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TARGETING INNATE AND ADAPTIVE IMMUNE RESPONSES TO ACHIEVE LONG-TERM ALLOGRAFT ACCEPTANCE FOLLOWING TRANSPLANTATION

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Targeting innate and adaptive immune responses to achieve long-term allograft acceptance following transplantation
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my mother

and my beloved Johanna

POPULAR SCIENCE SUMMARY OF THE THESIS

Organ transplantation is regarded as one of the most successful advances in medicine over the last century and is now regarded as standard treatment for organ failure. Heart, lung or liver transplantation are life-saving measures, while kidney transplantation increases life expectancy and quality of life in patients with renal failure. Cell transplantation has also been implemented, for instance, pancreatic islet transplantation, which is a way of treating type 1 diabetes by infusing insulin-producing tissue from a deceased donor into the recipient's liver. Islet transplantation can improve the quality of life of type-1 diabetic patients with blood sugar that is difficult to control. Most organs used for transplantation are from non-related deceased donors and medication is needed to suppress the recipient's immune system to prevent it from rejecting the transplanted organ. Current medication to suppress the immune system cannot fully protect transplanted organs from initial and long-term damage from the recipient's immune system. It may also cause side effects such as kidney toxicity, heart and vascular disease and an increased risk of cancer. Today, life-long medication is needed to prevent rejection and the risk of side effects increases the longer a patient is treated with such medication.

The immune system is divided into parts: a general part called the innate immune system that is inherent and non-specific, and another part called the adaptive immune system that is directed at specific foreign tissue. Moreover, there are parts of the immune system that modulate the response and accept specific foreign agents, called *regulatory cells*. Regulatory cells may enable tolerance of foreign proteins, for example, cells or organs from non-related individuals.

If we could develop novel methods or medication that could reduce the initial damage caused by the innate immune system and use regulatory cells to accept the proteins of a transplanted organ, for example, make the immune system tolerate the transplanted organ, it may allow better organ function and the possibility to reduce, or in the best case, abolish the requirement of immunosuppressive drugs for the transplanted patient.

In the field of pancreatic islet transplantation, it is known that a lot of islets are lost directly after transplantation through the effects of the innate immune system. In the first part of the thesis, we evaluated the effects of a potential new drug called cibinetide on pancreatic islet transplantation using preclinical models. The drug reduced the damage inflicted on transplanted islets by the innate immune system and subsequently reduced/delayed the effect of the adaptive immune system. This led to improved and prolonged function of the transplanted islets.

We then evaluated the safety of administering regulatory cells to the transplant recipient together with pancreatic islets from deceased donors in five patients. This way of delivering regulatory cells was shown to be safe and provides opportunities to evaluate other regulatory cell products or stem cells directly to the transplantation site.

Encouraging results in terms of tolerance induction has recently emerged in the field of living donor transplantation. However, most organs used for transplantation in the West are from deceased donors. In the final part of this thesis, we conducted preparatory studies for clinical tolerance induction trials in transplantation when organs from deceased donors are used. In these future studies we hope to be able to reduce or even eliminate the need for life-long immunosuppression for these patients. Here, we evaluated the efficacy of a tolerance-inducing cell therapy using clinically approved reagents in a preclinical setting. This cell product is based on a previously evaluated product that has shown promise in living donor liver transplantation but includes reagents that are not approved in Sweden. We also compared different methods of obtaining white blood cells from deceased donors in parallel with organ procurement to be used in creating regulatory cells that are specifically programmed to instruct the transplanted patient's immune system to accept donor organs.

In summary, the studies included in this thesis address the need to modulate both the innate and adaptive immune systems in order to improve results after transplantation and prepare these concepts for clinical implementation.

ABSTRACT

Organ transplantation outcomes have gradually improved over the last 50 years.
The development of more effective immunosuppressive drugs has been a major contributor to this improvement. However, current immunosuppressive regimens may cause significant side effects such as kidney failure, opportunistic infections, cardiovascular morbidity and increase the risk of tumor formation. These side effects cause increased mortality, morbidity and reduce the patient's quality of life, which obscures the long-term outcomes following transplantation. As an example, patient survival after liver transplantation has improved significantly since the 1980s, but this is mainly due to a lower mortality rate in the first year. Thus, one-year censored patient survival has not improved, which is thought to be at least partially attributable side effects of chronic immunosuppression. In addition, current immunosuppressive protocols still do not optimally protect transplanted organ grafts from the recipient's immune responses.

When a graft is transplanted, immediate non-specific innate immune reactions occur. These reactions not only could result in direct damage to the graft, but also sequentially potentiate the activation of adaptive immunity that could result in subsequent rejection. Innate reactions are particularly devastating in the field of cell transplantation, for example, pancreatic islet transplantation. When cells are transplanted into the bloodstream, an *instant blood-mediated inflammatory reaction* (IBMIR) occurs, which includes severe inflammation, platelet and leukocyte infiltration, resulting in thrombus formation around the grafts. Such reactions can result in more than half of the transplanted grafts being lost. Despite the need to mitigate these reactions, they are not fully accounted for in the current arsenal of immunosuppression.

Primary concerns about the adaptive immunity relate to its rejection activity. However, the adaptive immune system also contains a regulatory part that may promote acceptance and tolerance of specific antigens. If these regulatory properties could be utilized to the benefit of transplanted grafts, it may be possible to minimize or, in the best case, even abolish the requirement for life-long immunosuppressive treatment. Regulatory cell therapies have recently been evaluated in pilot clinical trials and have enabled immunosuppression dose reduction in living donor kidney recipients and the possibility of complete weaning of immunosuppression in living donor liver transplantation. Further development of these protocols, including the possibility to apply them in deceased donor transplantation setting is desired.

This thesis has investigated novel immune modulating transplantation protocols to target innate immunity and harness the potentially beneficial regulatory part of the adaptive immune system.

Paper I investigated the efficacy of the non-hematopoietic erythropoietin analogue cibinetide in an mouse allogenic intra-portal pancreatic islet transplantation model. We showed that induction therapy using cibinetide could protect islet grafts from initial damage from the innate immunity, reducing activation of adaptive immunity and significantly prolonging long-

term graft survival. As a step towards clinical implementation, **Paper II** focused on evaluating the protective effect of cibinetide on human islets. The results showed that cibinetide maintained the robustness and function of human islets in a pro-inflammatory environment and that cibinetide tends to mitigate IBMIR related platelet consumption. The results of the first two papers suggests that cibinetide has the potential to abrogate innate immune responses and thereby improve the short and long-term results of pancreatic islet transplantation.

In **Paper III** we performed a safety study of intra-portal co-infusion of autologous regulatory T cells and allogenic pancreatic islets. We concluded that it was safe to deliver regulatory cells together with islets.

The latter part of this thesis focused on preparatory studies using Good Manufacturing Practice (GMP) compatible reagents and methods, applying regulatory cell therapy to recipients of deceased donor liver transplantation to minimize, or in the best case, abolish the requirement for immunosuppression. In **Paper IV**, we evaluated whether the clinically approved drug Cytotoxic T-lymphocyte-associated protein-4-Ig (CTLA-4-Ig, Belatacept) could be used instead of the non-approved and previously used mouse monoclonal antibodies against human CD80 and CD86 (2D10.4/IT2.2) as a costimulatory blockade in the generation of a donor-specific immunomodulatory cell product. We conclude that CTLA-4-Ig or 2D10.4/IT2.2 could be used to produce a cell product with similar cell composition and donor-specific immunomodulatory efficacy. CTLA-4-Ig, which is a clinically approved drug, is therefore preferable to use to produce donor-specific immunomodulatory cells in a GMP-compatible tolerance induction protocol. In **Paper V**, we evaluated three methods of procuring peripheral blood mononuclear cells from deceased donors to be used as donor antigen stimulator in the production of donor-specific immunomodulatory cells. We showed that all the methods we evaluated were feasible. However, bedside leukapheresis before an organ donation procedure had tendency of higher cell numbers procured and allows the procurement of mononuclear cells in a sterile and controlled manner, compared to the other methods.

LIST OF SCIENTIFIC PAPERS

I. **Yao M**, Watanabe M, Sun S, Tokodai K, Cerami A, Brines M, Östenson CG, Ericzon BG, Lundgren T, Kumagai-Braesch M. Improvement of Islet Allograft Function Using Cibinetide, an Innate Repair Receptor Ligand. Transplantation. 2020 Oct;104(10):2048-2058.
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II. **Yao M**, Domogatskaya A, Ågren N, Watanabe M, Tokodai K, Brines M, Cerami A, Ericzon BG, Kumagai-Braesch M, Lundgren T. Cibinetide Protects Isolated Human Islets in a Stressful Environment and Improves Engraftment in the Perspective of Intra Portal Islet Transplantation.
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III. Bergström M, **Yao M**, Müller M, Korsgren O, von Zur-Mühlen B, Lundgren T. Autologous regulatory T cells in clinical intraportal allogenic pancreatic islet transplantation.
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IV. Watanabe M, Kumagai-Braesch M, **Yao M**, Thunberg S, Berglund D, Sellberg F, Jorns C, Enoksson SL, Henriksson J, Lundgren T, Uhlin M, Berglund E, Ericzon BG. Ex Vivo Generation of Donor Antigen-Specific Immunomodulatory Cells: A Comparison Study of Anti-CD80/86 mAbs and CTLA4-lg Costimulatory Blockade. Cell Transplant. 2018 Nov;27(11):1692-1704.
PMID: 30261751

V. **Yao M**, Henriksson J, Fahlander H, Guisti Coitinho P, Lundgren T, Ågren N, Ericzon BG, Kumagai-Braesch M. Evaluation of methods to obtain peripheral blood mononuclear cells from deceased donors adjacent to organ procurement for tolerance-induction protocols. *Manuscript*

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cells
ATMP	Advanced therapy medicinal products
ATP	Adenosine triphosphate
BCL	Blood procurement during cold perfusion of the organ donor followed by leukapheresis of the procured blood
BCS	Blood collection before cold perfusion and isolation of PBMC using SEPAX
BM	Bone marrow
BMDC	Bone marrow derived dendritic cells
B-reg	Regulatory B-cells
CD	Cluster of differentiation
CKD	Chronic kidney disease
CMRL	Connaught medical research labs
CNI	Calcineurin inhibitor
CTLA	Cytotoxic T-lymphocyte-associated protein
CXCL1	The chemokine (C-X-C motif) ligand 1
CXCR1	C-X-C Motif Chemokine Receptor 1
CXCR2	C-X-C Motif Chemokine Receptor 2
DAMPS	Damage-associated molecular patterns
DC	Dendritic cells
DCreg	Regulatory dendritic cell
DSIMC	Donor-specific immunomodulatory cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPO	Erythropoietin
EPOR	Eythropoietin receptor
FACS	Fluorescence-activated cell sorting
FOXP3	Forkhead box protein P3
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Good manufacturing practice

GvHD	Graft versus host diease
Gy	Gray
HBSS	Hanks balanced salt solution
HLA	human leukocyte antigen
HSC	Hematopoietic stem cell
HSCOT	Hematopoietic stem cell transplantation in parallel to organ transplantation
IBMIR	Instant blood-mediated inflammatory reaction
IFN-γ	Interferon-γ
IL	Interleukin
IP	Intra-peritoneal
IRR	Innate repair receptor
IS	Immunosuppression
LEU	Bedside leukapheresis of the donor prior to organ procurement
LMWDS	Low molecular weight dextran sulfate
LPS	Lipopolysaccharides
MACS	Magnetic activated cell sorting
MCP-I	Monocyte chemoattractant protein-I
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein-1β
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
MMTT	Mixed meal tolerance test
mTOR	Mammalian target of rapamycin
NHP	Non-human primate
NK-cells	Natural killer cells
NODAT	New-onset diabetes after transplantation
OT	Operational tolerance
PAMPS	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PITx	Pancreatic islet transplantation
POD	Post operative day
PVC	Poly vinyl chloride
RBC	Red blood cells
ROS	Reactive oxygen species
RPMI 1640	Roswell park memorial institute 1640 medium
RT	Room temperature
SOT	Solid organ transplantation
TCR	T-cell receptor
TGF-β	Transforming growth factor- β
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T-cells

1 INTRODUCTION

1.1 IMPROVEMENT OF ORGAN TRANSPLANTION

Solid organ transplantation (SOT) has revolutionized the treatment of end-stage organ failure. Heart, lung, or liver transplantation is regarded as a directly life-saving measure. Even though a patient with end-stage kidney failure can survive on dialysis treatment, it has repeatedly been shown that a successful kidney transplant not only result in reduced morbidity and socio-economic costs, but also increased patient survival (1, 2). The field of transplantation has also developed pancreas transplantation and pancreatic islet transplantation (PITx) as treatments for type-1 diabetes (3). Procedures to increase the quality of life such as face transplantation and the transplantation of lost limbs, also known as composite transplantations, are currently being evaluated (4-6).

Over the last five decades, the outcomes of transplantation have significantly improved (7, 8). Although the development of more effective immunosuppressive agents has contributed to these improved outcomes, they still do not adequately provide optimal graft acceptance and may cause severe side effects for organ recipients.

1.2 IMMUNE RESPONSE TO ALLOGRAFTS

Immediately after an organ is transplanted, the innate immunity recognizes danger-associated molecular patterns (DAMPS) via pattern recognition receptors, which results in severe innate reactions including infiltration of granulocytes and the release of pro-inflammatory cytokines and chemokines (9, 10). During the pro-inflammatory innate reactions, the maturation of antigen-presenting cells (APC), mainly dendritic cells (DC), occurs. The maturation and activation of APCs that leads to antigen presentation, is the link between the innate and adaptive immune responses (11). Consequently, the early innate reactions also increase the risk of subsequent allograft rejection (10, 12). The innate reactions are particularly devastating in the field of cell transplantation, for example PITx, due to a small graft volume and the lack of vascularization. Pancreatic islets express tissue factor, which activates the coagulation cascade via the extrinsic pathway (13) and results in the formation of thrombus and leukocytes that encapsulate and destroy many of the transplanted islets. This reaction is called the *instant blood-mediated inflammatory reaction* (IBMIR) and occurs within minutes after transplantation (14, 15). Imaging studies of PITx indicate that innate reactions, including IBMIR, can cause the immediate destruction of 50% of the transplanted graft (16, 17).

1.3 IMMUNOSUPPRESSIVE TREATMENT AND SIDE EFFECTS

The immunosuppression (IS) used during the early years of allogenic kidney transplantation consisted of cortisone and azathioprine and had variable results (18-20). After calcineurin inhibitors (CNI) were introduced in the 1980s, graft survival dramatically improved (21-23). More improvements were made in recent decades with the introduction of effective combinations of CNIs and antimetabolites to allow dose reduction with an acceptable rate of rejection (24-26).

The main disadvantage of current IS in the long term is their significant side effects, such as cardiovascular morbidity (27-30), nephrotoxicity and the increased risk of malignancy (31-34). Their non-specific suppression of the immune system also increases the risk of opportunistic infections (35, 36). The treatment of IS-related side effects rather than the function of the graft can obscure the follow-up of transplant recipients. Despite this, current IS lacks optimal coverage of the damage caused by initial innate responses and a significant number of transplant recipients suffer graft loss due to chronic rejection (37, 38).

1.4 TOLERANCE INDUCTION STRATEGIES

The adaptive immune system contains regulatory parts that antagonize the effector part that causes rejection (39). The phenomenon of tolerance (acceptance of an organ graft without any external administration of IS) of a non-related transplanted organ has been sought for since the beginning of modern transplantation. Deliberate weaning and/or cessation of IS has been attempted and has even shown some success in liver recipients (40-42). It is more successful long after transplantation when the morbidity of side effects has already occurred. It is also difficult to predict which patients may be successfully weaned off IS.

The use of donor-specific hematopoietic stem cell (HSC) transplantation in parallel to organ transplantation (HSCOT) to induce transient or permanent donor chimerism has been performed in the USA and South Korea and has shown successful results in living donor kidney transplantation settings (43-45). Since a strenuous partial myeloablative conditioning regimen is needed as a minimum to allow the engraftment of donor HSC, these strategies may not be suitable for the many severely ill recipients on the waiting list (46). Moreover, protocols that use HSCOT have not shown success in complete IS withdrawal in liver transplant recipients.

In recent decades, regulatory T-cells (Treg) have been extensively studied and are believed to be the main cell line to mediate tolerogenic effects (47-51). Regulatory cell therapies have recently been evaluated in clinical trials and have enabled IS dose reduction in living donor kidney recipients (52, 53). A clinical pilot study from Hokkaido University Hospital in Japan demonstrated the possibility of total withdrawal of IS in seven out of ten living donor liver transplantation recipients (54). In that study, regulatory cells were generated using recipient peripheral blood mononuclear cells (PBMCs) stimulated with irradiated donor PBMCs in the presence of costimulatory blockade, anti-CD80 and CD86 antibodies (mouse antibodies). Based on these encouraging results, our transplant center initiated the development of a regulatory cell tolerance induction protocol suitable for deceased donor liver transplantation. However, there are several challenges. The costimulatory blockade, CD80 and CD86 antibodies (also named 2D10.4/IT2.2) used in the study performed at Hokkaido University are not clinically approved drugs in Europe and the fact that the procurement of start and raw material for cell production must be performed in non-elective settings (due to deceased donors) is an obstacle that must be overcomed.

Identifying novel ways of reducing the impact of innate immune responses immediately after transplantation and developing protocols to minimize or, in the best case, abolish the requirement of chronic IS after transplantation may be one of the most significant research directions for transplant recipients. This thesis evaluated the benefits of a novel potential drug to inhibit innate immunity/inflammation and conducted preparatory studies for a clinical immunosuppression minimizing study using donor-specific immunomodulatory cells.

2 LITERATURE OVERVIEW

2.1 HISTORICAL DEVELOPMENT OF TRANSPLANT IMMUNOLOGY

Since Alexis Carrel developed the technique of vascular anastomosis, the surgical aspect of transplantation became a clinical reality (55). During the first part of the 20th century, knowledge of transplantation immunology was sparse. Sir Peter Medawar provided the basis for transplant immunology during his service for Britain during World War II when he discovered that the rejection of a foreign skin graft was an immunological reaction (56, 57). Later, Owen discovered the presence of blood chimerism in dizygotic twin freemartin calves that had shared blood circulation in the uterus (58). Jean Dausset's experiments on blood agglutination by anti-leukocyte antibodies, Georges Snell's extensive studies of histocompatibility (59, 60), and the contribution of many more immunological pioneers led to the knowledge of major histocompatibility complex (MHC), also called human leucocyte antigens (HLA) in human setting and the current understanding of transplant immunology.

2.2 THE PATHWAY TO REJECTION

2.2.1 Innate immunity

The innate immune system in humans is the first line of defense against pathogen invasion and has its origins in the early stages of human evolution. It functions without any need for priming. This part of the immune system mainly comprises granulocytes, dendritic cells, macrophages, and partially includes natural killer cells (NK cells), the complement system and coagulation. They detect either pathogen-associated molecular patterns (PAMPS), for example, from bacteria, or DAMPS (patterns that form during cell damage) (61). Activation of these leads to the secretion of pro-inflammatory cytokines and chemokines that attract other immune cells to the location that causes inflammation. These cytokines also affect, for instance, DCs by promoting their maturation and antigen presentation capabilities. When an organ graft is transplanted into another individual, inflammation occurs, activating the innate immunity (10). Deceased donor organs are cut off from the blood supply in cold storage and are subjected to ischemic injury. Depletion of adenosine triphosphate (ATP) and subsequent accumulation of reactive oxygen species (ROS) leads to cell death (62, 63). The surgical trauma and these dying cells release DAMPS that promote severe inflammation in the tissue and activate macrophages and APCs. Monocytes and macrophages (granulocytes, endothelial cells, damaged tissue are also important sources) secrete proinflammatory cytokines, that signal DCs to become inflammatory and upregulate their expression of antigen-presenting molecules. The upregulation of CD80 and CD86 molecules of the B7 family on DCs promotes the activation of naïve T cells through signaling via the CD28 receptor on T-cells (61). DCs migrate to the secondary lymphoid organs to interact with T-cells, which initiates the adaptive immune response (10, 64). In contrast, if the B7 family has not been upregulated by DAMPs, B7 (on APC) will favor the Cytotoxic Lymphocyte associated protein-4 (CTLA-4) with higher affinity, instead of the CD28 receptor on T-cells. This interaction impairs T cell activation (65).

2.2.2 Adaptive immunity

Adaptive immunity is the more evolved part of the immune system that can more effectively target specific antigens. It carries memory, meaning that the second time an antigen is recognized, the defenses may mobilize in a swifter and more effective way (66). Important components are T and B cells. $CD4^+$ T cells are activated by APCs presenting matching antigens on the MHC class II molecule. The de-novo activation of the adaptive immunity (mainly activation of T cells) requires three signals. First signal is activation of the T-cell receptor (TCR), The second signal of co-stimulation will determine the fate of the T-cell, while the third signal is the surrounding cytokine milieu (67). Activation of T-cells leads to colonial expansion, differentiation to cytotoxic $CD8^+$ T-lymphocytes that recognize donor MHC class-I antigens and migration to the transplanted graft. $CD8^+$T cells are also directly activated by donor antigens expressed on MHC class I of APC. $CD4^+$ T cells can also activate B cells via CD40L and CD40. These B cells may differentiate into antigen-specific antibody-producing plasma cells that afflict a humoral rejection response (66) or form memory cells. Within the immune system, regulatory parts exist that promote the acceptance of specific antigens, which will be further described in 2.6.

2.3 CURRENT IMMUNOSUPPRESSION

Even after improvement of the knowledge of transplantation immunology, it has been a struggle to develop immunosuppressive medication to effectively prevent graft rejection with acceptable side effects. Until the late 1970s, the available immunosuppression was limited to the early antimetabolite Azathioprine and corticosteroids. Graft survival was often limited (18-20) compared to today's standard and transplant physicians struggled to find new ways to improve the outcomes of transplantation. There was also an element of having to convince the public at large of its efficacy. The discovery and introduction of the CNI cyclosporine in the late 1970s became a gamechanger in the arsenal of immunosuppression, improving the one-year kidney graft survival rate from <50% to over 80% (68). Subsequently, Cyclosporine was largely replaced by the more effective CNI tacrolimus (69).

In most transplant centers, the standard maintenance immunosuppressive protocol after organ transplantation comprises a CNI (often tacrolimus), an antimetabolite drug (often mycophenolate mofetil (MMF) and corticosteroids (24, 68). Lymphocyte-depleting agents, are utilized as induction or rejection therapies (70). Alternatively, interleukin-2 (IL-2) receptor blocking agents such as basiliximab (71) can be used as induction treatment.

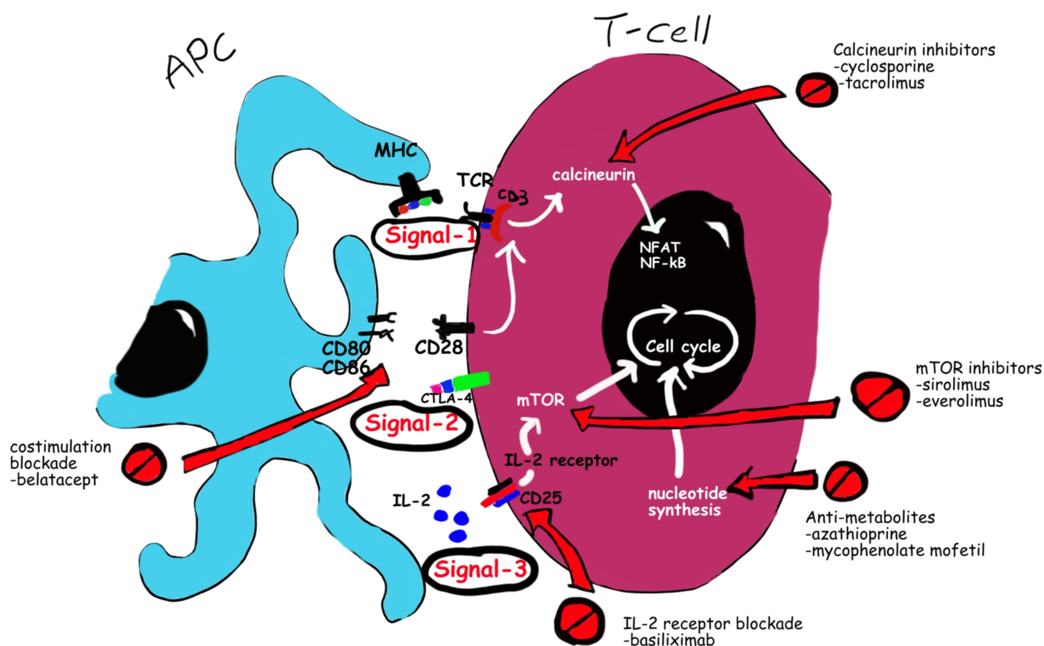

Figure 1. Mechanism of immunosuppression

2.3.1 The side effects of immunosuppression

Even though these drugs provide an acceptable level of protection for transplanted organs from immune rejection, immunosuppressive therapy has significant side-effects. Cardiovascular disease is a common cause of mortality in the general population. However, it is even more common and severe in transplant patients on immunosuppressive drugs. Following the introduction of CNIs, the incidence of hypertension in transplant recipients increased from 20% to 40–90% (72). Concordantly, hyperlipidemia occurs in 60–70% of kidney transplant recipients and 45% after liver transplantation (29). New-onset diabetes after transplantation (NODAT) is a common medical complication following immunosuppressive therapy and occurs in 5–25% of recipients after SOT (27, 73). High-dose corticosteroid treatment at induction or rejection, and beta cell toxicity of CNI are factors that contribute the development of NODAT (72, 74). This condition further promotes the development of arteriosclerosis (75) and, together with the above-mentioned negative cardiovascular side effects, increases the risk of cardiovascular events in transplant recipients.

CNIs, which are key components in the immunosuppressive arsenal, are nephrotoxic. Histologically, CNI treatment may cause vascular hyalinosis, tubular atrophy and interstitial fibrosis (31, 34). With a mean follow up of 36 months in non-renal transplant recipients, the incidence of chronic kidney disease (CKD) is around 16.5%. One third of the transplant patients with CDK progress to end-stage-renal disease and require dialysis treatment or kidney transplantation. Liver transplant recipients are particularly at risk of developing CKD with an incidence of 25% during follow up of 120 months post-transplant (32).

Since current immunosuppressive therapy is non-specific, recipients will generally become immunocompromised and more susceptible to bacterial, viral and fungal infections (36). If untreated, the cytomegalovirus is one of the most devastating opportunistic infections during immunosuppressive therapy (35) and may lead to severe illness and increase the risk of transplanted organs being rejected. The Epstein-Barr virus may cause morbidity but can also lead to the development of post-transplant lymphoproliferative disease, a serious complication in immunocompromised patients (76). In general, the malignancy risk is several times higher in transplant recipients compared to the general population. The risk increases with the higher dose of maintenance IS (77). The additional risk varies substantially between different types of cancer (33).

2.3.2 Novel immunosuppressive drugs

In recent decades, new drugs have been evaluated to replace CNIs. The randomized ORION trial evaluated whether the use of the mammalian target of rapamycin (mTOR) inhibitor sirolimus could eliminate the dependency on CNI as maintenance IS in kidney transplant recipients. However, the study showed inferior results in both the de-novo treatment group and delayed CNI switch to the sirolimus group. The sirolimus treated group had a much higher incidence of rejection and side effects such as delayed wound healing (78). The TRANSFORM study utilized the mTOR inhibitor (everolimus) with a reduced CNI dose in kidney transplant recipients and has recently been shown to have a lower incidence of viral infections and proven to be non-inferior compared to standard-of-care treatment in terms of rejection episodes at two-year follow up. The disadvantage of this protocol was its troublesome side-effect profile (79).

Another plausible CNI replacing drug is costimulation blockade with the CTLA-4-Ig, Belatacept. It was approved for kidney transplantation one decade ago and thought to be a good replacement for CNI since it lacks the nephrotoxic side effects. CTLA-4-Ig’s intermittent dosing regimen may also be beneficial for transplant recipients with compliance problems. CTLA-4-Ig has been evaluated and has shown encouraging results in clinical PITx as a replacement for beta-cell toxic CNIs (80). However, in the field of kidney transplantation, follow-up studies have shown an increased incidence of severe rejection episodes. This was suggested to be caused by CTLA-4-Igs in-effectivity against already existing memory lymphocytes and by the blocking of the immune inhibiting CTLA-4 to B7 pathway (81). Because of this, together with recent problems concerning drug manufacturing and distribution, the CTLA-4-Ig has not widely replaced previous IS regimens.

Although some of the above-mentioned drugs are still under investigation, the standard-of-care IS currently still highly CNI dependent.

2.4 GRAFT DESTRUCTION BY INNATE IMMUNITY

Another drawback of the current arsenal of IS the lack of focus on the innate immunity. Tissue damage caused by the transplant procedure induce severe inflammatory responses, which activate macrophages and dendritic cells. These immunological pathways lead to the

activation of adaptive immunity and subsequent rejection. This aspect is being increasingly recognized (10).

2.4.1 The innate immune systems effect in pancreatic islet transplantation

The effects of the innate immunity are of special concern during cell transplantation (mainly PITx and hepatocyte transplantation). The standard method of PITx is through infusion via the portal vein to the liver (3). Even though PITx offers a minimal invasive method with the promise of reducing hypoglycemic events or even insulin independence, the procedure has immunological drawbacks. Isolated islets of Langerhans are unprotected and will come into direct contact with the recipient's blood and innate immune cells. The low blood flow in the portal vein and the clots created also induce optimal conditions for cell-to-cell contact (14). When islets are embolized into the liver, tissue factor expression on the islets activates the coagulation and complement cascade, resulting in severe inflammation at the transplantation site, causing tissue injury and the production of pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-12 (82, 83). Macrophages secrete macrophage inflammatory protein-1β (MIP-1β) and monocyte chemoattractant protein-I (MCP-I), which potentiate a devastating inflammatory reaction to the islet grafts (84). The activation of the coagulation pathway and p-selectin promotes thrombocyte activation and leads to clot formation around the islet grafts, also called IBMIR (14). Studies using positron emission tomography in both preclinical large animal models and humans reveal that more than half of the transplanted islets may be destroyed immediately after transplantation (16, 17, 85, 86). This graft loss is believed to be caused by the above-mentioned innate immune reactions. This is a major obstacle since islets that constitute only 1% (around 1g) of the total pancreatic mass have already been affected by the isolation procedure and are placed in an environment with problematic initial abilities to pick up oxygen and nutrients. The remaining islets that engraft may also be rejected, and/or being impinged by beta cell toxicity caused by maintenance IS (for example CNI) (74).

Insulin independence following islet transplantation gradually decreases following PITx and is reported to be at 10–40% five years after transplantation (87). Islet recipients often require multiple re-transplantations to achieve and maintain an insulin-independent state. Given the shortage of pancreas grafts for islet isolation and the risk of immunizing the recipient via multiple islet transplant procedures, novel strategies are required to decrease the initial islet loss caused by innate immune reactions. In order to achieve long-lasting graft survival, there is also a need to avoid threat of the adaptive immunity and the beta cell toxicity caused by the current IS arsenal.

2.5 NOVEL THERAPEUTICS TO REDUCE INNATE IMMUNE RESPONSES IN PANCREATIC ISLET TRANSPLANTATION

Several additional induction agents have recently been evaluated with the aim of mitigating early innate immune reactions.

2.5.1 Anti-inflammatory and anti-coagulant treatment

A perioperative TNF-α blocker has been adopted by most islet transplant centers (88). However, the evidence for its use was a single preclinical syngeneic mouse model (89) and lacks the supporting controlled efficacy trials.

In 2011, Matsumoto et al. attempted to use the IL-1 receptor antagonist anakinra in combination with TNF-α blocker in a controlled study with three patients in each study arm. Only one patient in the control group became insulin independent, compared to two in the intervention group (90). The use of anakinra has since been evaluated in a preclinical "human islet to immune deficient mouse" transplantation model, which also showed that combination treatment of anakinra and TNF-α was more effective than either of the drugs on their own (91).

Another suggested pharmacological target has been interaction between the chemokine C-X-C motif ligand 1 (CXCL1) and C-X-C Motif Chemokine Receptor 2 (CXCR2). Cultured islet releases CXCL1, which binds to the chemokine receptor CXCR1/2 and has hazardous effects towards islet grafts. In preclinical models, blocking this pathway has been shown to improve islet engraftment (92) , but the benefits were not evident in a later randomized controlled clinical trial (93).

As specific protection against IBMIR beyond the co-administration of heparin during islet transplantation, in preclinical models, low molecular weight dextran sulfate (LMWDS) has been shown to reduce the IBMIR reaction (94). However, a recently reported clinical trial using low dose LMWDS has shown no significant improvement of the outcomes (95). An ongoing study is evaluating the efficacy of LMWDS in a higher dose [NCT03867851].

2.5.2 Erythropoietin receptor agonist

A naturally occurring anti-inflammatory agent is erythropoietin (EPO). In addition to its hematopoietic properties, EPO has anti-inflammatory, anti-apoptotic and cytoprotective effects. CD131, which also called *cytokine receptor common subunit beta, receptor for granulocyte-macrophage-colony stimulating factor (GM-CSF)/IL-3/IL-5,* are expressed highly under inflammation on the most hematopoietic and non-hematopoietic cells. One of the dimers of the heterodimeric erythropoietin receptor (EPOR) can merge with CD131 and form a heterodimeric receptor called the *innate repair receptor* (IRR) (96). Activation of the IRR requires higher amounts of EPO compared to the EPOR, and upon activation of IRR, the cell switches its energy expenditure to cytoprotective and anti-apoptotic properties. One of the anti-inflammatory mechanisms is the reduction of binding affinity of NF-κB to DNA, which leads to a reduced secretion of downstream mediators, such as MCP-1 and MIP1β, resulting in reduced macrophage infiltration (97). It has also been suggested that EPO may accelerate the revascularization of rodent pancreatic islets (98). However, the use of EPO clinically can be harmful due to its hematopoietic and prothrombotic properties. Cibinetide (originally named as ARA290) is a recently engineered EPO analogue that displays affinity with IRR but lacks hematopoietic properties. Our group recently reported that cibinetide

treatment could protect transplanted islets and improve engraftment in a syngeneic mouse pancreatic islet transplantation model (99). Cibinetide also has a favorable side-effect profile, shown in clinical trials in type-2 diabetes and sarcoidosis patients (100, 101). In this thesis we have evaluated this drug in new preclinical settings.

2.6 TARGETING ADAPTIVE IMMUNE RESPONSES TO INDUCE TOLERANCE

There are several definitions of tolerance in an allogenic setting. Originally, Billingham, Brent and Medawar described tolerance as an immunological unresponsiveness to specific foreign antigens, based on their preclinical animal studies (102). In clinical transplantation, however, operational tolerance (OT), defined as stable and acceptable graft function without external administration of IS over one year, has been more clinically applied in recent decades (103). Within a host's immune system, the phenomenon of tolerance can be divided into central and peripheral mechanisms.

Central tolerance involves the negative selection of immature unwanted T-cells in the thymus. Normally, thymic APCs direct autoreactive T cells (with a TCR specific for autoantigen) into programmed cell death (104). The natural Tregs that will be discussed later are also involved in this mechanism. After immune manipulation, the thymic negative selection process can also expel specific alloreactive T-cells via tolerogenic DCs (103).

Peripheral tolerance involves mature lymphocytes in the tissues outside the thymus and includes several mechanisms that lead to the inability of mature T-cells to be activated by the TCR-specific antigen. One key component of peripheral tolerance is the inhibitory function of Tregs (105). Immature DC that presents antigens weakly on MHC-II and expresses low levels of CD80 and CD86, which leads to a diminished activation of signal 1 and 2, are also a suggested mechanism (106). Auto or alloreactive T cells that are not activated or only partially activated (for example, TCR activation with no co-stimulation signal) can also undergo programmed cell death.

Currently, **three** principal ways of achieving a state of immune acceptance of an allogenic graft without any external administration of IS drugs are being considered.

2.6.1 Deliberate weaning of immunosuppression

The **first** way is via weaning and withdrawal of IS without any manipulation of the immune system. Few organ transplant patients have spontaneously ceased to intake IS and not rejected their grafts.

Historically, only around 250 spontaneous tolerant cases of kidney transplant have been reported, as well as around 100 patients after heart transplantation.

Since the liver is the most immune privileged organ, this state of spontaneous tolerance is more common in liver transplant recipients (103). Few prospective studies have been conducted of deliberate IS weaning and cessation. Existing studies suggest that older recipients, male gender and long duration after liver transplantation is beneficial to successful

IS weaning (40, 41, 107). A longer duration after transplantation also means that IS caused morbidity has often already occurred. Moreover, due to the complexity of the immune system, it is not known which recipients can be weaned or withdrawn from IS and which recipients will reject and possibly lose their grafts.

Two major ongoing trials OPTIMAL (USA) [NCT02533180] and LIFT (GB) [NCT02498977] are currently evaluating the efficacy and exploring the possible biomarkers to identify suitable patients for IS weaning.

2.6.2 Induction of mixed chimerism

The **second** strategy is inducing a donor-specific chimerism in the recipient in parallel to organ transplantation. The state of chimerism is defined as donor HSC that can peacefully co-exist in the recipient. Chimerism can be achieved via HSC transplantation (108). The mechanism is believed to be central tolerance. Over the last two decades, three transplant centers in the USA have developed and evaluated this strategy, mainly in living donor kidney transplantation.

1. At **Massachusetts General Hospital**, the strategy is to induce transient mixed chimerism using non-modified donor bone marrow with thymic irradiation, anti-CD2 antibodies, cyclophosphamide and rituximab induction in haplo-identical living kidney donor-recipient pairs. Their long-term follow up reveal that 7/10 living donor kidney recipients had achieved the definition of operational tolerance. However, in long-term follow up, only four of these 10 recipients has remained off IS (109, 110).
2. The **Stanford University** group focused on both HLA-matched and mismatched recipient and donor pairs. Their induction protocol comprises total lymphoid irradiation and lymphocyte-depleting polyclonal antibodies. The chimerism was induced by CD34 and CD3 dependent HSC. Recent follow up data reveal that 24/27 HLA-matched recipients could be long-term IS free. However, the Stanford group failed to induce long-term tolerance in the HLA-mismatched recipient groups (45). It is planned to initiate the Stanford protocol of tolerance induction in HLA-identical recipient-donor living-kidney transplantation at Karolinska University Hospital, Sweden. However, no suitable recipient-donor pairs have been identified for the protocol this far.
3. The **Northwestern** group from Chicago (US) utilized a non-myeloablative conditioning of fludarabine, cyclophosphamide and low-dose total body irradiation before the infusion of donor-specific $CD8^+$ TCR^- HSC, also called “the Facilitating cell”. The recipient’s IS was weaned during one-year post transplantation if no signs of rejection were detected in renal biopsies. Due to the strenous conditioning before the HSC, 26/37 HLA-mismatched living-kidney recipients achieved a stable (>50%) mixed chimerism, while 23 of these recipients achieved “full” chimierism of >98% donor HSC in peripheral blood. All 26 recipients have been withdrawn from IS therapy and experienced no rejection episodes. However, the risk of post-transplant

infections and graft versus host diease (GvHD) is an increased problem using this tough strategy (44).

Limited attempts have also been made to induce tolerance in liver transplant recipients using HSC. In the early 2000s, the University of Miami attempted post-operative transplantation of unmodified donor-derived bone marrow (BM) cells and subsequent weaning of IS in deceased donor liver transplantation recipients. They showed no difference in the percentage of operational tolerant patients compared to a group without BM infusion at 60-months follow up (111). Donckier et al. reported two studies of two and three recipients undergoing living donor partial liver transplantation due to liver malignancy. In the first study, two recipients were subjected to immune conditioning with cyclophosphamide and thymoglobulin, while in the second study, thymoglobulin, sirolimus and steroids were administered before HSC transplantation. Even though the weaning of IS was successful in 4/5 cases, all the patients that could be weaned off IS had tumor recurrence, while the only patient who was still on maintenance IS had no signs of tumor recurrence (112, 113). Attempts to perform HSCOT from a living related donor after a deceased donor liver transplantation in hepatocellular carcinoma and cholangiocarcinoma patients have shown variable results. However, this protocol was designed to evaluate the graft versus tumor effect rather than attempts at IS minimization (114).

Experience of living donor kidney transplantation reveals that achieving a higher ratio of donor vs. recipient chimerism (close to full donor chimerism), that require more strenuous myeloablative conditioning, is beneficial for tolerance induction (43). However, the higher percentage of chimerism also presents an increased risk of GvHD and compromises the immunity against infections. Facilitating mixed chimerism is a less risky path, leading to the possibility of operational tolerance towards the transplanted graft (even if the chimerism is transient), and a preserved more competent recipient immune system. However, if this involves a higher risk of patients remaining on maintenance IS, there are less grounds for tolerance induction.

As previously described, clinical protocols to induce chimerism are dependent on at least partial myeloablative conditioning before an already invasive transplant procedure. This is unsuitable for many transplant recipients (46). An important finding in patients who underwent HSCOT for the purpose of tolerance induction, was a higher amount of circulating Tregs (44, 46, 109, 110). Transplant researchers have recently attempted to harness these Tregs or other regulatory cell populations to use as regulatory cell therapy, which is also the **third** strategy of tolerance induction.

2.6.3 Regulatory cell therapy

Regulatory cell therapy in contrast to HSCOT involves peripheral immunomodulation pathways rather than central immunomodulatory pathways.

2.6.3.1 *The regulatory T-cell*

The idea of regulatory cell therapy originates in early attempts at blood transfusion to improve the graft survival rate. Blood transfusions are traditionally avoided in recipients who need an organ transplant due to the risk of immunization. However, previous trials have shown that pre-transplant blood transfusions improved the graft survival rate after kidney transplantation (115). The benefits were thought to be higher when the blood donor and recipient shared at least an HLR-DR gene (116). This effect is also believed to be associated with an increased Treg population in the recipient (117). In the last three decades, Tregs have come to be seen as a key component in immunomodulation and tolerance induction.

The Tregs immunomodulatory effect has been suggested since the 1970s and its discovery was contributed to several research groups (48, 49, 51). The most accepted current definition of Tregs is the $CD4^{+}CD25^{+}CD127^{low}FOXP3^{+}$ population. They are classified as either being thymus derived natural Tregs (nTreg) or induced iTregs (47). Several immunomodulatory mechanisms of the Tregs have been suggested. Tregs express CTLA-4 which signals B7 on DCs and leads to a down regulation of CD80 and CD86 (B7 molecules are crucial as signal 2 in T-cell and DC interaction) (50). Tregs secrete IL-10, Transforming Growth Factor-β (TGF-β) and IL-35, which inhibit T-effector cell function, down regulate interferon-γ (IFN-γ) production and dampen the effect of the adaptive immune reactions (118). Tregs also express a high amount of CD25, which is part of the IL-2 receptor and consumes IL-2, which leads to reduced activation of signal 3 on effector T-cells (119), and several more mechanisms have been suggested. Dysfunction of Treg-specific genes is associated with autoimmune disease, for example, mutation of the Forkhead Box Protein P3 (FOXP3) gene leads to autoimmune lymphoproliferative disease, seen in both humans and rodents (120, 121). Rodent models have shown that Treg infusion can regulate graft rejection in an allogenic setting (122) and that infused Tregs travel to the site of transplanted grafts and secondary lymphoid organs in the periphery (123). In 2005, Bashuda et al. reported that generation and post-transplant infusion of donor-specific Tregs could prolong allogenic graft survival in a non-human primate (NHP) kidney transplantation model, even after cessation of immunosuppressive drugs (124).

The first clinical trials of Treg treatment were in the field of HSC transplantation for treatment of GvHD (125). This has subsequently proven to be effective and feasible (126).

2.6.3.2 *Other suggested regulatory cell types*

The regulation of adaptive immune responses, both stimulatory and inhibitory pathways, generally involves multiple cell types and mechanisms. Even though Tregs are still believed to be master regulators of adaptive immune responses, several other cell populations have been suggested to contribute to peripheral immune acceptance in recent decades. One example is the regulatory dendritic cell (DCreg). It is suggested that DCregs promote Treg function, attenuate T-effector cell mechanisms and have an IL-10 and TGF-β secreting capacity (127). The feasibility of using DCregs has been shown in rodent and NHP models

(128, 129). Another cell type suggested to be involved in immune acceptance is the regulatory B-cell (B-reg). Biomarker assessments have shown that tolerant kidney recipients show higher granzyme B positive B-cell signatures (130).

2.6.3.3 Clinical applications of ex-vivo generated donor-specific immunomodulatory cells

One of the major turning points of regulatory cell therapy in the field of organ transplantation were the encouraging results reported by Todo in 2016 (54). Ten living donor liver transplantation recipients underwent partial liver transplantation and regulatory cell treatment 14 days post-operative. The cell treatment comprised 5×10^9 PBMCs from the recipients, who were cultured with 2×10^9 irradiated donor PBMCs in the presence of CD80 and CD86 antibodies for 14 days. During the transplant procedure, a splenectomy was performed. The recipients initially received standard CNI, MMF and steroid treatment, in which the MMF and steroids were withdrawn during the first month after liver transplantation. Five days after liver transplantation, the recipients received 40mg/kg cyclophosphamide. Six months after liver transplantation a controlled weaning of CNI was started and was completely ended at 18 months post-transplant if no signs of rejection were seen biochemically or in protocol biopsies. Seven out of 10 recipients were successfully weaned off IS without any cell treatment-related side effects for over six years. It is interesting to note that all three recipients who experienced acute rejection episodes and who could not be weaned off IS had an autoimmune liver disease. All the recipients had functioning liver grafts (54, 131). One of the recipients died in 2019 due to a brain infarction. This was not considered to be related to the treatment (*personal communication Dr Watanabe, Hokkaido University Hospital 2022-02*).

Although the cell product was regarded as Treg treatment, no enrichment of Tregs took place before or after the cell culture and the recipients received the full dose of these "donor-specific immunomodulatory cells" (DSIMC). Release criteria for the DSIMC were: $>10^8$ cells with viability >70% and $CD4^+CD25^+FOXP3^+$ Tregs $> 10^6$.

A similar protocol was evaluated in kidney transplantation and reported by Koyama et al. in 2020 in nine living donor kidney recipients. None of the patients could be completely weaned off IS due to acute rejection during the weaning period. However, IS could be reduced to around 50% (52). All the recipients had functioning kidney grafts at the end of the follow-up period.

Two groups from the USA have reported the use of expanded Tregs in a kidney transplantation setting. One of these is a phase I study using magnetic activated cell sorting (MACS) enriched polyclonal expanded Tregs in living donor kidney transplantation (TRACT study) and the second study (TASK study) using fluorescence-activated cell sorting (FACS) sorted polyclonal Tregs in kidney recipients (132, 133). The goal of these is however not to achieve IS withdrawal, but are phase I safety studies, and only preliminary encouraging results have been published focusing on safety. Concordantly, a large EU and US-based research consortium "The ONE Study group" has evaluated the safety and feasibility of the

adoptive transfer of various regulatory cell therapies (Treg, M-reg, B-reg, DC-reg) in living donor kidney recipients in seven separate phase 1/2 studies. The recipients in the cell therapy groups had an overall rejection rate of 16% but could all be weaned off MMF and continue tacrolimus monotherapy with functioning kidney grafts (53).

2.7 ONWARD IMPROVEMENTS

Newly suggested induction agents that target innate immunity have showed promising results in reducing initial graft loss and may potentially reduce the subsequent activation of adaptive immunity in the field of preclinical islet transplantation models. However, these agents still need further investigation with a step ward approach to prove themselves effective in clinical settings.

By taking advantage of the adaptive immunity using tolerance induction strategies, the use of regulatory cell therapy is under evaluation. However, controlled production in GMP-facilities and the use of clinically approved reagents are crucial to vouch for the safety of these trials. Since these protocols have mostly been studied in an elective living donor transplantation setting, logistical hurdles must be overcomed to also include deceased donor transplantation recipients.

3 RESEARCH AIMS

The overall aims of the studies included in this thesis are:

(1) To evaluate the ability of the anti-inflammatory erythropoietin receptor agonist cibinetide to reduce initial damage and the subsequent activation of adaptive immunity caused by the innate immune response.

(2) To develop methods to facilitate the use of regulatory cells in order to minimize immunosuppression.

Specific aims of the study:

I. Characterize the short and long-term effects of cibinetide treatment in an allogenic mouse pancreatic islet transplantation model.
II. Evaluate the effects of cibinetide on human islets and to clarify its effect on IBMIR from the perspective of intra-portal pancreatic islet transplantation.
III. Investigate the safety and feasibility of intra-portal co-infusion of autologous Tregs and allogenic islets in clinical islet transplantation.
IV. Evaluate whether CTLA-4-Ig can be used as a co-stimulation blockade in the generation of a donor-specific immunomodulatory cell product instead of anti CD80/CD86 antibodies.
V. Evaluate the feasibility of three different methods of procuring donor PBMCs for the generation of donor-specific immunomodulatory cell products for tolerance induction therapy in a deceased donor liver transplantation program.

4 MATERIALS AND METHODS

4.1 ETHICAL CONSIDERATIONS

4.1.1 Animal experiments (*studies I–II*)

The animal models in studies I–II were used under the Guidelines for the Use of Laboratory Animals of the Karolinska Institute. All research methods have been approved by the Swedish Board of Agriculture (S30–15 and 78–15). The animals were purchased from Charles River Inc. (Sulzfeld, Germany) and were acclimatized according to the ethical guidelines and kept in pathogen-free animal rooms.

4.1.2 Experiments with human tissue (*studies II, III, IV, V*)

The use of human pancreatic islets from deceased donors in study II was approved by the Swedish Ethical Review Authority (2015/715-31/1 and 2016/1883-31/1). The co-infusion of Tregs in PITx was approved by the Swedish Ethical Review Authority (2017/1026-31, 2018/1419-32 and amendment 2019-02412)

The use of blood from healthy volunteers for the experiments in study IV and the procurement of blood from deceased donors in study V were approved by the Swedish Ethical Review Authority (2014/1565-31/4). The procurement of PBMCs from deceased donors via bedside leukapheresis was approved by the Swedish Ethical Review Authority via the additional amendment application (2019-04445).

4.2 CHARACTERIZING THE EFFECT OF CIBINETIDE ON PANCREATIC ISLET TRANSPLANTATION IN PRECLINICAL MODELS (*STUDIES I–II*)

The first two studies in this thesis focused on evaluating the beneficial effect of the non-hematopoietic EPO analogue cibinetide on both mice and human pancreatic islets. The methods were designed to characterize the anti-inflammatory and anti-apoptotic function in both in-vitro systems and from a composite perspective using an in-vivo mouse model of allogenic PITx. As IBMIR is one of the feared reactions that can cause the loss of over half of the islet grafts after an intra-portal transplantation, it is important to characterize the effect of cibinetide on IBMIR, particularly when conventional EPO has the potential side effect of promoting a prothrombotic state (134). Therefore, we also reintroduced the in-vitro IBMIR model, using rotating heparinized tubing (14) with blood from healthy volunteers to observe the effects of cibinetide on IBMIR.

4.2.1 Mouse pancreatic islet isolation (*study I*)

BALB/c mice were euthanized according to the procedure described in the ethical guidelines. A laparotomy was performed, and the duodenum was exposed. The papilla vateri was clamped using the micro hemostat “Herc”. The liver was retracted and the ductus choleducus was visualized under 10x magnification using (Stereo microscope OSE-40, KERN & Sohn

GmbH, Balingen Germany). A 27-gauge butterfly needle was used to puncture the ventral side of the bile duct, followed by the insertion of an injection needle-shaped PE-10 tubing in the bile flow direction towards the pancreatic duct. 2–2.5 ml collagenase P (2 mg/mL; Roche, Stockholm, Sweden) was injected under microscopic visualization of the expanding pancreas until the pancreatic tissue stopped expanding. The pancreas was then surgically explanted and put in ice.

The islets were incubated at 37ºC for 20 minutes for collagenase digestion. Collagenase activity was then stopped using cold Hanks balanced salt solution (HBSS). The pancreas was filtered through a metal mesh to remove large clumps (fat and fibrotic structures) and the islets were isolated via gradient centrifugation using Histopaque 1.119 and 1.077 (Sigma-Aldrich, Missouri, USA). The islets were transferred out of the layers using a glass pipette, washed and extracted by hand under the microscope at 10-20x magnification (Leica Zoom 2000 stereomicroscope).

4.2.2 Mouse pancreatic islet transplantation (*studies I–II*)

C57BL/6N mice were rendered diabetic via streptozotocin injection (150 mg/kg). A blood glucose level of >25 mmol/L over two consecutive days was considered as diabetes. The mice were anesthetized using inhalation anesthesia. A laparotomy was performed, and the bowel was mobilized to the left to expose the portal vein and pancreatic head. A 27-gauge butterfly needle was used to puncture the portal vein just below its entrance under the pancreatic neck (the needle punctured the portal vein via the pancreatic tissue). The allogeneic mice islets (320 in the marginal model, 450 in the standard model) or 1250 IEQ human islets (in human islet to NMRI mice transplantation) were suspended in medium to a total volume of 150 μL and slowly injected during direct visualization through the anterior portal vein of the diabetic C57BL/6 mice or NMRI athymic mice (figure 2). The needle was retracted, followed by compression of the puncture site over the pancreatic neck using a clean hemostatic stick on a micro crile peang for one minute for hemostasis. The abdominal incision was then closed using 6-0 PDS, followed by end of anesthesia.

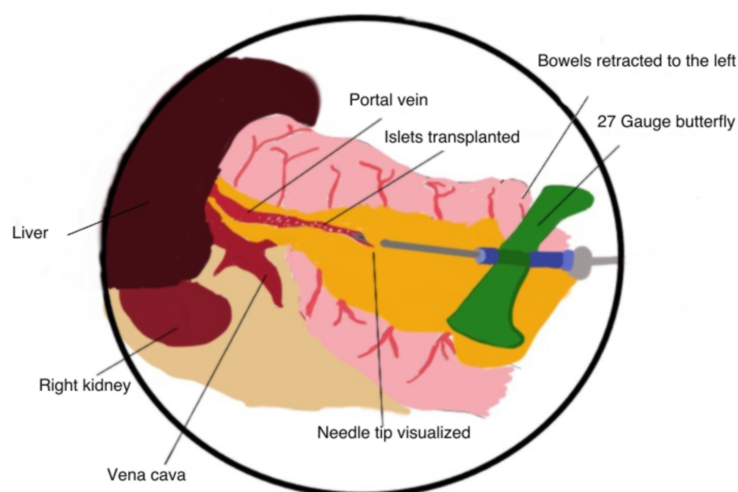

Figure 2. Transplantation of pancreatic islets in mouse was performed under a surgical microscope with 10x magnification.

4.2.3 Pancreatic islet transplantation treatment protocol (*studies I–II*)

In standard model islet transplantation (450 allogenic islets), the mice were treated with four different treatment protocols. Group 1 (control group) were treated just before PITx, at PITx, six hours after PITx and once daily for 14 consecutive days post operatively (POD) with intra-peritoneal (IP) injections of phosphate-buffered saline (PBS) in the same volume as group 2. Group 2 (cibinetide group) received 120 µg/kg cibinetide IP just before PITx, at PITx, six hours after PITx, followed by daily injections from POD 1 to 14. Group 3 (tacrolimus treatment group) received 0.4 mg/kg Prograf (Astellas, Tokyo, Japan) in daily IP injections from POD 4–14. Group 4 (combination treatment) received both cibinetide and tacrolimus as described above (figure 3). In marginal PITx (320 allogenic islets), the mice were treated according to group 1 (control group) or 2 (cibinetide group). Daily measurement of non-fasting blood glucose was performed. Blood glucose of >19.5 mmol/L for two consecutive days was defined as graft loss.

The NMRI mice that were transplanted with 1250 IEQ human islets received either PBS (control group) or 120 µg/kg cibinetide (cibinetide group) IP just before PITx, at PITx, and 6 hours after PITx.

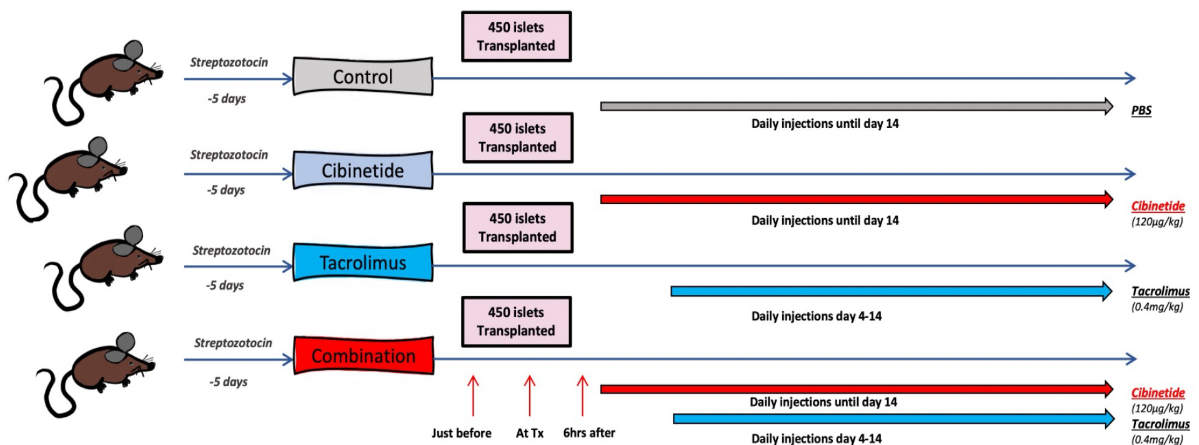

Figure 3. Allogenic mouse PITx treatment protocols.

4.2.4 Polymerase chain reaction analysis of liver (*studies I–II*)

The livers extracted from the recipient mice were frozen and homogenized. RNA extraction was performed using TRIzol reagent (Ambion Life Technologies, Waltham, MA, USA) in accordance with the manufacturer's protocols. RNA was then dissolved in RNase-free water (50 µL). DNA was synthesized from 1 µg RNA using Applied Biosystems high-capacity

complementary DNA reverse transcription. Targeted mRNA quantification was performed using polymerase chain reaction (PCR) TaqMan real-time PCR and Applied Biosystems 7500 Fast Real-Time PCR (Thermo Fisher, Waltham, MA, USA). To quantify the relative concentration of the mRNA, the Ct values were compared to the housekeeping gene (peptidyl-prolyl cis-trans isomerase A).

4.2.5 Effect of cibinetide on the development of allogeneic immune responses *in-vivo (study I)*

Mice transplanted with allogeneic pancreatic islets were euthanatized on day five. Splenocytes were used to examine allogeneic responses. Cell proliferation and IFN-γ production was measured by thymidine-incorporation assays and ELISpot assays, respectively. In proliferation assays, $2x10^5$ recipient splenocytes (C57BL/6N mice with or without cibinetide treatment) was stimulated with donor (BALB/c $2x10^5$ irradiated splenocytes). These cells were cultured for 28-72 hours at 37ºC. In ELISpot assays, cells were incubated on the ELISpot plates and spot forming units were measured after 24 hours incubation.

The liver was homogenized to analyze cytokines and infiltrating dendritic cells using qPCR (see 4.2.4).

4.2.6 Engraftment of human islets in athymic mice *(study II)*

Six days after transplantation, the mice were euthanatized. Plasma and islet-bearing liver were harvested. Livers were homogenized with acid ethanol and insulin was extracted from the liver. The extract was neutralized using tris buffer (pH10) and human insulin concentration was measured using enzyme linked immunosorbent assay (ELISA) (Invitrogen, CA. USA). Human C-peptides were measured in the plasma of athymic mice using enzyme linked ELISA (Mercodia, Uppsala, Sweden)

4.2.7 Mouse dendritic cell isolation and maturation *(study I)*

Mouse dendritic cells were extracted from the bone marrow from the femur of C57BL/6N mice. The muscles and soft tissue were removed and washed with PBS. The bone ends were cut, and the bone marrow was flushed out using 2ml PBS with a 25-gauge needle. The flush was then strained through a 75 µm pore nylon mesh to remove debris. Red blood cells (RBC) were lysed by short incubation (20 seconds) of sterile H_2O and immediately diluted with 10 parts of PBS. The remaining cells were cultured in a DC medium* in 24 well plates for seven days in a humidified atmosphere of 37ºC with 5% CO_2. During the second and fourth day, non-adherent cells were removed when the DC medium* (with or without 100 nm/ml) cibinetide was replaced. At day six, briefly adherent DCs were collected and transferred into 24 new well plates ($5x10^5$ cells/well). Lipopolysaccharides (LPS) (100ng/mL) was added for final stimuli of 20 hours in order to complete DC maturation. In the cibinetide-treated group, cibinetide was added 60 minutes before LPS stimulation.

*The DC medium comprises Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal bovine serum, including (50 μmol/L 2-mercaptoethanol), 100 U/mL penicillin, 100 μg/mL streptomycin (all from Invitrogen, Waltham, MA, USA), 10ng/mL GM-CSF and 10ng/mL IL-4 (PeproTech Inc., Rocky Hill, NJ, USA).

4.2.8 Human pancreatic islets (*studies II–III*)

Procured pancreases from brain dead donors within the Scandiatransplant region were transported to Rudbeck laboratory (Uppsala, Sweden) where the pancreatic islets were isolated using previous reported protocols (135). The islets were kept in platelet transfusion bags suspended in islet culture medium: Connaught Medical Research Labs 1066 (CMRL1066, Corning, Manassas VA, USA) and kept in a humidified atmosphere of 5% CO_2 at 27°C until use.

4.2.9 Characterizing islets in pro-inflammatory environment (*study II*)

Human pancreatic islets were picked up in a microscope. 20 islets were seeded into every well of 24-well non-attaching culture plates (Sigma-Aldrich, Missouri, USA) suspended in 0.5 mL islet culture medium in the presence or absence of a pro-inflammatory cytokine cocktail of 50 IU/mL IL-1β, 1000 IU/mL TNF-a and 1000 IU/mL IFN-γ (PeproTech Inc. Rocky Hill, NJ, USA) and with or without 100 nmol/L cibinetide for 18 hours.

4.2.10 Islets' ATP content and caspase activity (*study II*)

After culture (4.2.10), the islets were placed in Eppendorf tubes, suspended in 80 μL HBSS and carefully mixed with the same volume of CellTiter-Glo® reagent (for the ATP content assay) or Caspase-Glo® reagent (Promega Corp, Madison, USA). The solution was aliquoted 60 μL in duplicates into half size 96-well white plates (Corning, NY, USA). For the ATP assay the islets were incubated for 15 minutes and for the caspase 3/7 assay, for 30 minutes. The luciferase activity was then measured using a Biotek FLx800 luminometer. To indicate the cell number in each well, double-stranded DNA was measured using PicoGreen dsDNA quantitation assay (Thermo Fisher Scientific, Waltham, MA, USA). After measuring ATP activities or caspase 3/7 activities by luminometer, a 60 μL/well of PicoGreen solution was added and fluorescence was measured using a Biotek FLx800 fluorometer. ATP content and caspase activities were indicated by luminescence/min/μg dsDNA.

4.2.11 Dynamic glucose perfusion assay (*study II*)

After culture (4.2.10), the islets were subjected to an insulin secretion test using a dynamic perfusion assay. 20 islets were picked up and placed in perfusion filter closed chambers and perfused first with 1.67 mmol/L glucose (low glucose) for 36 minutes followed by 20 mmol/L (high glucose) for 42 minutes and then again with 1.67 mmol/L glucose. The perfusate was collected every four minutes and the insulin levels in the perfusate were measured using ELISA human insulin (Mabtech AB, Nacka, Sweden).

4.2.12 In-vitro IBMIR model (*study II*)

To characterize the effect of cibinetide on IBMIR, an in-vitro system of heparinized poly vinyl chloride (PVC) tubing (Corline Biomedical, Uppsala, Sweden) was used. The tubing had a width of 6 mm diameter and were cut to 20 cm and connected to loops with a specially made heparinized stainless-steel connector (engineered at Karolinska University Hospital Huddinge, Sweden). The system was used to mimic the portal flow. The loops contained 4 mL AB0 compatible whole blood and approximately 2 ml air. Human islets were washed in HBSS and a 3µL islet pellet was injected into each loop. The loops were rotated at 22 rpm for 60 minutes.

The treatment groups were (1) blood only (2) blood and islets, (3) blood, islets and 0.4 units/mL heparin, (4) blood, islets, 0.4 units/mL heparin and 100 nm/L cibinetide.

After 60 minutes incubation, 16 µmole Ethylenediaminetetraacetic acid (EDTA) was added to each loop to stop the coagulation (final concentration was 4 mmol/L). The blood from the loops was analyzed for hemoglobin, platelets and white blood cells using Sysmex XP-300 (Sysmex Europe, GmbH, Germany).

4.3 SAFETY EVALUATION OF TREG CO-INFUSION DURING PANCREATIC ISLET TRANSPLANTATION (*STUDY III*)

To improve long-term graft survival and to avoid drawbacks of IS, tolerance induction therapies using regulatory cell therapies are under development. Clinical PITx would especially benefit from this kind of treatment, due to the beta-cell toxicity of conventional IS. PITx offers the possibility to directly deliver regulatory cells into the transplant site. Study III evaluated the safety of infusion of enriched autologous polyclonal Tregs in conjunction with allogenic PITx. The Tregs were infused together with the islets via an intra-portal catheter placed in the portal vein. The primary outcomes were safety parameters, secondary outcomes were efficacy of the islet transplantation.

4.3.1 Leukapheresis (*studies III, V*)

Patients received peripheral cannulas (*study III*) or a central dialysis cathether (*study V*). Apheresis was performed by specialized nurse using a Spectra Optia (TerumoBCT, USA) machine with the continuous mononuclear cell collection protocol.

4.3.2 Enrichment of Tregs (*study III*)

Leukapheresis was performed and the product was stored overnight at room temperature (RT). The depletion of CD8 and CD19 positive cells was first performed via negative selection. CD25 positive cells were enriched using positive selection in a sterile CliniMACS system (Milteny Biotec, Bergish-Gladbach, Germany). The cells were suspended in freezing medium (80% pooled plasma and 20% dimethyl sulfoxide). The release criteria were set at >60% $CD4^{+}CD25^{+}$ cells with a viability >70% with no signs of bacterial contamination.

4.3.3 Combined Treg and pancreatic islet transplantation (*study III*)

Islet isolation from deceased donor pancreases followed standard procedures and islets were kept in culture awaiting transplantation (135).
The Tregs were thawed and thereafter washed with sodium chloride solution supplemented with 2.5% albumin on the day of transplantation. They were centrifuged and the supernatant was removed. Minor portions were taken for analysis of phenotype, viability and cell counting.

Islets and Tregs were transported in separate platelet transfusion bags to the transplantation site. 70 U/Kg (body weight of the recipient) heparin was injected into the islet transfusion bag. The islets and Tregs were infused into the portal vein by gravity. Blood glucose was carefully monitored and kept between 4–8 mmol/L during the procedure.

4.3.4 Islet transplantation recipients (*study III*)

Patients were 18-65 years of age with type 1 diabetes for more than 5 years. If not transplanted before they should have <0.1 nmol/L c-peptide in a mixed meal tolerance test (MMTT). They were included in the study after signing an informed consent. Primary outcomes of safety were recorded until POD 90. Outcome measurements of the graft function: Clarke-score (136), HbA1c, stimulated C-peptide levels and daily insulin dose were recorded at 75 days after transplantation. Basiliximab was used for immunosuppressive induction and the preferred maintenance was tacrolimus/everolimus.

4.4 COMPARISON OF CTLA-4-IG AND 2D10.4-IT2.2 (*STUDY IV*)

Based on the encouraging results of antigen-specific regulatory cell treatment abolishing the need for life-long IS after living donor-liver transplantation (54), we started the development of a tolerance induction protocol suitable for deceased donor liver transplantation.
Study IV characterized the donor-specific effect of DSIMC generated by either CTLA-4-Ig or 2D10.4/IT2.2 (anti CD80 and CD86 antibodies). Although the 2D10.4/IT2.2 generated DSIMC has shown encouraging efficacy in the total withdrawal of IS in living donor liver transplantation, the 2D10.4/IT2.2 are non-registered mouse antibodies that are not approved for clinical use. The CTLA-4-Ig is a clinically approved IS agent that blocks the interaction between CD80/CD86 and CD28, which is the same targets as 2D10.4-IT2.2 in the previous DSIMC cell production methods (54). We generated DSIMC in a 100-fold scale-down culture setting. To characterize the reactivity of the recipients PBMCs to donor-specific antigens, a mixed lymphocyte reaction (MLR) assay was set up using freshly isolated recipient PBMCs, stimulated with irradiated donors or third-party antigens (positive controls). A proliferation assay using a thymidine incorporation assay and functional assays (ELISA and ELISpot assays) to detect cytokine production was used.

4.4.1 Isolation of human PBMCs (*study IV*)

After receiving written consent, blood was drawn (up to 200 ml) from healthy volunteers in 8 ml acid citrate dextrose tubes. The tubes were centrifuged at 300 G for 10 minutes and the

serum was then removed. This was followed by density gradient centrifugation for 35 minutes at 450 G using Lymphoprep™ (Axis-Shield, Oslo, Norway). The PBMC layer was then extracted with a pipette, washed with PBS and suspended in RPMI 1640 medium.

4.4.2 Generation of donor-specific immunomodulatory cells (*study IV*)

In order to produce DSIMC in a small scale, $25cm^2$ culture flasks (Corning, NY, USA) were used. The HLA-non matched recipient and donor PBMCs were isolated. The donor PBMCs were irradiated at 30 Gray (Gy). The recipients PBMCs ($50x10^6$) were stimulated with $20x10^6$ irradiated donor PBMCs in the presence of a costimulator blockade as follows:

1: No costimulator blockade (sham).

2: 40µg CTLA-4-Ig /10^6 recipient PBMCs.

3: 3µg 2D10.4 /10^6 and 3µg IT2.2 /10^6 recipient PBMCs.

The cells were cultured for 14 days in RPMI 1640 medium containing 1% heat inactivated recipient serum. At day 7, the medium, costimulatory blockade and irradiated donor PBMC were replenished. At day 14, the cultured cells were harvested and suspended in RPMI 1640 medium (figure 4). The composition of lineage, DC population and Treg population were examined using FACS.

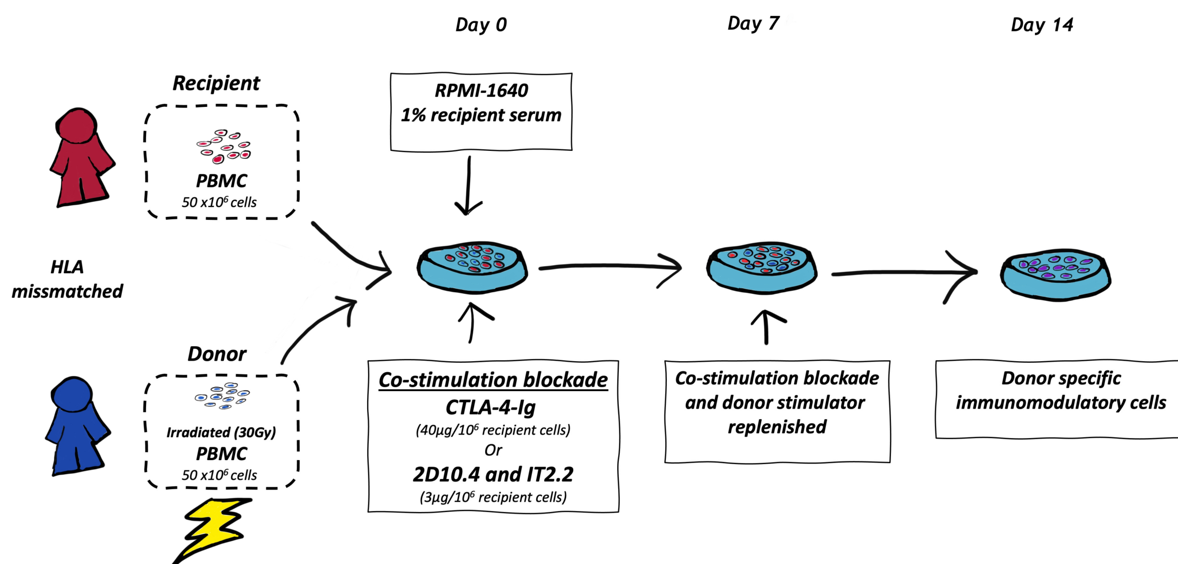

Figure 4. Generation of donor-specific immunomodulatory cells using CTLA-4-Ig or 2D10.4 and IT2.2. As control, recipient cells were also cultured without costimulatory blockers (no antibody group).

4.4.3 Mixed lymphocyte reaction (*study IV*)

Freshly isolated recipient PBMCs ($1x10^5$ responder cells) were stimulated with $1x10^5$ irradiated donor or third-party (positive control) PBMCs in round bottom 96-well plates. DSIMC generated in presence of costimulatory blockade (either CTLA-4-Ig or 2D10.4-

IT2.2) or no antibody (control group) was added in a dose-escalation manner at a ratio of 1:16 (responder cells: DSIMC), 1:8, 1:4 and 1:2. The cell were cultured at 37ºC during seven days for the thymidine incorporation assay, and at 37ºC during 24 hours for the IL-10 ELIspot assay.

4.4.4 Thymidine incorporation assay (*study IV*)

As a measurement of donor-specific reactivity of responder cells, the proliferative activity of PBMCs from the recipients who had been stimulated with donor-specific antigens was measured using a thymidine incorporation assay.

Radioactive thymidine (H^3-thymidine 1µCi/well) was added to each well during the final part of the MLR. After 18 hours of incubation, the plate was analyzed using a ß-counter (Wallac Sverige AB, Upplands Väsby, Sweden).

4.4.5 ELISpot assay (*study IV*)

IFN-γ ELISpot assay were used to detect donor-specific memory T-cell activities, while IL-10, was used to indicate tolerogenic effects.

4.5 DECEASED DONOR SELECTION FOR PROCUREMENT OF PERIPHERAL BLOOD MONONUCLEAR CELLS (*STUDY V*)

It will be necessary to overcome additional logistical obstacles in order to develop a donor-specific regulatory cell therapy for deceased donor liver transplantation. Obtaining start material (recipient- derived cells) to produce regulatory cell treatment can often take place just before the transplantation procedure at the transplant center. However, in donor-specific regulatory cell protocols, donor antigens need to be procured, which can be challenging in the deceased donor setting. In study V, we evaluated three methods of procuring PBMCs from deceased donors in a non-elective setting.

All donors included in study V were declared brain dead and had reported earlier positive to organ donation including the donation of organs and tissues for research purposes, either according to relatives or a previous report in the Swedish Organ Donation Registry. The donor PBMCs were procured using the three methods below:

4.5.1 Blood procurement during cold perfusion of the organ donor followed by leukapheresis of the procured blood at the transplant center – (BCL) (*study V*)

During the organ procurement procedure, just before the start of cold perfusion via the aorta or iliac arteries, a 20 Fr tubing appurtenant to a 10-liter blood collection bag (Thermo Fischer Waltham, MA, USA) was inserted into the vena cava abdominis using ligatures. The blood and perfusion solution were drained into the blood collection bag during cold perfusion. The bag was then kept in cold storage at 2–6° C and transported to Karolinska University Hospital, Huddinge. A leukapheresis procedure was performed to enrich the mononuclear cells. The cell composition of the product was evaluated using FACS.

4.5.2 Blood collection before cold perfusion and isolation of PBMC using SEPAX at the transplant center – (BCS) (*study V*)

During an organ procurement procedure, before the abdominal cold perfusion of the donor, blood was collected directly from the vena cava using the cannula included in a standard blood donation bag (NPT6280LE Quintruble Bag LCRD-S, MacoPharma, Mouvaux, France). The bag volume was aimed to reach >450 ml. This procedure was repeated using an additional bag. The blood bags were kept at room temperature and transported to Karolinska University Hospital, Huddinge. The blood was separated into buffy coat using Macospin (MacoPharma, Mouvaux, France). The leukocytes were then separated by gradient centrifugation using SEPAX (Biosafe, Switzerland). The cell composition of the final product was examined using FACS.

4.5.3 Bedside leukapheresis of the donor prior to organ procurement – (LEU) (*study V*)

A Spectra Optia (Terumo BCT, USA) was transported to the organ donation site.
A central dialysis catheter was inserted into the internal jugular vein or the femoral vein of the donor under ultrasound guidance.
Leukapheresis was performed during monitoring of the donor's vital parameters. 50–100 ml plasma was also collected at the end of the apheresis. The cells were transported to Karolinska University Hospital, Huddinge at 2–6° C. The composition of the product was examined using FACS.

4.5.4 Cell counting and leukocyte characterization.

All blood products obtained were counted by Sysmex-XP300 (Sysmex Europe, GmbH, Germany) and further analyzed for lymphocyte subsets using FACS (DxFlex, Beckman Coulter Biotechnology, Suzhou, China).

4.6 STATISTICAL ANALYSIS

Descriptive data were presented as mean values ± standard deviation (SD). Mann-Whitney U-test was used to compare one outcome without normal distribution between two groups.
A student-t test was used to compare one outcome with expected normal distribution between two groups. A Wilcoxon's test was used to compare non-normal distributed data between two paired groups with one outcome. One-way ANOVA was used to compare >two groups with one outcome variable. Two-way ANOVA was used to compare >2 groups with >two categories of outcomes. Spearman's test was used to detect whether there was any correlation between two categories of data. Survival analysis was performed via a log-rank test using the Kaplan Meier method. A p value of <0.05 was considered statistically significant.

GraphPad Prism versions 8 and 9 (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis and the creation of figures. Microsoft Excel version 16.17 (Microsoft Corporation, Redond, WA, USA) was used to create tables.

5 RESULTS AND DISCUSSION

5.1 PAPERS I–II

5.1.1 Cibinetide treatment reduced the blood glucose levels of diabetic mice after marginal PITx (*methods 4.2.2 – 4.2.3*)

Using a marginal PITx model of 320 islets to streptozotocin-induced diabetic mice, the blood glucose values of the vehicle-control group did not decrease. The blood glucose levels of the cibinetide-treated diabetic mice that received a marginal number of islets had significantly decreased during the 15-day follow-up (figure 5).

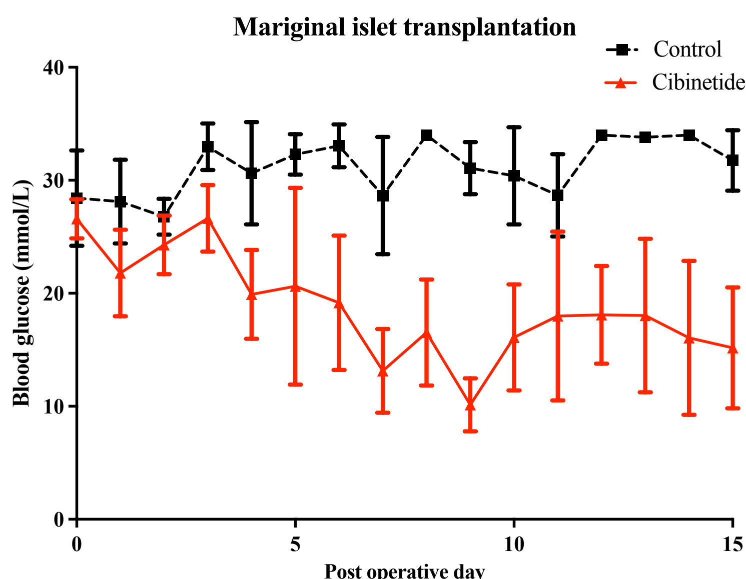

Figure 5. Marginal PITx using 320 BALB/c islets in diabetic C57BL/6N mice. The figure depicts non-fasting blood glucose levels in mmol/L (mean ± SD). The control group showed no reduction in blood glucose levels. The cibinetide-treated group showed significantly reduced blood glucose levels during the 15 days follow up. ($p<0.0001$, 2-way ANOVA) n = 3 in each group.

5.1.2 Local inflammation at the transplant site was mitigated by cibinetide treatment (*method 4.2.4*)

Five recipients after standard model PITx from both the control and cibinetide group were euthanized at 16 hours after PITx. The livers from the mice were explanted and examined. The relative gene expression of IL-1ß, IL-6 was significantly lower in the cibinetide-treated group compared to the control group (figure 6), which indicates a reduced level of local inflammation at the transplant site.

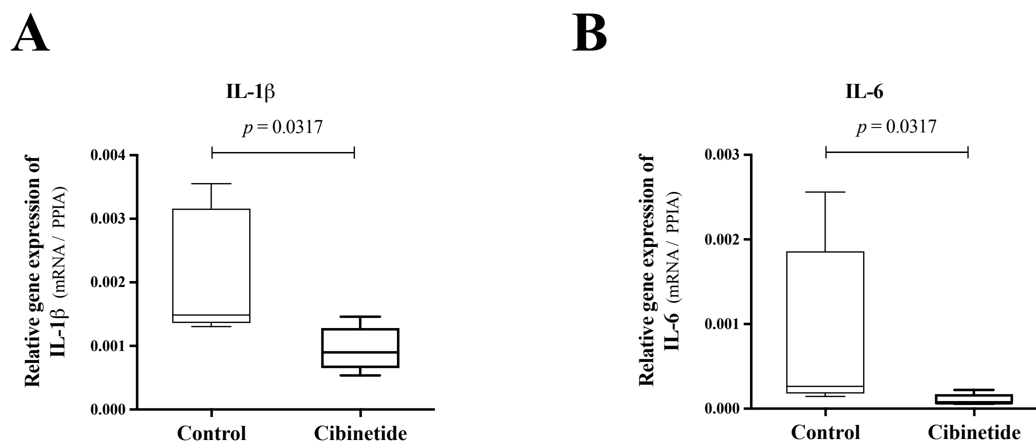

Figure 6. The relative gene expression was measured by PCR. Box roof and floor represent the 25 and 75th percentile, while the whiskers show the max and min values. The extirpated livers of cibinetide-treated recipients showed a significantly reduced level of IL-1β (*p=0.0317, Mann-Whitney U-test*) and IL-6 (*p=0.0317*) at 16 hours after PITx compared to the livers from the control group.

5.1.3 Cibinetide maintained the robustness and insulin secretion of human pancreatic islets in a pro-inflammatory milieu (*methods 4.2.9 – 4.2.11*)

Similar results as (5.1.2) were observed in-vitro when isolated human islets were incubated in a cocktail of pro-inflammatory cytokines with or without cibinetide. The presence of cibinetide in the culture maintained the ATP content and reduced the caspase 3/7 activity compared to the islets that were cultured without cibinetide (figure 7).

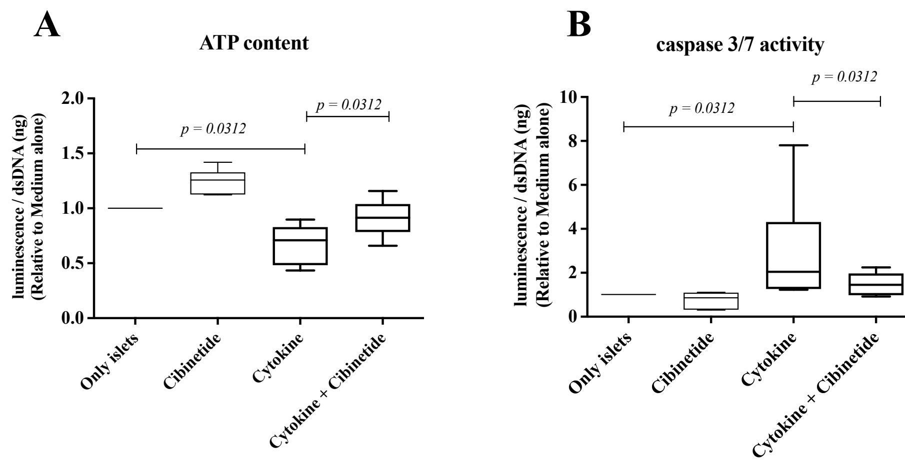

Figure 7. ATP content and caspase 3/7 activity were measured using CellTiter-Glo and Caspase-Glo 3/7. Box floor and roof represent the 25th and 75th percentile, while the whiskers show the max and min values. When 20 islets were cultured in pro-inflammatory cytokines (50 IU/mL of IL-1b, 1000 IU/mL of TNF-a, 1000 IU/mL, and 1000 IU/mL of IFN-g), the addition of cibinetide to the medium maintained the ATP content (*p = 0.0312, Wilcoxon test*) **(A)** and reduced the capase 3/7 activity (*p = 0.0312*) **(B)**.

In a dynamic glucose perfusion assay, the islets that were cultured in pro-inflammatory cytokines in the presence of cibinetide had significantly higher level of insulin secretion in response to high glucose compared to the islets that were cultured in the cytokine cocktail without cibinetide (figure 8). This suggests that the presence of cibinetide maintained the insulin secretion capabilities of the islets in a stressful environment, thereby improving the robustness of the isolated islets.

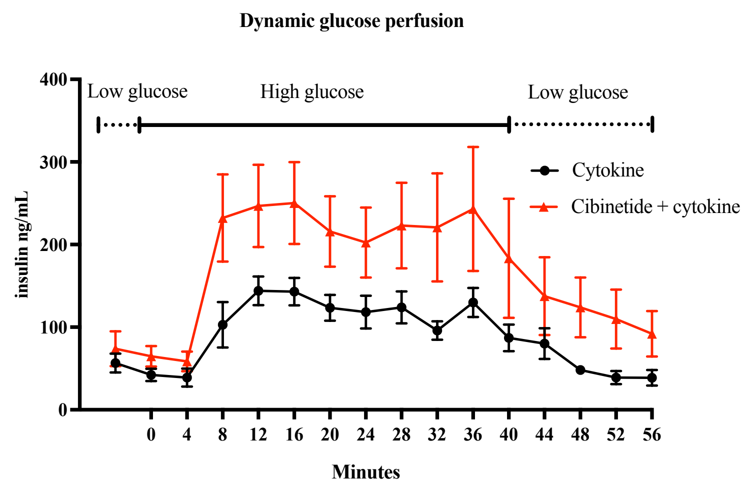

Figure 8. After 18 hours of incubation in proinflammatory cytokines with or without 100nmol cibinetide in the medium, the islets were subjected to a dynamic perfusion assay of low-high-low glucose stimulation (n=5 in both groups). First, a low glucose perfusate of 1.67 mmol/L was used, followed by 42 minutes of 20 mmol/L high glucose perfusate and finally with low glucose of 1.67 mmol/L for 48 minutes. The human insulin in the outcoming perfusate was collected every four minutes and analyzed using ELISA. The cibinetide-treated islets showed a significantly higher insulin production in response to high glucose compared to the islets that were only cultured in cytokines. (AUC *p= 0.0062*, two-way ANOVA. data shown in mean ± SD).

5.1.4 Cibinetide and heparin treatment reduced platelet consumption in the in-vitro IBMIR model (*method 4.2.12*)

To mimic the portal system, we used human AB0-compatible blood in heparinized PVC loops rotated for 60 minutes. The hematocrit and white blood cell levels in the loops did not differ across the groups. As expected, the isolated islets that were rotated in the loops with AB0-compatible blood without any treatment had decreased levels of platelets due to the platelet consuming clot formation, a central characteristic of IBMIR. When cibinetide was administered in the loops together with 0.4 units/ml heparin, the platelet levels were maintained (figure 9), which suggests a mitigation of IBMIR related platelet consumption.

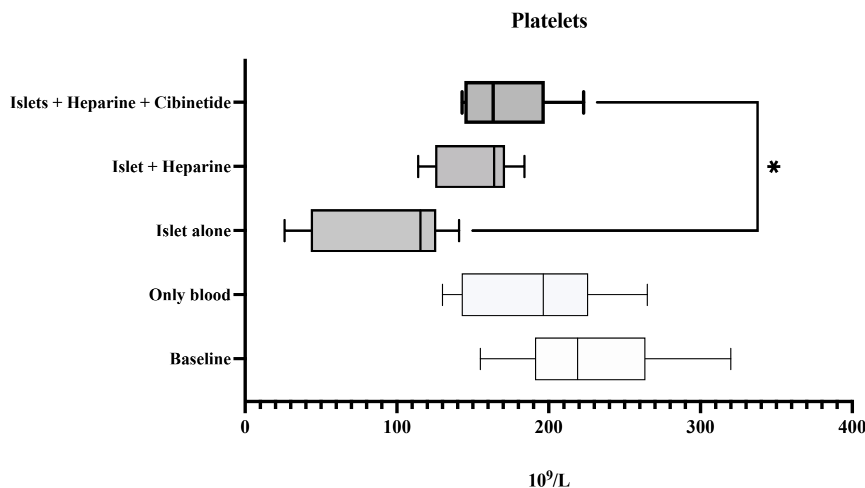

Figure 9. The platelet's concentration was analyzed after 60 minutes of rotation in the in-vitro IBMIR model using PVC loops (n=6). Baselines represent the platelet value of the whole blood donated from healthy volunteers. Values are depicted in median, and the boxes represent the 25th and 75th quartile, while the whiskers symbolize the min and max values. Treatment with both cibinetide and heparin showed a mitigation of IBMIR related platelet consumption compared to islets alone (*$p = 0.0363$ One-way ANOVA).

5.1.5 Treatment with cibinetide improved the engraftment of human islets transplanted to the livers of athymic mice (*methods 4.2.2 – 4.2.3, 4.2.8*)

Evaluating if the observed in-vitro results could impact the outcomes in an in-vivo model. We performed xeno transplantation of isolated human islets to NMRI (athymic) mice with or without cibinetide treatment to characterize the short-term benefits in-vivo. Due to the infrequent availability of isolated human islets from deceased donors, these NMRI mice were not rendered diabetic. We therefore evaluated the levels of human insulin in the graft bearing liver and human C-peptide in the recipient serum to assess the graft function. After transplantation of 1250 IEQ human islets, the mice were sacrificed five days after transplantation and their livers and blood serum analyzed. The cibinetide-treated mice showed significantly higher human-insulin per extracted liver and significantly higher levels of human C-peptide in the procured serum compared to the mice in the control group that received vehicle treatment.

5.1.6 Cibinetide reduced the cross talk between the innate and adaptive immune system (*methods 4.2.5, 4.2.7*)

Given that the innate and adaptive immune responses occur with reciprocity, we also evaluated whether targeting the innate immunity the effect of cibinetide would indirectly affect the adaptive immunity. In-vitro, cibinetide did not affect the function of T-cells in mixed lymphocyte reaction.

5.1.6.1 Cibinetide interfered DC differentiation and antigenpresenting capacity in vitro

The translation of innate responses to adaptive immunity occurs through the cross talk between APC and T-cells, in which DCs are considered the most potent (9). We therefore evaluated whether cibinetide could affect the maturation and antigen-presenting capacity of DCs. Bone marrow derived DCs (BMDC) from C57BL/6 mice, with or without cibinetide pretreatment, were cultured in LPS to stimulate maturation. The BMDC that were cibinetide pretreated produced a significantly lower amount of IL-12 and expressed significantly lower levels of MHC-class II on cell surface compared to the BMDC that were LPS stimulated without pretreatment of cibinetide.

5.1.6.2 Cibinetide delayed alloreactive memory cell generation in vivo

After allogenic PITx using 450 islets, extracted splenocytes at POD 5 showed reduced IFN-γ producing cell frequency and delayed proliferation when stimulated with donor-specific antigens compared to the control group (figure 10).

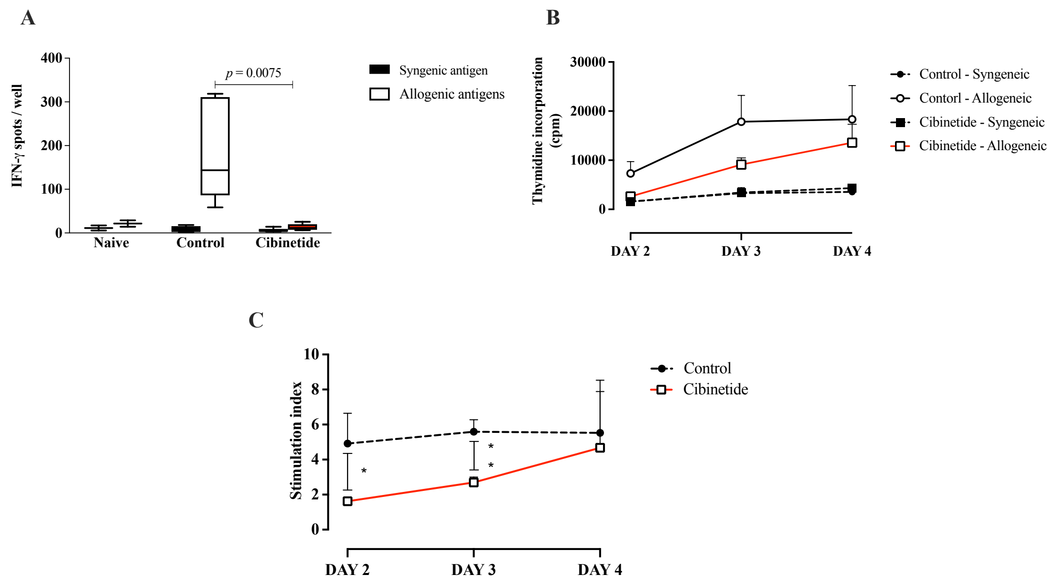

Figure 10. Five days after allogenic PITx using 450 islets, the splenocytes of recipients from the cibinetide treated group and control group were examined in MLR against syngeneic or allogenic antigens. **(A)** The splenocytes from cibinetide treated recipients showed fewer IFN-γ producing precursors when stimulated with donor-specific (allogenic) antigens compared to the control group (*p = 0.0075 Mann-Whitney-U-test*) (n = 5). **(B)** The splenocytes from the cibinetide treated recipients showed lower proliferative response against donor antigens until day 3. **(C)** Stimulation index of the cibinetide group was significantly lower at day 2 and 3 compared to the control group (** p=0.0301, ** p=0.0025 student-t test*).

5.1.7 Combination treatment of cibinetide and tacrolimus significantly prolonged graft function (*methods 4.2.2 – 4.2.3*)

In allogenic PITx using 450 islets (standard model), administration of cibinetide together with conventional IS tacrolimus could significantly prolong the long-term (75 days) graft survival in mouse allogenic PITx compared to either of the drugs alone (figure 11).

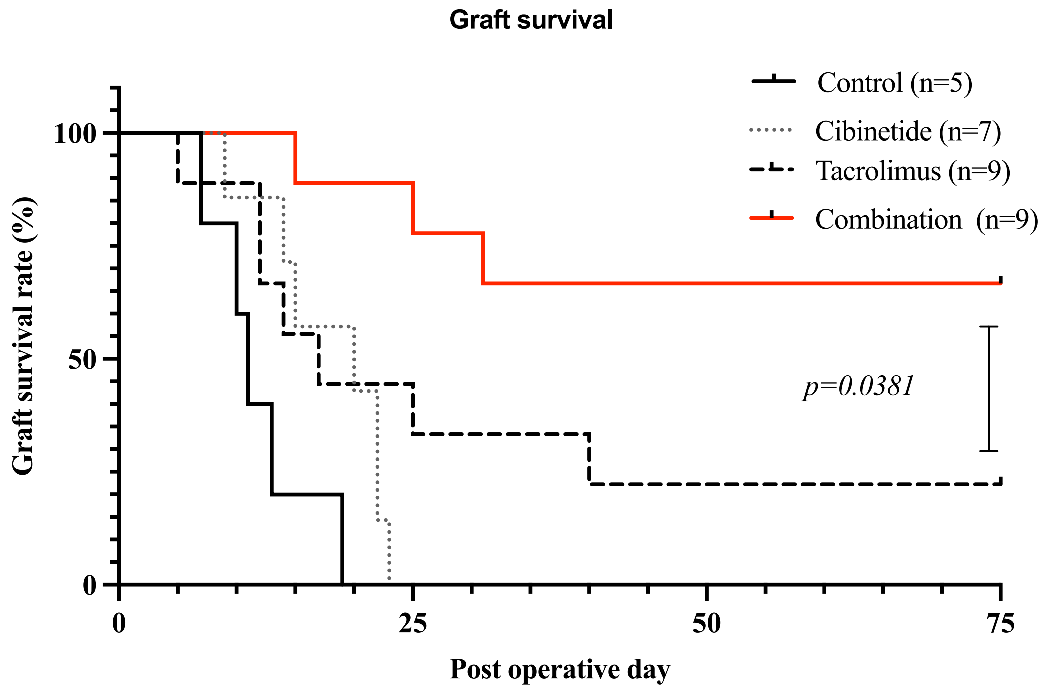

Figure 11. After allogenic PITx using 450 islets, daily non-fasting blood glucose was measured. Normoglycemia after transplantation was defined when blood glucose < 11.1 mmol/L. Graft loss was defined as non-fasting blood glucose >19.5 mmol/L for two consecutive days. The recipients who were treated with cibinetide and delayed onset tacrolimus had a significantly longer graft survival compared to the mice that were only treated with tacrolimus (In survival: $p = 0.0381$, Log-rank Mantel-Cox test).

Summarizing the first two papers, the multi-level methodology indicates that cibinetide has several beneficial effects from the perspective of intra-portal pancreatic islet transplantation. In a proinflammatory milieu, cibinetide maintained the robustness and function of the islets. Cibinetide was also capable of reducing DC maturation and activity, which may lead to reduced cross talk between the innate and adaptive immune cells, which subsequently tends to mitigate the rejection activity (figure 12).

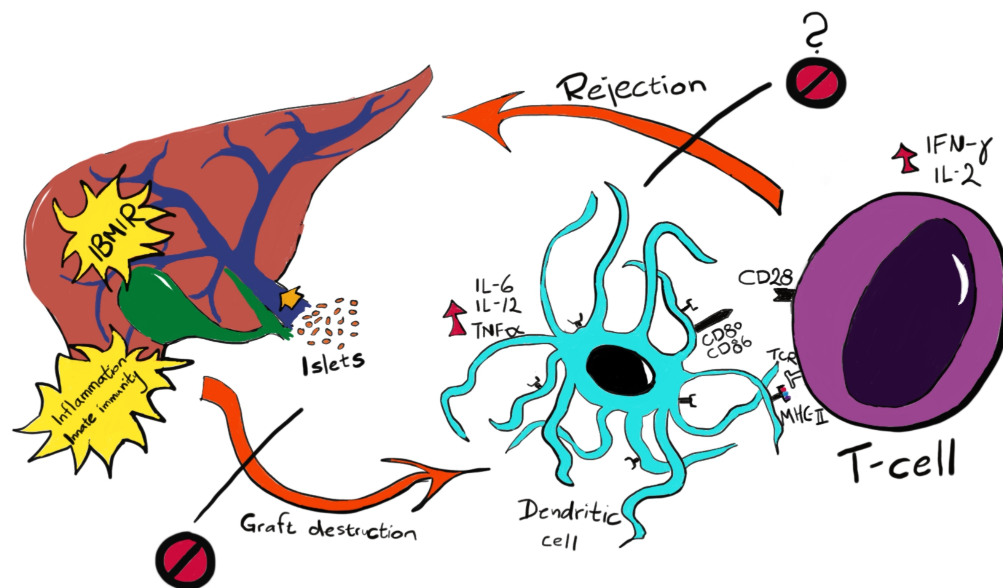

Figure 12. Cibinetide reduced the islet injury caused by the innate immune system and reduced the maturation and antigen presenting capabilities of DCs. This may subsequently reduce/delay the adaptive immune responses.

All these mechanisms may synergistically benefit intra-portal islet transplantation. One of the limitations of these studies is that several mechanisms responsible for the demonstrated beneficial effect have yet to be elucidated on a molecular level. Even though the results are promising both in-vitro and in-vivo, there are no results from clinical application, which is suggested as the next step forward.

5.2 PAPER III

Five patients were enrolled in this safety study. The leukapheresis of the recipients yielded 106.48–223.94 (x10^6 cells/kg). After MACS enrichment, freezing and thawing, the flow cytometric data of the cells to be transplanted showed 34–79% $CD4^+CD25^+FOXP3$ positivity. A total of 0.14–1.27 enriched Tregs (x10^6 cells/kg body weight) were transplanted with the islets into the portal vein.

No immediate complications occurred during the transplantation procedures. On POD 1, one patient had a minor intra-peritoneal hemorrhage found with ultrasound. However, this was treated conservatively, without any need for transfusion. Another patient had oral ulcers, presumably related to the immunosuppressive treatment, which led to hospitalization and switching from everolimus to mycophenolate mofetil. None of the patients became insulin independent. All the patients had reductions in HbA1c, from 7% (range 6.2–8.3) to 6.3% (5.7–6.6) and insulin requirements from 32 U (17–76) to 16 U (8–30). There were no hypoglycemic events during the follow-up period. There was no formation of de-novo donor-specific antibodies recorded.

It is believed that Tregs migrate to the transplanted organ and adjacent lymph nodes, reside in these adjacent tissues, and serve as immune accepting. The transplantation of Tregs directly into the portal vein together with the islets may be a more optimal transplant route compared to the conventional systemic administration of regulatory cells that most other protocols entail (52-54). In this respect, we have shown that co-transplantation of Tregs into the portal vein is a safe method and did not cause any thromboembolic side effects. Due to the small number of patients in this safety study, no conclusions can be drawn concerning the effect of Tregs on graft function outcomes. Intra-portal administration for Tregs together with PITx appears to be a safe method and should be evaluated in further developed cell products, for example, antigen-specific regulatory cell products and with IS minimization and/or withdrawal protocols.

5.3 PAPER IV

5.3.1 2D10.4/IT2.2 and CTLA-4-Ig generated immunomodulatory cells have comparable cell numbers, viability and Treg increment (*method 4.4.2*)

Our results show that cell number and viability were comparable between the CTLA-4-Ig and 2D10.4/IT2.2 groups. During the culture period of cell production, the IFN-γ were comparable between the CTLA-4-Ig and the 2D10.4/IT2.2 groups' culture supernatant at day 7 and 14, which were both significantly lower compared to the control group cells with no costimulation blockade. The Treg population in the CD4+ cells increased in all three groups.

5.3.2 The immunomodulatory cells from both groups using costimulatory blockade had a comparable donor-specific immunosuppressive capacity (*methods 4.4.3 – 4.4.5*)

The cells generated with CTLA-4-Ig and 2D10.4/IT2.2 groups showed a dose-dependent donor-specific immune suppressor effect towards the freshly isolated recipient PBMCs shown in the mixed lymphocyte reaction and thymidine incorporation assay (figure 13).

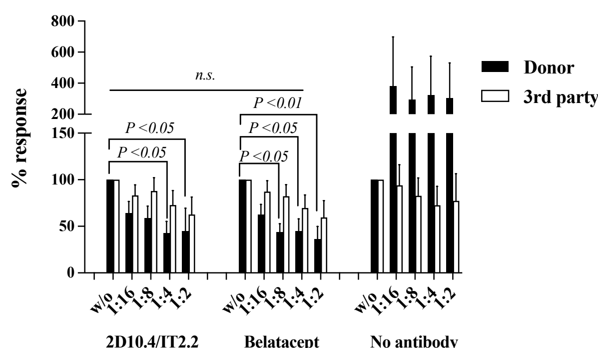

Figure 13. The inhibition of proliferation in the mixed lymphocyte reaction of freshy isolated responder cells stimulated with either donor or 3rd party antigens when 2D10.4/IT2.2 or CTLA-4-Ig generated donor-specific immunomodulatory cells were added in a dose-escalating fashion (responder cell: generated cell ratio of 1:16, 1:8, 1:4 and 1:2) (n=8). The bars show mean value, while the top whisker symbolizes SD. Both the 2D10.4/IT2.2 and CTLA-4-Ig generated cells had a comparable donor-specific immunosuppressive effect shown in a generated cell dose-dependent fashion. This effect was not seen in the control group in which the cell product was generated without a costimulatory blockade. (Two-way ANOVA. A Tukey's multiple comparison test was used to compare the proliferative response between the groups).

The cells generated from both groups in which a costimulation blockade was used also showed higher levels of IL-10 when the cell product was restimulated with donor-specific antigens. This effect was not seen during re-exposure of donor antigen to cell products that were generated without a costimulation blockade (figure 14).

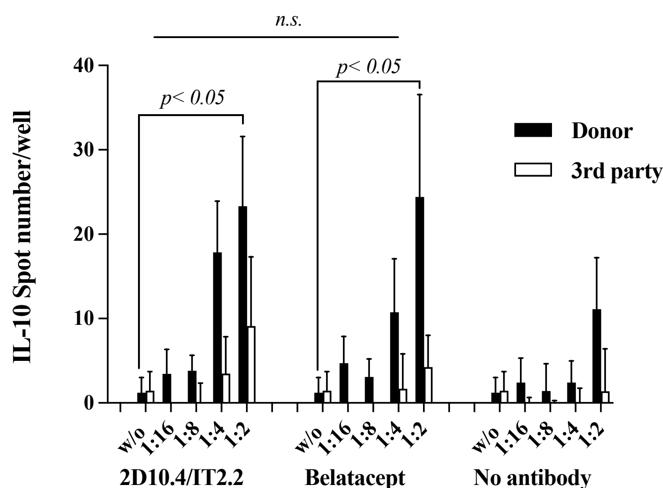

Figure 14. A dose escalation (generated: stimulator ratio of 1:16, 1:8, 1:4, and 1:2) of the cell product generated with 2D10.4/IT2.2, CTLA-4-Ig or the control group (cell product generated without a costimulatory blockade) was stimulated with either donor or 3rd party antigens. The production of IL-10 from immunomodulatory cells was assessed using ELISpot assay. The 2D10.4/IT2.2 and CTLA-4-Ig generated immunomodulatory cells showed a donor-specific dose-dependent increment in IL-10 production that was significantly higher in a concentration of 1:2. (Two-way ANOVA. Tukey's multiple comparison test was used to compare the IL-10 production between the groups).

The results of this study suggest that CTLA-4 Ig could replace 2D10.4/IT2.2 antibodies during the production of DSIMC. CTLA4-Ig is a clinically approved IS for kidney transplantation and is more suitable for inclusion in the development of an advanced therapy medicinal products (ATMP). One of the limitations of this study is that the evaluation of both cell products has been performed only in in-vitro settings using isolated PBMCs from heathy human subjects as responder cells. It would require non-human primates to obtain credible pre-clinical in-vivo results, due to the difference in immune systems between the species. Considering logistics, ethical and animal welfare questions, we have to decide if we are ready to move to a clinical study without this step.

5.4 PAPER V

Of the included donors, there were no significant differences between age, weight, height, white blood cell and hemoglobin values between the different procurement methods. LEU tends to have the highest cell yield/kg, while the viability of the procured cells was comparable across the methods (figure 15).

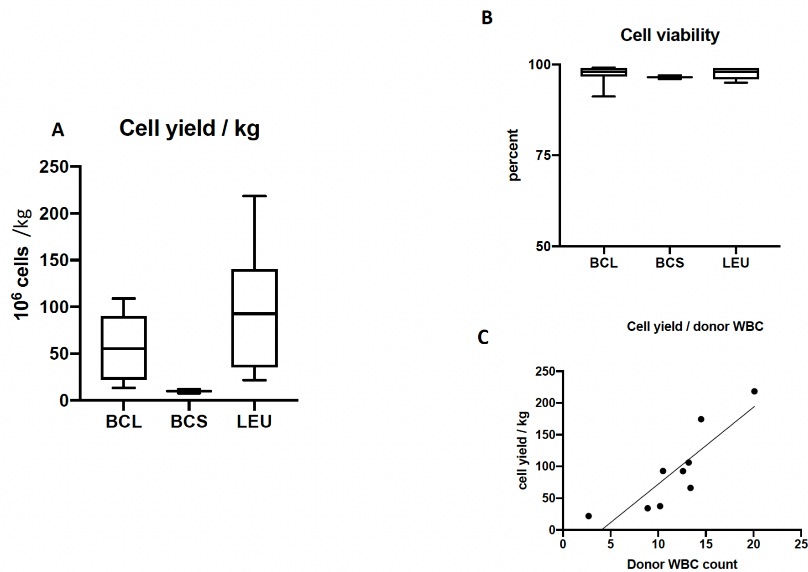

Figure 15. The yield/kg **(A)** and viability **(B)** of the cells procured using the three methods. The boxes symbolize the lower and upper quartile while the whiskers show the min and max values. The middle line shows the mean values. LEU has a tendency of higher cell yield /kg compared to BCL and BCS, while the viability is comparable between the groups. There was a correlation between the donor peripheral white blood cell count and procured cell yield/kg using LEU **(C)** (*Spearman's p*). This correlation was not seen in the two other methods.

Of these methods, BCS tends to result in a higher percentage of granulocyte contamination, compared to BCL and LEU, while BCL tends to result in a higher percentage of NK cells (figure 16).

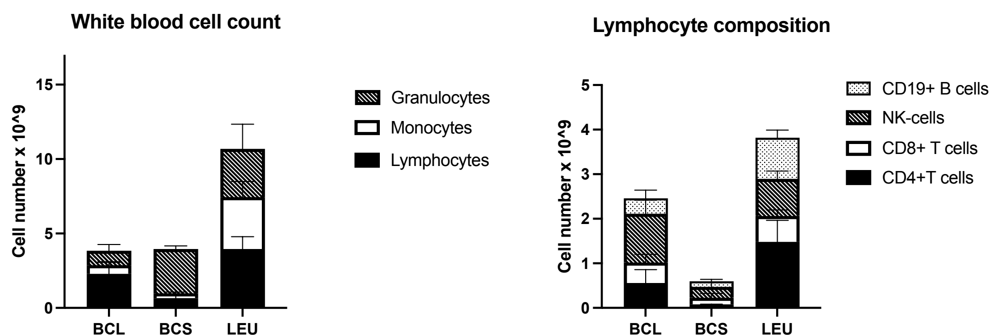

Figure 16. The composition of the white blood cells in fractions of granulocytes, monocytes and lymphocytes **(A)** and the composition of lymphocytes **(B)**. The box size represents mean values; whiskers show the SD.

All three methods appear to be feasible in obtaining PBMCs from deceased donors. However, considering the higher percentage of granulocytes and NK cells using BCS and BCL, together with the fact that LEU tends to yield a higher cell procurement rate/kg in a controlled manner, we would suggest using LEU in the development of tolerance induction protocols in our transplantation center. However, the disadvantage of LEU are the resources

required to maintain an on-call team comprising an apheresis nurse and access surgeon, and that a special vehicle with a suitable on and off-loading system is required for the heavy leukapheresis machine.

6 CONCLUSIONS

The main conclusions of this thesis are as follows:

- Studies I–II: Cibinetide mitigated the intial damage of transplanted islets by reducing inflammation at the transplant site and mitigated IBMIR related platelet consumption.

- Studies I–II: The reduced activity of innate immune system subsequently reduced the activation of the adaptive immune responses and, in doing so, may have improved the long-term graft function.

- Study III: Intra-portal infusion of autologous Tregs together with allogenic pancreatic islets is a safe method and could be used to administer regulatory cells in tolerance induction protocols.

- Study IV: In the production of a donor-specific regulatory cell product, the costimulatory blockade CTLA4-Ig is as effective as 2D10.4/IT2.2 (CD80 and CD86 mouse antibodies).

- Study V: Bedside leukapheresis before organ donation tends to yield a higher cell procurement rate/kg with lower percentage of granulocyte and NK-cell contamination compared to the other evaluated methods (BCL and BCS).

7 POINTS OF PERSPECTIVE

The studies included in this thesis can be seen as a "rite of passage" that takes encouraging concepts and prepares them for a clinical reality. Although the studies show promising results, important measures beyond this thesis will be necessary to demonstrate whether these concepts might improve the outcomes and the lives of transplant patients.

Studies I–II show that the non-hematopoietic EPO analogue cibinetide ameliorated the local innate immune responses towards the graft after islet transplantation, reduced IBMIR related platelet consumption and could improve both short and long-term graft function in a rodent PITx model. Importantly, even though cibinetide is included in the EPO analogue family, we have not detected any side effects related to thromboembolism. Cibinetide has previously been evaluated in clinical trials and has proven to be safe with no clinically relevant side effects (97, 100). Although we have shown improved outcomes from the perspective of PITx both in-vitro and in-vivo and are aware of some of the intra and inter-cellular mechanisms of cibinetide, there is still a lack of knowledge regarding cibinetides mechanism towards other cell lines in the immune system, for example, B cells or neutrophils. Further research focusing on cell mechanistics should be performed, not only to increase knowledge of the compound, but also to uncover additional beneficial aspects of this drug. At a later stage we would like to evaluate cibinetide as an induction agent in clinical PITx in addition to conventional IS. This promising drug might also be beneficial in intra-portal hepatocyte transplantation, which takes place in a similar immunological setting as PITx. Given that the effect of innate immune responses also impacts whole organ transplants, even if not as violently as in cell transplantation, a non-hematopoietic EPO analogue could also be beneficial, for instance, as an addition in cold perfusion solutions during deceased donor organ procurement procedures, or for use in novel warm or cold ex-vivo organ perfusion devices. These ideas could offer interesting research opportunities in the future. The effect of innate immune responses and its synergistic connection to the adaptive immunity, is increasingly recognized. If we could perform organ transplantation with no inflammation and innate immune responses at all, would there still be a subsequent risk of rejection? Moreover, cibinetide is only one of several potential agents that could mitigate initial immune reactions. These reactions also go hand in hand with the ischemia-reperfusion effect that should be further highlighted in the field of transplantation research.

Study III addresses the concept of regulatory cell therapy using enriched Tregs administered directly to the transplant site, via the same intra-portal pathway to the liver as the islet graft. This method was found to be safe. The concept can be used to further evaluate co-infusion beyond the framework of this safety trial. Continued research evaluating a more effective cell product, for example, DSIMC (evaluated in study IV) and with a protocol of IS reduction or weaning, should be prioritized in future research. PITx is a field in which IS minimization/tolerance induction strategies may benefit the most since the current IS standard

(CNI) is beta-cell toxic. PITx also has the advantage in tolerance induction trials, because a possible rejection of a graft is not directly life threatening in the same way that the rejection of a liver or heart could be. However, since islet transplantations are rare, carefully coordinated multicenter studies are required to optimally evaluate these kinds of regulatory cell protocols.

Over the last two decades, the development of regulatory cell therapies has accelerated and shown encouraging results in living donor organ transplantation settings (52-54, 131). Thus, it is of great importance and a natural step further to develop protocols suitable for deceased donor organ transplant recipients, since most of the organs used for transplantation in Western countries are from deceased donors. The latter studies IV–V in this thesis depict the preparatory work prerequisite for the clinical implementation of a regulatory cell tolerance induction trial using DSIMC in a deceased donor liver transplantation setting. This has included evaluating cell production protocols from a GMP perspective and obtaining the required raw material for cell production in a non-elective setting. The results of these studies have allowed our center to initiate the planning to perform a phase I/IIa clinical evaluation using DSIMC.
Globally, several research centers are presently conducting pilot/safety studies of regulatory cells to induce tolerance after allogenic organ transplantation. Many of these research centers focus on purifying a single cell line, for example, Tregs, regulatory DCs and regulatory macrophages (53). The DSIMC product we suggest using, comprises a "cocktail" of a donor de-sensitized cell mix that has not been subject to any purification. Our preliminary lab results have shown that the enrichment of Treg in our protocol may hamper donor-specific immunomodulation. Further preclinical mechanistic studies to characterize various regulatory cell lines and, most importantly, the interaction of these cell lines, are required. This will allow us to understand on a more detailed level the mechanisms of immunological tolerance and to refine future precision medicine cell therapies.

Although the age of precision medicine provides opportunities to make great leaps towards individualized health care, novel challenges have arisen. In cell therapy projects, classed as ATMPs, the issue of reimbursement may inhibit the progress of research, especially for academic research groups. GMP facilities are required for safe and consistent cell production. Since one batch of cell product may only be used for one patient, mass production is not possible, which makes it very costly and may be a less attractive option for pharmaceutical companies. These kinds of treatment options have obstacles to overcome that are not only academic in nature. There are also regulatory and political issues that need addressing in order to allow future specialized health care to be shaped.

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