactive cells (e.g., rapamune) and not with a drug that blocks signal 1 (T-cell receptor recognition of antigen) like cyclosporine A [270]. Despite the shortcomings of the CD154 mAb, the
CD40/CD154 pathway is still a promising target in both xeno- and allotransplantation. There are several upcoming drugs directed toward CD40 that are explored at different levels [98]. Of these only Chi220 (anti-CD40 mAb) has so far been reported to be effective in the pig islet-to-NHP model [271].

“Not to see the wood for the trees” (paper V)

“Not to see the forest for the trees” is a useful description of the issue with multi-analyzing tools. Presented in this thesis summary (Figs. 23-25) is an attempt to illustrate pathways and functional groups of genes, pointing out specific cellular responses. This way of presenting data will always be somewhat subjective; however, it is a complement to the gene ontology groups and more strictly chosen genes presented in our manuscript (paper V). When pointing to a single gene as being truly differentially expressed, a high fold change value helps corroborate a true up-/down-regulation. However, the biological relevance of a fold change cut off can always be questioned. Confirmation using another method, such as real-time PCR or proteomics, is preferred. Up-regulation of set of genes involved in the same pathway strengthens the significance for individual genes.

Aside from being an established model of insulitis, cytokine exposure of islets also mimics the inflammatory response after islet transplantation [272]. Inflammatory cytokines from activated macrophages (IL-1β, TNF-α) and T cells (IFN-γ) are mediators of graft rejection. Superior graft survival in animals treated with CD154 mAb and leflunomide was associated with lower expression of IL-1β and TNF-α mRNA in graft tissue (paper IV), which goes hand in hand with the deleterious effects of these cytokines on islets in culture (paper V). Gene expression studies, such as ours, have identified genes that later have been shown useful as targets for islet protection in islet transplantation, such as NFκB, STAT-1 and indoleamine 2,3-dioxygenase (IDO) [178, 273-275].

Another potential application for mapping these stress response genes is to identify genes reflecting islet quality. The long-term goal would be to be able to predict transplantation outcome. Can the gene pattern identify islets in a bad condition prior to transplantation? So far only sporadic reports have stated a predictive value for single proteins/genes (e.g., MCP-1); most likely because of the multifactorial genesis of transplantation outcomes [109, 276]. A recent study suggested the JNK3 protein and the c-fos gene as potential markers for islet quality [277]. mRNA expression is temporal and the timing of islet sampling for gene expression analysis is important for the evaluation of the results.

We evaluated the effect of elevated glucose and a cocktail of inflammatory cytokines (IL-1β, TNF-α and IFN-γ) on the gene expression pattern in porcine islets. Our data revealed an overall low fold change difference in response to hyperglycemia and not much of a deleterious effect (paper V). The time of
exposure was most likely too short (48 h) for fully induced “glucotoxicity” [171, 278]. As compared to the work of Eizirik and his group, we used lower concentrations of IL-1β in our experiments (0.13 U/ml versus 10-50 U/ml) and also experienced a less prominent IL-1β response based on the gene pattern. We worked with recombinant porcine cytokines and the concentrations we used were based on previous experiences with adult porcine islets [202]. We could clearly identify genes defined as IFN-γ-induced in previous studies [165, 167] and could also trace the effect of IL-1β and TNF-α. STAT-1 and NFκB transcription factors have been described as key players in apoptosis caused by cytokines in β cells [163]. Several genes connected to these two pathways were up-regulated in our model when we used a cutoff of p<0.01 (Fig. 23). In summary, our results were overall consistent with previous findings of cytokine exposure (Eizirik and coworkers, 2000-2005; INS-1, rodent islets, human islets) and significant changes in gene expression patterns coincided with or preceded physiological changes.

The future is now! Over the years, new technology has evolved for multiple gene expression analysis, partly leaving microarray technology behind. The next generation sequencing (NGS) technology today provides affordable whole transcriptome analysis with considerably higher sensitivity and specificity [279]. Avoiding hybridization to arrays significantly reduces assay variation. Eizirik et al recently presented a study using RNA sequencing (RNA-seq) technology to identify transcripts (including splice variants), expressed in human islets in response to inflammatory cytokines [280]. Proteomics has also evolved to offer techniques for multiplex protein analysis (paper II). Considering the translational regulation of protein expression, measuring protein concentrations in addition to mRNA expression seems attractive [281, 282].
Conclusions

- A novel whole-blood model for long-term incubations was developed. This model is useful for prolonged studies of the immune response to cellular transplants, with application also to other fields (papers I-II).

- Intragraft gene expression analysis of liver tissue bearing islet grafts is a useful tool for exploring immune mechanisms in the NHP model of intraportal islet transplantation (papers III-IV).

- The kinetics of coagulation/complement activation, cytokine/chemo-kine production and cellular recruitment in response to human islets and exocrine tissue was described for up to 48 h in an allotransplant whole-blood model. Increased production of chemokines targeting the CXCR1/2, CCR2 and CXCR3 receptors was observed, as well as high levels of IL-6. Immunohistochemistry revealed a massive infiltration of neutrophils over the 48 h, whereas monocytes/macrophages were found infiltrating the clotted graft tissue in only few to moderate numbers (paper II).

- Exocrine/acinar tissue triggered an innate immune response of similar magnitude as an equal volume of human islets in ABO-compatible blood (paper II).

- Gene expression analysis of liver tissue with islet xenografts identified up-regulation of chemokine mRNA (mainly CXCR3, with ligands IP-10 and Mig, together with MIP-1α) and cytotoxic effector molecule transcripts within 48-72 h post-transplant in non-immunosuppressed NHPs. These findings were associated with histological observations of T-cell and macrophage infiltration at 48-72 h (paper III).

- Long-term survival (>100 days) of adult porcine islets in the NHP model was demonstrated using a T-cell-based immunosuppression protocol including co-stimulatory blockade (anti-CD154 mAb). Severe side effects included thromboembolic events. Graft rejection was associated with increased levels of circulating, indirectly activated T
cells and non-Gal pig-specific IgG antibodies. Treatment with co-stimulatory blockade was associated with suppression of IL-1β mRNA and lower levels of cytotoxic effector molecule transcripts (paper IV).

- Microarray analysis of the response to inflammatory cytokines (IL-1β, TNF-α and IFN-γ) in cultured porcine islets over 48 h identified groups of genes involved in cell death, immune response, stress response and oxidative stress. Genes in the JAK-STAT pathway were up-regulated (paper V).

- Microarray analysis of the response to hyperglycemia resulted in up-regulation of genes involved in metabolic processes. The gene thioredoxin-interacting protein was increased as a sign of oxidative stress (paper V).

- Changes in gene expression, in response to cytokine stress and hyperglycemia, coincided with or proceeded physiological changes in cultured porcine islets (decrease in insulin and ATP content; paper V).
Future perspectives

I here provide my summary of the expansive field of β cell replacement, together with some ideas for upcoming studies.

*The ultimate cure*

The ultimate cure for diabetes through β cell replacement would require an *endless source of insulin-producing cells* and *no need for immunosuppression*. If we could meet these two requirements the patient population eligible for treatment would grow substantially, illustrating the need and potential within this research field (Fig. 28). Xenotransplantation and β cell regeneration (differentiation of insulin-producing cells from progenitor cells) have been two competing directions in the race to find the “endless” source of insulin-producing cells. “No need for immunosuppression” would demand complete tolerance to the graft or shielding from the recipient’s immune system.

*Figure 28.* Patient population eligible today and for future β cell replacement therapies. Modified from Matsumoto et al.; copyright © 2010 Ruijin Hospital, Shanghai Jiaotong University School of Medicine and Blackwell Publishing Asia Pty Ltd.
The endless source

Xenogeneic islets

Our evaluation of long-term survival (>6 months) of intraportally transplanted porcine islets in NHPs was a-proof-of concept study; however, it demanded heavy immunosuppression not justifiable in clinical practice (paper IV). The rationale behind using porcine islets has been previously discussed (see Introduction). Despite the species barrier, porcine islets can offer potential immunological advantages. Pig islets are less prone to recurrent autoimmunity, and there is the possibility of pre-treating the donor with known donor antigens (to induce hypo-responsiveness) and the ability to create genetic modifications [283]. The hunt for the perfect donor pig is ongoing [284]! A diverse set of genetically modified pigs has been developed over the last 10 years to protect the islets from immune activation. The first α-galactosyltransferase knockout (Gal-KO) pig was cloned in 2003 and has been followed by generations of pigs with islets expressing different immune protection genes, such as human complement regulatory proteins (hCRPs, e.g., CD46) and human cytotoxic T-lymphocyte antigen 4 (CTLA4) Ig [285] [124]. The Pittsburgh team has reported ongoing studies in NHPs involving transplanting islets with a combination of these traits [124]. Thus far, Gal-KO and the Gal-KO/hCD46 combination have showed the most beneficial effects [122, 259].

β cell engineering

The ultimate source of β cells would theoretically be engineered insulin-producing cells derived from the patient’s own cells. This ideal situation would, in immunological terms, mean an autotransplantation with no need for immunosuppression; however, there would remain a risk of autoimmunity. Intense work on the differentiation of stem cells of embryonic or adult origin into stable insulin-producing cells is in progress [286, 287]. Even though it might sound straightforward this is a somewhat “science fiction”-like task, recreating nature in a Petri dish. Pluripotent cells are pushed through the stages of embryonic pancreatic development. The production of cells capable of synthesizing all five pancreatic hormones from human embryonic stem cells (hESC) has been achieved [288, 289]. However, these cells lack the ability of glucose-stimulated insulin secretion in vitro. Interestingly, several months after their transplantation into immunedeficient mice, the hESCs started to secrete insulin. This suggests the need of in vivo factors for final differentiation [288, 290]. Also, induced pluripotent stem cells (iPS), derived from adult cells (e.g., fibroblasts), have been differentiated into insulin-producing cells [291]. A concern is how to define a successful differentiation: A cell expressing insulin but unable to store and secrete it, is that a true β cell? [292]. In addition to low cell numbers and uncertain function in vivo, a major concern is the risk of abnormal cell growth [286]. ESCs have demonstrated a high risk of teratoma formation [293]. Genetic manipulation used to generate iPS comes along
with potentially carcinogenic traits [293]. There are numerous of challenges related to the transplantation of engineered cells. How do β cells perform without the other endocrine neighbors in the islet cluster? Will they be able to develop the vascular support they need [294]?

**Tolerance or immunoisolation**

**Inducing tolerance or avoiding non-tolerance**

Can we dupe the immune system, or shall we hide the islets? To create selective immune tolerance, making the recipient capable of accepting the transplanted graft without immunosuppression, is the ultimate goal of the field of transplantation. Operational tolerance (ad hoc stable graft function after immunosuppressive treatment has ended) is occasionally experienced in transplanted patients, establishing the existence of immune tolerance [295]. Even though the mechanisms behind immune tolerance are far from understood, there are situations in the CD4+ T cells’ life that are known to induce tolerance: negative central/peripheral selection, a lack of co-stimulation after MHC-peptide signal and suppression when encountering a regulatory immune cell (e.g., Tregs) [296]. Mixed chimerism, when the recipient’s hematopoetic system comprises a mixture of donor and recipient cells, is a known status of tolerance. Certain immunosuppressive drugs (e.g., anti-CD154 mAb, anti-CD3 Ab) and drug combinations have shown to induce tolerance [297]. Although several subpopulations of lymphocytes might possess suppressive functions, the best characterized and consistently suppressive T cells are the CD4+CD25+Foxp3+ T cells (the Tregs) [296]. Work on isolating and expanding this graft tolerant pool of cells for subsequent immunotherapy is ongoing [298].

The “danger theory” presented by Polly Matzinger in 1994 states that it is not only the presence of “non-self” that triggers an immune response but also the combination of “non-self” and “danger signals” [299]. Among the endogenous danger signals she mentions are CD40L and dissolution of the extracellular matrix – components highly expressed during the IBMIR. To hide the islets from the initial thrombo-inflammatory response might not only protect them from immediate complement destruction. It might also help to induce tolerance or at least attenuate the developing adaptive immune response.

**Hiding and coating islets**

Immunoisolation of the islets has been intensively investigated, including vascular perfusion devices, macro- and microencapsulation [300]. The capsule sizes, materials and implantation sites differ. The various techniques have different advantages and drawbacks that relate to the diffusion distance for oxygen, nutrients, and insulin (capsules with thicker walls); fibrosis and inflam-
mation around the capsules/device; thrombosis/bleeding (intravascular capsules); clustering of microcapsules, leaving the central portion less well-oxygenated. Microencapsulation using alginate capsules transplanted intraperitoneally has thus far been the predominate approach in animal models. Even though thinner capsules are under development, the volume of alginate capsules so far (~600-800 μm compared to an islet ~150μm) has made the graft too large for intraportal transplantation [301] [302]. In a clinical setting, the graft volume needed would also be too large for other implantation sites. Success in mice, returning to temporal normoglycemia with encapsulated allo/xenogeneic islets has occasionally also been demonstrated in NHPs [123, 300, 303]. Only a few clinical studies with microencapsulated islets have been performed, with varied results; most have resulted in transient effects on glycemic control or no effects at all [128, 304, 305]. A controversial study with porcine islets and Sertoli cells transplanted within a collagen-generating device into Mexican children is, to my knowledge, the most successful clinical attempt published using an “artificial pancreas,” resulting in periods of insulin independence (two patients) and highly reduced insulin doses [126].

In addition to traditional encapsulation, cell-surface coating of islets is an intriguing research direction. The coating could serve both as protection from (at least) the initial immune response and also enable us to “dress” the islets with suitable proteins to inhibit coagulation/complement activation. Two examples of coating are the biotin/avidin binding of heparin complexes [306] and polyethylene glycol (PEG)-conjugated phospholipid [307], both of which have demonstrated protective features in in vivo transplant models [307, 308]. These coatings have been further bound with active proteins (e.g., heparin to vascular growth factor; PEG-lipid to factor H-binding peptide) to help engraftment and inhibit initial destruction [309, 310]. An issue with smaller capsules and coatings is the left-over artificial material in the body after the islets have lost their function.

**Cells helping cells**

Transplantation of islets, together with or coated by other cell types (such as endothelial cells and/or MSCs) is another approach that has been investigated [311-313]. The rationale behind composite grafts is to enhance compatibility with the new environment (blood or other site) and to enhance revascularization and promote immunogenicity. Co-transplantation together with Sertoli cells, which have been shown to have immunoregulatory activity, has also been tested [314]. Composite grafts are larger and not possible to transplant intraportally. This demands finding alternative transplantation sites and ways to disperse the graft cells for optimal oxygenation and engraftment.
IBMIR modulation

Improved immunomodulation is necessary to bring clinical islet allotransplantation forward. In addition to achieving steroid-free maintenance protocols [49], we also need to taper down the calcineurin inhibitors and find alternative drugs/approaches for tolerance. I will not go further into the upcoming alternatives regarding T-cell modulating drugs and maintenance treatment but will instead focus on approaches for IBMIR modulation. My work on characterizing the early innate immune response has emphasized the importance of early interventions, such as coagulation/complement blockade and inhibition of chemokine-mediated chemotaxis (papers II and III). I see, with our novel whole-blood model, the possibility to evaluate thrombo-inflammation, IBMIR, for up to 48 h (maybe even further) and to intervene by different approaches. I believe a combination of drugs or approaches will be needed to inhibit the highly cross-talking coagulation, complement and innate immune systems. To the measurement of cellular infiltration, coagulation/complement activation and cytokine/chemokine expression, I would add measurements of islet viability/destruction (e.g. C peptide release).
Drugs for systemic administration:
There are several new drugs used in clinical practice for platelet- and coagulation inhibition (e.g., P2Y12 inhibitors and factor X inhibitors) that would be of interest to evaluate in the context of the IBMIR.

Platelets: P2Y12 inhibitors (e.g. ticagrelor, prasugrel). Abciximab, binding GPIIb/IIIa and inhibiting platelet aggregation. Anti-CD40mAb (see Discussion).

Coagulation: Low molecular weight dextran sulfate (LMW-DS) has been demonstrated to inhibit both coagulation and complement activation in the IBMIR; a clinical study is ongoing [315]. Factor Xa inhibitors (e.g. apixaban, rivaroxaban, fondaparinux) could be alternatives.

Complement inhibition: Eculizimab (Soliris®), a mAb inhibiting the cleavage of C5 and preventing the generation of C5b-9.

Chemokine receptor blockade: CXCR1/2: Reparaxin, a dual CXCR1/2-antagonistic drug. IL-8 is known to be involved in angiogenesis and one could speculate that administration beyond an induction period could impair engraftment [316]. To my knowledge, there are no clinical marketed drugs for inhibition of CCR2 and CXCR3 with ligands. AMG487 (CXCR3 antagonist) has been evaluated in animals.

IL-6: Pre-culturing islets with tocilizumab (anti-IL6 rec mAb) has shown beneficial effects in experimental models of islet transplantation [317] and the drug is currently being evaluated in clinical islet transplantation.

Pre-treating islets in culture:
NFkB inhibitors (withaferin and CAY10512) have shown protective effects in a blood-model of autolog islet transplantation up to 6 h [178]. It would be interesting to repeat and extend these experiments in an allogeneic setting.

Coatings
PEGylation of islets has opened up the possibility of binding different proteins to the islet surface [310]. Binding of complement inhibitors (e.g., CD46) or CD39 (NTPDase-1; an enzyme degrading ADP) to the islet surface would potentially provide an anti-complement/coagulant acting locally in the islet surroundings. To evaluate these coated islets over 2-3 days in ABO-compatible blood using my whole-blood model would be an intriguing project.
**My take on the future**

When asking fellow researchers which approach for β cell replacement they think will ultimately succeed, I get a variety of answers. I believe in keeping a broad research front and collaboration. A combination of different approaches may prove to be the final solution (Fig. 29). Different approaches may also be beneficial for different patient groups. In such an innovative field, it is important to keep the diabetic patient in focus. We must never neglect that the treatment must be safe and beneficial for our patients.

Transplanting cells in a device provides the possibility of removing the graft. In the case of engineered insulin-producing cells with the potential risk of teratoma formation, this ability is crucial. Compared to stem-cell derived cells, porcine islets are already fully functional, insulin-producing cells. However, with porcine islets comes the problem with the species barrier. Even if transgenic approaches can decrease the immunogenicity, the porcine cells include an endless source of antigens that are slightly different from the corresponding human antigens. Also, for porcine islets, a working device or encapsulation method would potentially solve many issues. The unsolved problem with larger devices is obtaining adequate oxygenation of the graft, and the large diffusion distance for glucose and insulin. There are more science-fiction-like approaches coming along with genetic manipulation. With the technique of blastocyst complementation it is possible today to grow a pancreas, within a large animal, derived from blastomeres of another individual (another species?) [318]. A pig factory for growing human organs? Obviously there are many ethical considerations surrounding these steps.

In the years to come, further development of clinical cadaver-islet allotransplantation is an important goal. This development would potentially also benefit other directions within the field. To this end, this thesis has emphasized protection of the islets from the early thrombo-inflammatory, innate destruction.

In general trying to *play with nature* instead of trying to *change nature* appeals to me. Here the ways to induce tolerance to the transplanted graft is attractive. If we can understand the mechanisms behind tolerance induction, we can play with the immune system and make it accept the newly transplanted cells.
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“No man is an island entire of itself; 
every man is a piece of the continent, a part of the main; 
if a clod be washed away by the sea, 
Europe is the less, 
as well as if a promontory were, 
as well as any manner of thy friends or of thine own were; 
any man's death diminishes me, 
because I am involved in mankind. 
And therefore never send to know for whom the bell tolls; 
it tolls for thee.”

"Ingen människa är en ö, hel och fullständig i sig själv; 
varje människa är ett stycke av fastlandet, en del av det hela. 
Om en jordklump sköljs bort av havet, 
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_John Donne (1572-1631)_
References


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