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Boosting Beta-Cell Replacement Therapy For Treatment Of Type 1 Diabetes

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“I Hear And I Forget. I See And Remember. I do, And I Understand.”

By Confucius

(As Palavras convencem, mas os exemplos arrastam/Actions speak louder than words)

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ABBREVIATIONS

AICD	activation-induced cell death
APCs	antigen presenting cells
ATG	anti-thymocyte globulin
BB	BioBreeding
BCRs	B-cell receptors
BMP4	bone morphogenetic protein 4
BOECs	blood outgrowth endothelial cells
CAM	chick chorioallantoic membrane
Ctla-4	cytotoxic T lymphocyte antigen 4
DAISY	Diabetes Autoimmunity Study of the Young
DCs	dendritic cells
DiaMond	Diabetes Mondiale study
DPT-1	Diabetes Prevention Trial-1
dsRNA	double-stranded RNA
EGF	epidermal growth factor
EPCs	endothelial progenitor cells
ER	endoplasmic reticulum
ESC	embryonic stem cells
FGF	fibroblast growth factor
Foxp3	forkhead box p3
GAD	glutamic acid decarboxylase
GADA	GAD autoantibodies
GCSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GRP78	glucose regulated protein 78
GVHD	graft versus host disease
GWAS	genome wide association study
HGF	hepatocyte growth factor
HIPs	hybrid insulin peptides
HLA	human leukocyte antigen

HSCT	hematopoietic stem-cell transplantation
IA-2	insulinoma antigen-2
IAA	insulin autoantibodies
IBMIR	instant blood-mediated inflammatory reaction
ICA	islet cytoplasmic antibodies
IFN	interferon
IGF	insulin-like growth factor
IGRP	islet-specific glucose-6-phosphate catalytic subunit-related protein
Igs	immunoglobulins
IL	interleukin
IL2RA	IL2 receptor α chain
Ins	insulin
iPSCs	induced pluripotent stem cells
JNK	c-Jun N-terminal kinase
KGF	Keratinocyte growth factor
KLN	kidney draining lymph nodes
LADA	Latent Autoimmune Diabetes in Adults
L. lactis	*Lactococcus lactis*
mAb	monoclonal antibody
MAPC	multipotent adult progenitor cell
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
MMP	matrix metalloproteinase
MSCs	mesenchymal stem cells
mTOR	mammalian target of rapamycin
NCSCs	neural crest stem cells
Ngn3	neurogenin 3
NK	natural killer
NOD	non-obese diabetic
nPOD	pancreatic organ donors with Diabetes

PKC	protein kinase C
PAK	pancreas after kidney transplant
PDGF	platelet-derived growth factor
PLN	pancreatic lymph nodes
poly-(I:C)	polyinosinic-polycytidylic acid
PTA	pancreas transplant alone
PTM	posttranslational modification
Ptpn22	protein tyrosine phosphatase non-receptor type 22
OKT3	Ortho Kung T3
ROS	reactive oxygen species
RPL27	ribosomal protein L27
Shh	sonic hedgehog
SMA	α-smooth muscle actin
SNP	single nucleotide polymorphism
SPK	simultaneous pancreas-kidney transplant
T1D	type 1 diabetes
T2D	type 2 diabetes
T1D	type 1 diabetes
TCR	T-cell receptor
TEDDY	The Environmental Determinants of Diabetes in the Young
Teff	T effector cells
TGF	transforming growth factor
Tfh	T follicular helper
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
VEGF	vascular endothelial growth factor
WHO	World Health Organization
ZnT8	zinc transporter 8

CHAPTER 1

INTRODUCTION[1]

[1]partially based on João Paulo Monteiro Carvalho Mori Cunha, Conny Gysemans, Pieter Gillard, Chantal Mathieu. Stem Cell-Based Therapies For Improving Islet cell transplantation Outcomes In Type 1 Diabetes. Current Diabetes Reviews (in press)

1.1 DIABETES MELLITUS

Currently, diabetes mellitus affects 415 million people around the world, and this number is expected to increase to 642 million by 2040 (http://www.idf.org/). This metabolic disorder of multiple etiologies characterized by chronic hyperglycemia is the most common cause of nephropathy, retinopathy, neuropathy, and cardiovascular disease leading to heart attack and stroke (http://www.who.int/diabetes/en/). Diabetes mellitus is the fourth leading cause of death in most developed countries, and the disease diminishes not only the quality of life but also life expectancy [1]. While type 2 diabetes (T2D) is the most common form of diabetes, type 1 diabetes (T1D), the main focus of this thesis and previously classified as insulin-dependent diabetes mellitus and juvenile-onset diabetes, accounts for over 90% of diabetes in young people aged under 25 years and results from a selective autoimmune-mediated destruction of insulin-producing beta cells contained in the islets of Langerhans in the pancreas [2–5]. The incidence of T1D is increasing worldwide especially in the last three decades and in children less than five years of age, but the roots of this steady rise are poorly documented [6,7]. Up to date, the standard of care for all T1D patients but also for 25% of T2D patients is lifelong exogenous insulin administration as basal-bolus therapy either with several daily injections or continuous subcutaneous insulin infusion. Despite impressive improvements in insulin analogs, insulin pumps, and glucose sensors, current insulin therapies still do not match the insulin secretory profiles of endogenous pancreatic beta cells, and all pose risks of suboptimal glycemic control, hypoglycemia, and ketoacidosis, in particular in children and adolescents. Therefore, in patients with recurrent hypoglycemic episodes despite optimal insulin therapy and chronic macro- and microvascular complications, alternative approaches are required [5,8,9].

1.2 TYPE 1 DIABETES AS AN AUTOIMMUNE DISEASE

T1D is a T-cell-mediated autoimmune disease that induces selectively loss of functional beta-cell mass in the endocrine pancreas, leading to absolute insulin deficiency [10]. The destructive process of the beta cells by the immune system in the patients starts many years before clinical detection and presentation of the first symptoms of the disease. What triggers the autoimmune destruction of the beta cells in T1D remains unidentified, but genetic and environmental factors are believed to act together to precipitate the disease. Moreover, our understanding of the immunopathogenesis of the disease has increased tremendously during the last years and points towards major imbalances between multiple regulatory and effector immune mechanisms.

1.2.1 *Genetics*

The primary risk factor for T1D is genetic (reviewed in [11]) as evidenced by the strong disease concordance in monozygotic twin pairs (≥50%)[12]. Moreover, kids born in T1D families have different lifespan risks depending on whether the mother (3%), father (5%) or a brother/sister (8%) has the disease [13]. Four decades of studies have shown that immune genes, especially those that encode for the human leukocyte antigen (HLA) system, the major histocompatibility complex (MHC) proteins in humans, confer the strongest genetic risk for T1D [14]. The genes that encode the HLA molecules, the most polymorphic known in the human genome, are located on chromosome 6p21. HLA class I (i.e. HLA-A, B and C) molecules are found in almost all cells, while HLA class II (i.e. HLA-DR, DQ, and DP) molecules are confined to professional antigen-presenting cells (APCs), including macrophages, dendritic cells, and B cells, and in humans, activated T cells also express HLA class II. By the 1980s, the very high risk of having a heterozygous genotype consisting of a haplotype including HLA-DRB1*0301 on one chromosome and HLA-DRB1*0401 on the other (commonly referred to as HLA-DR3/4) was reported [15]. The combinations HLA-DR3-DQ2 (DQB1*0201) and HLA-DR4-DQ8 (DQB1*0302) are present in 90% of T1D children [16,17], but an environmental trigger is usually needed to further progress to beta-cell autoimmunity (see 1.2.2). In patients harboring the high-risk HLA-DR3 and HLA-DR4 class II alleles, the HLA-A*0201 allele has been shown to confer additional risk to T1D development [18]. HLA-A*0201 is one of the most prevalent HLA class I alleles, with a frequency of more than 60% in T1D patients [19]. The genotype combining two susceptibility haplotypes contributes to the highest risk of T1D and is most common in kids who develop the disease at a very early age [20]. Since HLA molecules are known to play a role in antigen presentation, the HLA linkage and association support the concept that T1D has an important autoimmune element. Interestingly, islet autoantibodies (see 1.2.3), at the time of T1D diagnosis, are known to be associated with HLA-DR-DQ haplotypes or alleles. Kids homozygous for HLA-DR3-DQ2 often have autoantibodies against the 65 kD isoform of glutamic acid decarboxylase (GAD65)(GADA) as the first autoantibody, while kids with the HLA-DR4-DQ8 haplotype tend to have insulin autoantibodies (IAA) as the first autoantibody.

Nowadays, it is clear from genome-wide association study (GWAS) meta-analyses and single nucleotide polymorphism (SNP) profiling studies that also other genes confer risk to T1D development (e.g. the insulin (*Ins*) gene on chromosome 11 is associated with T1D)[21]. The *Ins* gene was identified as the second most important genetic susceptibility factor, contributing 10% of the risk to T1D [22,23]. There is also evidence for another risk locus in human T1D,

which is mapped in the *Il2RA* region on chromosome 10p15.1 [24]. The interleukin-2 (IL2) receptor alpha (*Il2RA*, also known as *CD25*) encodes the α-chain of the IL2R complex and binds IL2 with high affinity. CD25 is expressed on T cells upon activation and also on natural regulatory T cells (Tregs) at baseline. Since IL2 is essential for Treg growth and survival, and IL2 signaling is mainly mediated by IL2RA [25], a reduction in the expression or function of *Il2RA* gene could contribute to autoimmune disease susceptibility. Two SNPs in the *Il2RA* gene were found to be significantly associated with T1D and suggested a potential role of *Il2RA* in the pathogenesis of T1D, likely involving Treg functionality [26–28]. Cytotoxic T-lymphocyte-associated protein 4 (*Ctla4*) is a down-regulator of the immune response. T1D association with *Ctla4* was noted in multiple ethnic groups, although with a variable degree of importance [29]. The immune regulator *Ptpn22* (gene located at chromosome 1p13) was shown to be associated with T1D in 2004 [30]. The *Ptpn22* gene encodes a protein tyrosine phosphatase important in down-regulation of the immune response. Since *Ctla4* and *Ptpn22* are also implicated in other autoimmune diseases including autoimmune thyroid disease [31], it supports the idea that similar or overlapping biological pathways for a different autoimmune disease may exist [32,33]. Interestingly, the susceptible T allele of the *Ptpn22* SNP can be linked with IAA or GADA. On the other hand, the susceptible A allele of the *INS* SNP has linkage with IAA, but not with GADA, whereas the minor G allele of the *Ctla4* SNP increased the risk for GADA positivity, yet not for IAA [34].

1.2.2 ***Environment***

Only environmental and lifestyle changes can clarify the steep rise in T1D incidence over the last three decades (reviewed in [35]). In Europe, T1D risk varies considerably in people who are genetically alike but separated by social and economic boundaries [36]. This hazard becomes more uniform in societies with the ability to travel [37]. Several hypotheses have been put forward, one of the most accepted is called the "hygiene hypothesis," which relates to improved sanitation and postulates that fewer early infant infections and less diverse symbiotic gut microflora deviate the immune system towards beta-cell autoimmunity [38]. Numerous viruses have been implicated in T1D etiology, with enteroviruses having the strongest association with T1D in both animal models [39] and men [40]. Several studies suggest a role for innate immune system activation in this model. Direct evidence for the involvement of viruses in T1D comes from the injection of polyinosinic-polycytidylic acid (poly-(I:C)), a mimic of viral double-stranded RNA (dsRNA) that induces interferon (IFN)-α secretion, which triggers insulitis and diabetes in normal non-diabetic rodents [41,42]. In humans, other

convincing evidence of associations between viral infections and T1D risk comes from the congenital rubella syndrome [43]. T1D developing in congenital rubella patients has the genetic and immunological features of classical T1D [44]. Other viral infections especially in the gastrointestinal tract (e.g. rotaviruses) have been reported to be associated with the appearance of serum autoantibodies to islet antigens [45]. However, the role of viral infections as a cause of beta-cell loss in human diabetes remains to be elucidated.

Another important environmental factor that is believed to influence T1D is diet. Several candidates have been described such as a short duration of breastfeeding [46,47], the feeding of cow's milk proteins during the early months of infants' life [48], and the early introduction of solid foods and cereals [47]. However, results of these studies must be interpreted with caution.

The involvement of the gastrointestinal system in T1D etiology is suggested by differences in intestinal microbiota composition observed in individuals diagnosed with T1D or with evidence for islet autoimmunity [49–51]. In addition, proof-of-concept studies conducted in NOD mice provide evidence to suggest that changes in the composition of intestinal microbiota prevent or reduce T1D incidence [52–54].

Numerous studies have found associations between T1D development and vitamin D (reviewed in [2,55]). The host lab has a great interest in the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3, as a natural immunomodulatory molecule [56]. 1,25-dihydroxyvitamin D_3 prevents insulitis and diabetes in NOD mice, although with serious calcemic side effects [57,58]. Interestingly, in mice, vitamin D deficiency predisposes to diabetes [59] while vitamin D supplementation prevents T1D [60]. Based on these observations, the host lab proposes that vitamin D deficiency should be avoided, especially in all at-risk people.

Besides "the hygiene hypothesis" also "the beta-cell stress hypothesis" exist which suggests that common environmental elements such as birth weight and infant growth, toxins and chemical compounds, dietary deficiencies or biological stress (leading to posttranslational modifications (PTMs) and the generation of islet neoantigens [61–63](see 1.2.3), alone or in combination, exhaust and damage the pancreatic beta cells, leading to secondary beta-cell autoimmunity. Several questions still remain about the environmental and lifestyle influence in T1D. However, some observational cohort studies such as The Environmental Determinants of Diabetes in the Young (TEDDY) study aim to ascertain environmental determinants that may trigger islet autoimmunity and either speed up or slow down the progression to clinical onset in subjects with evidence for persistent islet autoimmunity will bring new insights (https://teddy.epi.usf.edu/).

1.2.3 *Autoantibodies*

There are several islet autoantibodies and autoantigens related to T1D. In kids who are genetically at risk for developing T1D, the onset of T1D-related autoantibodies peaks between 9 and 24 months of age, with IAA appearing first [64]. Indeed, the most widely used in clinic and research are IAA, islet cell cytoplasmic autoantibodies (ICA), and autoantibodies directed against the four major pancreatic islet autoantigens: GAD, protein tyrosine phosphatase IA-2A and its isoform IA-2b/phogrin (IA-2bA) and zinc transporter 8 (ZnT8). Recently a fifth autoantigen has been described: tetraspanin-7, a multipass transmembrane glycoprotein with neuroendocrine expression [65]. Although tetraspanin 7 autoantibodies are a marker of T1D, they seem to provide minor additional value to existing autoantibodies in identifying ongoing beta-cell destruction [66]. Other T1D-related autoantibodies have also been described but are difficult to measure and/or are often not sufficiently sensitive. Recently, several groups proposed that PTMs within the endoplasmic reticulum (ER) of beta cells under stress might impact on the autoantigen T-cell epitope repertoire and T1D development [61–63]. For example, the host lab demonstrated that citrullinated glucose regulated protein 78 (GRP78) is a new autoantigen in T1D in NOD mice [67]. Lately, the group of K. Haskins demonstrated that through transpeptidation of insulin fragments a new class of naturally occurring autoantigens called hybrid insulin peptides (HIPs) can be formed. The resulting peptides are very immunogenic not only for diabetogenic $CD4^+$ T cells from the NOD mice but also for $CD4^+$ T-cell clones isolated from the residual islets of T1D patients [68,69].

From all the major autoantigens only insulin is unique to the beta-cell. IAA themselves do not cause the disease, but as IAA are detected in the initial pathogenesis of human and NOD mouse T1D. (Pro)-insulin has been identified as a dominant autoantigen in T1D and might trigger the process of beta-cell autoimmunity and destruction [70,71]. IAA in NOD mice is usually detected after 6 weeks of age and reach a maximum between 8 and 16 weeks and then decline [64]. NOD mice do not necessarily express positive IAA values when diagnosed with symptomatic diabetes. NOD mice expressing IAA at 8 weeks of age develop diabetes earlier. Interestingly, eliminating immune responses to insulin blocked not only generation of T1D and insulitis but also immune responses to downstream autoantigens, such as the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) in NOD mice [72]. Moreover, beta-cell autoimmunity in NOD mice is avoidable by tolerogenic expression of proinsulin long term in the APCs [73]. The host lab demonstrated that oral delivery of human proinsulin together with IL10 by genetically modified *Lactococcus lactis* (*L. lactis*) bacteria and a short induction course of anti-CD3 reversed established T1D in mice long term [74](see 1.3.3.1.1).

However, insulin-based trials to prevent or reverse diabetes in humans have failed to demonstrate a therapeutic benefit (see 1.3.1).

ICA was first described in the 70s [75] and are detected in 70 to 80% of individuals with new-onset T1D. ICA positivity declines after clinical diagnosis of T1D. After ten years, only 5% of the diabetic patients remain ICA positive. In the Diabetes Prevention Trial-1 (DPT-1), a multicenter study, non-affected relatives of people with T1D who were screened for ICA positivity and then found to have low first-phase insulin responses to intravenous glucose challenge had a 5-year risk of the development of T1D of 60% and a projected 10-year risk near 90%.

In the 90s, GAD was discovered to be the 64 kDa molecular weight islet autoantigen that was immunoprecipitated by diabetic (but not non-diabetic) sera [76]. GAD catalyzes the conversion of glutamic acid to the inhibitory neurotransmitter GABA (γ-amino butyric acid). Biochemical analysis has shown that GAD is found in a variety of tissues, with highest concentrations in the nervous system. GAD is an intracellular enzyme, and thus it is not normally expressed on the surface of the beta cell [77]. Although there are two major isoforms of GAD, GAD autoantibodies (GADA) which are more frequently detected are those directed against GAD65 (65 kDa molecular weight) instead of GAD67 (67 kDa molecular weight). GADA are found at similar rates (70 to 80%) as ICA in patients with new-onset T1D. GADA testing is preferred over ICA testing when the diagnosis of latent autoimmune diabetes of adulthood (LADA) is sought in individuals with long-standing diabetes since after the onset of T1D, GADA is more persistent than ICA [78,79].

IA-2A was detected by screening an insulinoma expression library for reactivity with sera from T1D patients. IA-2A is less common at the onset of T1D (around 60%) than either ICA or GADA [80]. Unquestionably, as the number of islet autoantibodies expressed by a non-diabetic individual rising, their risk for T1D also rises. Individuals positive for a single islet autoantibody are far less likely to develop T1D than individuals who are positive for multiple islet autoantibodies. In general, the number of islet autoantibodies expressed by an individual is a more important predictor of T1D than any specific combination of islet autoantibodies [81]. In DPT-1 per year of follow-up, T1D developed in approximately 15% of unaffected first-degree relatives who were repeatedly positive for ICA and had persistent low insulin secretion in response to intravenous glucose [82]. The greater the number of islet autoantibodies present, the greater the risk of developing T1D: the risk for T1D in 4-autoantibody-positive individuals: 50%; the risk of T1D in 3-autoantibody-positive individuals: 40%; the risk of T1D in 2-autoantibody-positive individuals: 16%; the risk of T1D in 1-autoantibody-positive individuals:

3%; and the risk of T1D in 0-autoantibody-positive individuals: 0.5%. For IAA determinations, the CVs are considerably larger (e.g. up to 20 – 25%).

ZnT8A were first described in 2007, and ZnT8A have the potential to be the fourth major biochemical autoantibody and the fifth overall major islet autoantibody[83]. In young patients with new-onset T1D, 63% were positive for ZnT8A (vs. 72% positive for GADA, 68% positive for IA-2A, and 55% positive for IAA). Following the onset of T1D, ZnT8A concentrations decline rapidly [84]. In young patients with new-onset T1D who were negative for ICA, GADA, IA-2A, and IAA, 26% were positive for ZnT8A [85]. Of new-onset T1D patients tested for GADA, IA-2A, and IAA, 6% were negative for all three autoantibodies. However, when ZnT8A testing was added to this panel, only 2% of new-onset T1D patients were islet autoantibody negative. Whereas 72% of new-onset T1D patients were positive for 2 or more autoantibodies when testing was limited to GADA, IA-2A, and IAA, the addition of ZnT8A testing increased the number of individuals positive for 2 or more autoantibodies to 82%. Whereas IAA prevalence at the onset of T1D declines with increasing age of the patient, ZnT8A becomes more common at onset with increasing age. This finding argues in favor of testing for all four biochemical autoantibodies when a marker of islet autoimmunity is sought. The predictive value of ZnT8A for T1D development was evaluated in 43 subjects in the Diabetes Autoimmunity Study of the Young (DAISY) study (30 first-degree relatives of T1D patients and 13 individuals from the general population; mean age at onset of T1D: 7 years). Based on HLA-DR and HLA-DQ genotype, or family history, DAISY consecutively follows children at increased risk for T1D. Comparable to other biochemical autoantibodies, ZnT8A were found in 63% of individuals before T1D development (vs. 70% positivity for GADA before T1D onset; 52% for IA-2A, and 85% for IAA). IAA were most commonly the first autoantibodies to be detected, followed by GADA, ZnT8A, and IA-2A. The later presence of ZnT8A suggests that reactivity to ZnT8 may reflect progressive beta-cell destruction. In 88 participants in the DAISY study who were more than three years old and were followed for more than one year, positivity for one biochemical autoantibody (excluding ZnT8A) yielded a 7% risk of T1D. However, when ZnT8A were present together with GADA, IA-2A, or IAA, the risk for T1D rose approximately 5-fold to 37%. As noted above, natural variants of ZnT8 exist.

1.2.4 *Immune cells*

During T1D progression immune cells – macrophages, B cells, natural killer (NK) cells and T cells (mostly $CD8^+$ T cells) – invade the pancreatic islets generating an inflammatory condition

called insulitis (Figure 1.1). More than 100 years ago, this condition was first described in a 6-year-old girl who died from ketoacidosis complications [86].

Macrophages and dendritic cells (DCs) are APCs being among the first cells to infiltrate the pancreatic islets during diabetes development in T1D animals [87]. Recent studies demonstrated that the myeloid cells found in NOD islets are typically composed of Batf3-dependent F4/80$^+$ macrophages with few CD103$^+$ DCs [88]. This infiltration precedes the invasion of the islets by B cells, T cells, and NK cells. The activation of autoreactive T cells may be initiated from the processing and presentation of pancreatic islet autoantigens by APCs. Inactivation of macrophages in T1D animals significantly prevents T1D development [89,90]. Further studies showed that macrophages are required for the generation of autoreactive T cells that destroy beta cells in NOD mice. T cells in the macrophage-depleted 8.3-T-cell receptor (TCR)-beta transgenic NOD mice did not destroy transplanted NOD islets, indicating that T cells in a macrophage-depleted environment lose their ability to differentiate into beta-cell-specific cytotoxic T cells [91]. While circulating monocyte-derived, tissue-infiltrating macrophages (Ly6C$^+$) have a proinflammatory phenotype and function in T1D [92,93], the role of their tissue-resident or controlling equivalents (Ly6C$^-$) is less known. Recently, a new class of immunoregulatory tissue-resident macrophages (CD39$^+$, CD73$^+$ and galectin-9$^+$) has been identified and found to be reduced in NOD islets [94](Figure 1.1). After tissue infiltration, Ly6C$^+$ monocytes can develop into either macrophages or DCs.

DCs are essential for both the priming of naive T cells for protective immune responses and the purging of the T-cell pool for autoreactive clones. DCs are the most potent APCs allowing them to professionally take-up and cross-present processed antigen fragments to T cells. Depending on the differentiation state of DCs, antigen cross-presentation can lead to functional activation, deletion, or anergy of T cells (reviewed in [95]). Several groups have demonstrated that compared with those from non-autoimmune-prone mice, NOD DCs do not mature normally in response to a variety of stimulatory signals [96,97]. This could be of significance because a higher level of stimulation is required to induce T-cell deletion by activation-induced cell death (AICD) than needed to trigger effector T-cell function. In NOD mice, microcytic DCs (CD11c$^+$CD11b$^{-/lo}$CD8α$^-$PDCA-1$^-$) which can mediate cross-presentation of islet antigens to CD4$^+$ and CD8$^+$ T cells in the draining lymph nodes are found in high numbers [98]. When loaded with antigen these DCs can rescue CD8$^+$ T cells from peripheral anergy and deletion, and stimulate autoreactive CD4$^+$ T cells [99]. On the other hand, pre-diabetic NOD mice have low numbers of tolerogenic CD8α$^+$CD103$^+$Langerin$^+$ DCs, with reduced expression of CCR5,

CLEC9A, and IL10, locally in the pancreas [100]. Collectively, these data suggest that imbalances between damaging and tolerogenic cells (i.e. macrophages, DCs but also T cells) may surely contribute to the initiation of the disease (Figure 1.1).

B cells have the ability to secrete antibodies and cytokines, and present antigens (Figure 1.1). The islet autoantibodies (for insulin, GAD65, IA-2, and ZnT8) secreted by B cells are currently used as diagnostic and prognostic criteria and also as surrogate markers for disease activity. These autoantibodies are not believed to instigate T1D but, rather, provide evidence of autoreactive T-cell/B-cell interactions. NOD mice in which B cells express membrane-bound (but not secretory) immunoglobulins (Igs) develop T1D with penetrance comparable to regular NOD mice [101]. Antigen-experienced B cells can present antigens and secrete cytokines which can either encourage or suppress immunity. Each B cell recognizes a single antigenic epitope on folded proteins via the antibody variable (V) region of its B-cell receptors (BCRs), internalizes that protein, and processes it into peptide fragments for presentation on the MHC-II molecules to T cells. Thus, autoreactive B cells are uniquely equipped to turn-on autoreactive T cells. In fact, several studies have irrefutably demonstrated that B-cell-deficient NOD mice do not develop T1D [102] in addition to NOD mice with a B-cell-restricted deficiency of the MHC-II (I-Ag7 in NOD) molecule [103]. NOD mice with transgenic B cells which do not recognize an islet antigen (i.e. insulin) fail to develop diabetes, while skewing the B-cell repertoire towards insulin promotes diabetes development [101,104]. Also, peripherin-autoreactive B cells are potent contributors to T1D pathogenesis [105]. While B cells likely play an important role in T1D pathogenesis via antigen cross-presentation to $CD4^+$ and $CD8^+$ T cells, these interactions seem not required for the initial recruitment of T cells to the pancreas. In new-onset diabetic NOD mice, long-term disease reversal was observed in mice treated with anti-CD20 [106]. Some initial clinical success was described with a B-cell targeting approach (i.e. rituximab) as T1D intervention strategy [107]. However, follow-up studies demonstrated that C-peptide improvement was limited to the early period after rituximab administration and disappeared [108]. Moreover, the appearance of new autoreactive B cells [109] and the depletion of IL10-producing regulatory B cells [110] after rituximab therapy question the usefulness of this approach as single agent therapy.

T cells are accepted as the most important players in a pancreatic beta-cell loss in T1D, with $CD4^+$ and $CD8^+$ T cells bearing autoreactive TCRs being critical for T1D pathogenesis (Figure 1.1). In humans, $CD8^+$ T cells primarily invade the pancreatic islets [111], yet their activation and proliferation probably require $CD4^+$ T-cell support. For example, studies performed within the framework of the Pancreatic Organ Donors with Diabetes (nPOD) network demonstrated

that islet-specific hyper-expression of HLA class I molecules is a consistent observation in post-mortem pancreatic samples from T1D patients but not from healthy individuals and that $CD8^+$ T cells dominate the lymphocytic infiltrate [111]. Not only do pancreatic islets of T1D patients have an increased antigen presentation capacity for $CD8^+$ T cells, they also promote infiltration of the chemokine receptor $CXCR3^+$ T cells via secretion of the interferon (IFN)-γ-inducible protein 10/CXCL10 [112,113]. The CXCR3 receptor can guide effector $CD8^+$ T cells toward activated DCs to receive further "help" from $CD4^+$ T cells or recruit them to inflamed tissues. $CD4^+$ T cells can pick from several differentiation fates following activation, and this choice has profound consequences for their cytokine production and migratory potential. Initial models of T-cell generation concentrated on the dichotomy between T helper-1 (Th1) and Th2 immune reactions, with T1D being viewed mainly as a Th1-mediated pathology. However, several extra fate choices have appeared, including Th17 cells and follicular helper T (Tfh) cells (reviewed in [114]). Under an IFN-γ signal transducer and activator of transcription (STAT) 1-dependent signal, naïve $CD4^+$ T cells develop into Th1 cells, through the activation of the transcription factor T-bet (T-box expressed in T cells). Th1 cells are important for macrophage and $CD8^+$ T-cell activation and clearance of intracellular pathogens. Increasing levels of IFN-γ correlated with diabetes progression in NOD mice [115], and IFN-γ is required for diabetes induction in a virus-induced T1D model [116]. In this view, Th1 has a strong relation with autoimmune T1D. Th2 cells are key coordinators of humoral immune responses. The key transcription factor in Th2 differentiation is GATA (GATA-binding protein) 3. Th2 cells stimulate IgM, IgG1, and IgE synthesis by B cells and activate eosinophils, thus promoting hypersensitivity responses due to their capacity to produce IL4, IL5, IL10, and IL13. The exogenous provision of IL4 was shown to inhibit diabetes in NOD mice [117], and transgenic expression of IL4 in the islets under the control of the insulin promoter completely prevented diabetes development [118]. The cornerstone of the Th1/Th2 dichotomy is that Th1 and Th2 cells negatively cross-regulate the function of one another through their characteristic cytokines [119]. Unlike Th1, Th2 cells are often described to have protective effects on beta-cell autoimmunity, although this view is oversimplified as Th2 cells and their mediators have been shown to facilitate the immune invasion of the islets [120] and accelerate beta-cell autoimmunity [121].

With the discovery of IL17-producing (Th17) cells, the Th1/Th2 paradigm was seriously revised and this opened the discussion that tissue-specific autoimmunity might be driven by Th17 cells rather than Th1 cells. The Th17 development requires TGF-β and STAT3 activation in response to IL6, which in turn triggers the transcription factor retinoic acid-related orphan receptor (ROR)-γt. To date, the role of Th17 cells in diabetes remains indeterminate. In murine

studies it is suggested that IL17 is upregulated in the early stages of diabetes development [122,123], however, it was also shown that silencing IL17 expression did not protect NOD mice from diabetes [124]. Th17 cells from BDC2.5-TCR transgenic mice were capable of inducing diabetes but appeared to achieve this by differentiating further to a Th1 phenotype [125,126]. Indeed, the subsequent pathology could be inhibited by antibodies to IFN-γ but not IL17 [125,126]. Conversely, two reports documented the ability of IFN-γ deficient Th17 cells to transfer diabetes successfully [127,128], arguing against the requirement for a Th1 transition. Fate mapping experiments in mice established that in autoimmunity, IL17-positive T cells can initiate IFN-γ production leading to the presence of a substantial number of T cells co-expressing both cytokines. Interestingly, cells co-producing IFN-γ and IL17 (Th1/Th17 cells) have been implicated in T1D development. By measuring IFN-γ transcripts within sorted IL17-producing cells, a study in T1D children found an increased frequency of IL17 cells to make IFN-γ compared with healthy controls [129]. Additionally, both IL17 and IFN-γ were up-regulated at the mRNA level within the pancreatic islets of an individual who died within 5 days of T1D diagnosis [123]. Besides Th1, Th2 and Th17 cells, Tfh cells have also been implicated in T1D pathology [130]. These cells are essential for the formation of germinal clusters, specialized structures in secondary lymphoid organs in which maturation of B cells into high-affinity plasma cells and long-lived memory B cells takes place. Both mice and patients with T1D seem to have an expansion of their Tfh cells. Moreover, Tfh cells might be the source of IL21 production in T1D [130]. IL21 is a cytokine with the capacity to act on different cell types, including $CD4^+$ and $CD8^+$ T cells, NK cells, B cells, macrophages and DCs. In a TCR transgenic diabetes model, a mutation in Roquin leading to aberrant Tfh development dramatically enhanced diabetes generation [131]. In a second TCR transgenic model, based on a different pancreatic antigen and different transgenic T cells, enriching for Tfh cells increased their capacity to cause diabetes upon adoptive transfer [130]. Although IL21 is the characteristic cytokine associated with Tfh cells, they are also known to be capable of producing other cytokines, including IFN-γ, which could explain the association of this cytokine with T1D [132]. Several studies have implicated repeated antigen presentation in Tfh development [133], a concept that fits with the inability of autoantigens to be cleared in a disease context.

Recent literature present new insights in T1D pathogenesis demonstrating that T cells specific for beta-cell antigens exist normally in healthy individuals, but are prevented from their autoreactivity by immunoregulatory mechanisms, especially through the actions of Tregs (reviewed in [134,135])(Figure 1.1). T1D may develop from a failure to suppress autoreactive T cells by immunoregulatory mechanisms, permitting them to become activated, expanded, and

finally enter a cascade of inflammatory processes, leading to progressive beta-cell destruction. Tregs are a diverse population of lymphocytes that can counterbalance adaptive immune responses. Tregs counteract autoreactive T cells, induce tolerance, and reduce inflammation [136–142]. Among these regulatory cell populations are natural Tregs (nTregs; $CD4^+Foxp3^+$ T cells) that arise in the thymus during early fetal development as a consequence of positive selection involving interactions of "intermediate affinity" [143,144], whereas conventional $CD4^+$ T cells are selected based on "low affinity" interactions. nTregs can suppress $CD4^+$ T cells, $CD8^+$ T cells, NK cells, DCs, monocytes/macrophages, B cells, basophils, eosinophils, and mast cells [144,145]. IL2 is essential for nTreg development, function, and homeostasis (i.e., CD25 is the α-chain of the high-affinity IL2R)[146]. Interestingly, $CD45RA^+$ naïve and $CD45RO^+$ memory Tregs respond to low IL2 concentrations, while other lymphoid populations (i.e. naïve and memory $CD4^+$ T cells, naïve and memory $CD8^+$ T cells and $CD56^{hi}$ and $CD56^{lo}$ NK cells) need much higher IL2 concentrations (3 vs. 100 pmol/l)[147]. The suppressive function of Treg cells is directed by the transcription factor X-linked gene forkhead box p3 (Foxp3). Although Foxp3 expression is widely used as a marker of the Treg lineage, Treg fate seems to be regulated by a multifactorial signaling cascade, involving cytokines, transcription factors, and epigenetic modifications [148,149]. Foxp3 expression and the Treg phenotype can also be attained by peripheral T cells, exemplifying that Treg fate is not automatically conferred during thymic development. Indeed, besides the thymus-derived nTregs, there are also peripherally-generated adaptive or induced Tregs (iTregs) of variable phenotype and function. Both subsets have comparable phenotypic features and similar regulatory function against T-cell-mediated immune responses and diseases. Both Tregs express the Treg markers *IL2RA* (IL-2R α-chain; also known as CD25), *FOXP3*, *CTLA4*, *TNFRSF18* (TNFR superfamily member 18; also known as GITR) and *ICOS* (inducible T-cell co-stimulator), but nTregs exhibit a higher expression of *PD-1* (programmed cell death-1), *NRP1* (neuropilin-1), *HELIOS*, and *CD73* compared with iTregs [150]. The most important iTregs are the Tr1 cells that secrete IL10 [151–153], the Th3 cells that secrete TGF-β and IL10 [152], and $Foxp3^+$ iTregs). Tr1 cells are capable of secreting high levels of IL10 and TGF-β in the human and mouse and also secrete low levels of IL2, IL5, and IFN-γ [151,153]. An important growth factor for Tr1 cells is IL15, which can support Tr1 cell proliferation even without TCR activation [151,153]. Tr1 cells are anergic and proliferate poorly upon antigen-specific activation which is likely due to the autocrine production of IL10 leading to suppression of proliferation [151,153]. The mechanism of suppressive effects with Tr1 cells is soluble factor-based (i.e., IL10), and the suppressive effects of Tr1 cells are annulled by anti-IL10 neutralizing antibody *in vitro* [151,153]. Th3 cells are

induced from naive $CD4^+$ T cells by TGF-β and have an important role in oral tolerance to non-self antigens and counterbalancing autoimmune reactions [154]. Th3 cells secrete TGF-β, which has immunosuppressive effects acting through a soluble factor mechanism. Th3 cells have a reciprocal relationship with Th17 cells [155,156].

To date, it is clear that defects in the frequency [157] and especially in the suppressive capacity of Tregs from T1D patients [122,157,158] exist.

NK cells were originally described as large granular lymphocytes with natural cytotoxicity against tumor cells and provide early and rapid responses to viral infections. NK cells were later recognized as a separate lymphocyte lineage, with both cytotoxicity and cytokine-producing effector functions, particularly IFN-γ. The role for NK cells in T1D is conflicting and so far, inconclusive. Since some studies in islets from T1D patients are not concordant, with a few reporting NK-cell infiltrates and altered frequencies in peripheral blood of new-onset T1D patients [159,160] and others reporting no presence of these cells [161]. Animal studies are also inconsistent, with some showing that NK-cell depletion [162] or attenuation of their function reduces disease [163]. A pathogenic role for NK cells has more recently been supported in a model of coxsackie B4-induced autoimmunity, where NK cells were important for disease progression [163]. Probably the presence of NK cells indicates a distinct etiology of disease, since there is a correlation between virus infection and T1D with virus infection being the initiating event [164].

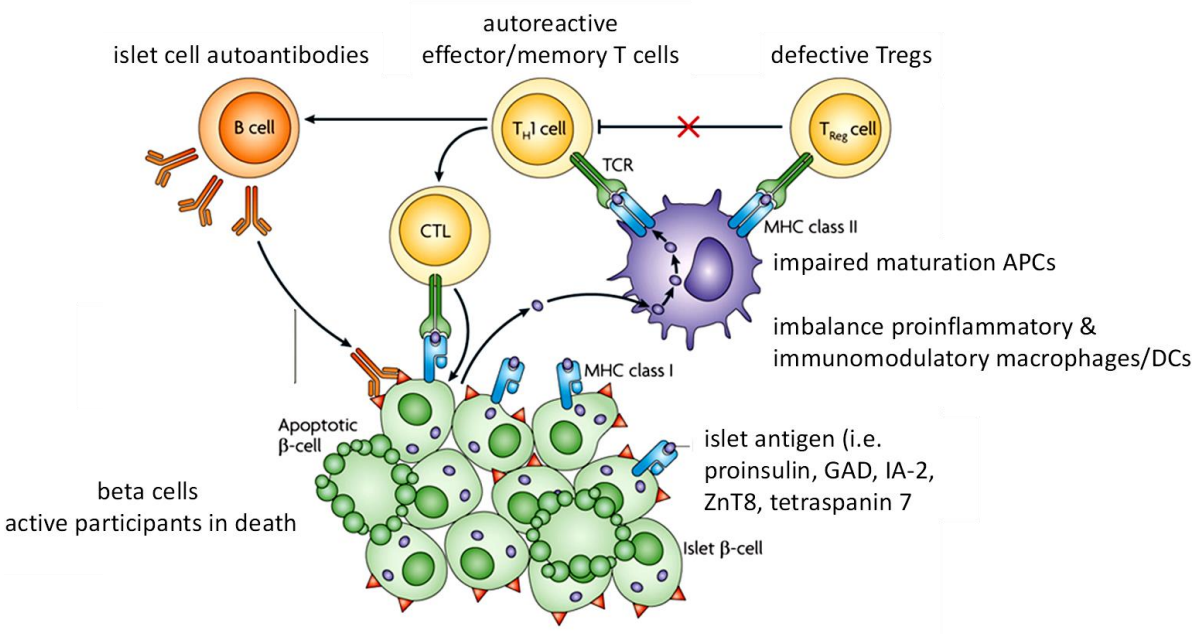

Figure 1.1. Immunopathogenesis of T1D. Islet-cell fragments are phagocytosed by APCs, such as macrophages and DCs. APCs process these islet-specific proteins to peptides that are presented by MHC class II molecules to proinflammatory Th1/Th17 cells, which in turn trigger several immune responses, including activation of B cells (producing islet autoantibodies) and islet antigen-specific (effector/memory) T cells that can directly kill beta cells presenting islet peptides by MHC class I molecules. Alternatively, APCs can present islet antigenic peptides to Tregs but these are often defective in suppressing the proinflammatory cascade, preventing beta cells from being destroyed (adapted from [165]).

1.3 **PREVENTION AND INTERVENTION**

Longitudinal studies in individuals at risk of developing T1D have clearly indicated that the pathology is a continuous process that progresses at anticipated steps through distinctly classifiable stages prior to the onset of symptoms. Stage 1 is defined as the presence of beta-cell autoimmunity as evidenced by the presence of two or more islet autoantibodies with normoglycemia and is presymptomatic, stage 2 as the presence of beta-cell autoimmunity with dysglycemia and is presymptomatic, and stage 3 as the onset of symptomatic disease [166]. Thus, prevention and intervention studies will depend on the stage of the disease (Figure 1.2). These cure-targeted strategies can be divided in primary prevention (before the functional loss of beta cells), secondary prevention (before the onset of symptomatic disease) and tertiary prevention and intervention (after the onset of symptomatic disease).

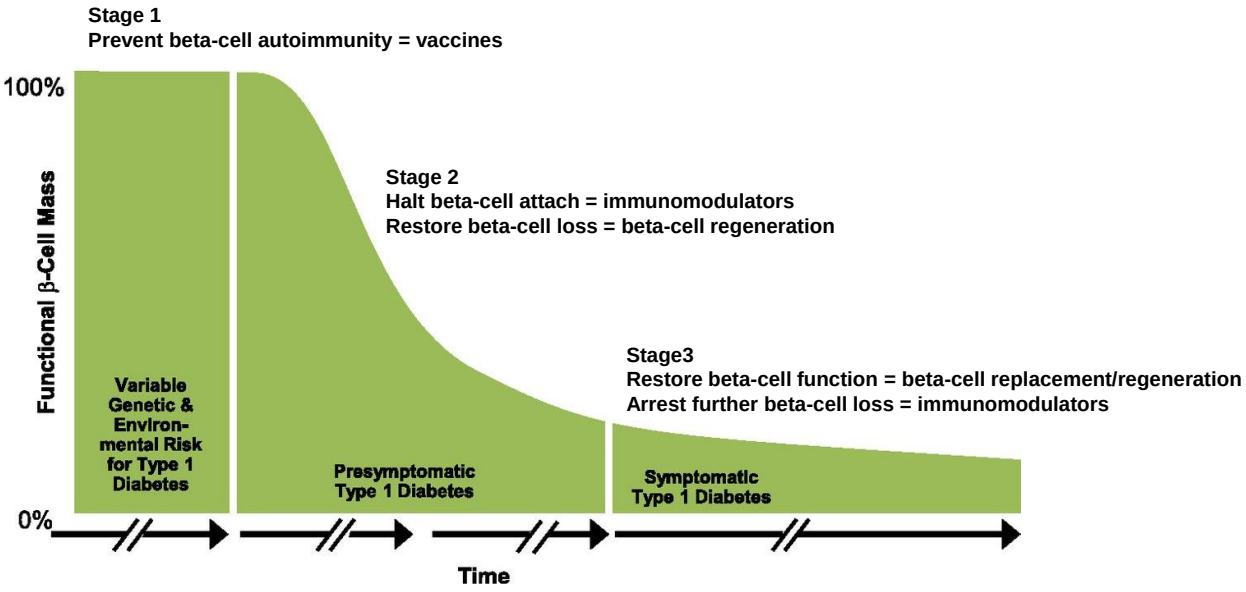

Figure 1.2. Curative strategies and interventions for T1D split up by disease stage (adapted from [11]).

1.3.1 *Primary prevention*

Primary prevention trials focus on young children with high genetic susceptibility for T1D. However, finding these kids in the general population is severely hindered by the low specificity of the genotypes described in the literature. On the other hand, the specificity is much higher in populations with a higher a priori risk of T1D, i.e., first-degree relatives. These strategies attempt to "cure" the core of the pathology and aim at modifying or modulating the imbalances in the (auto)immune system. It is known that during the early pre-diabetic state, beta cells can already show abnormal phenotypes with one pathognomonic mark being the increase in HLA class I expression in both insulin-deficient and -containing islets [111,167]. Currently, primary prevention trials include largely low-risk dietary modifications: elimination of cow's milk (ClinicalTrials.gov Identifier: NCT01055080; ClinicalTrials.gov Identifier: NCT00570102) or gluten (BABYDIET-Study – ClinicalTrials.gov Identifier: NCT01115621; TEFA family prevention – ClinicalTrials.gov Identifier: NCT02605148) and supplementation of diet with ω-3 fatty acids (ClinicalTrials.gov Identifier: NCT00333554) or vitamin D (2,000 IU/day; ClinicalTrials.gov Identifier: NCT00141986; [168]). In addition, trials of antigen-specific immunomodulation are giving insulin (DPT-1 – ClinicalTrials.gov Identifier: NCT00004984; oral insulin – ClinicalTrials.gov Identifier: NCT00419562; Pre-POINT-Early Study – ClinicalTrials.gov Identifier: NCT02547519) as this was found previously to be safe in large secondary prevention trials (see 1.3.2).

1.3.2 *Secondary prevention*

The patients included in secondary prevention trials are those with high genetic risk and having positivity for two or more islet autoantibodies. The majority of young individuals with multiple islet autoantibody positivities progresses to diabetes in 5 to 10 years; however, the rate of progression decreases with age. As the pathology progresses, immune cells (i.e. mainly $CD8^+$ T cells, macrophages, B cells and $CD4^+$ T cells) start to invade the islets of Langerhans and selectively destroy the insulin-secreting beta cells. At this stage, one needs to target the diseased immune system that attacks the beta cells but also needs to restore the functional beta-cell loss. Some prevention trials have found no effect on the rate of progression to symptomatic T1D (reviewed in [169]): insulin administered parenterally (i.e. subcutaneous; [170], orally [171] or intranasally [172] in addition to nicotinamide administered orally [173]. Interestingly, a post hoc analysis of data from the DPT-1 study of oral insulin suggested that relatives with high levels of IAA appeared to experience a delay in the progression to disease by approximately 4 years [171]. Even if the secondary prevention trials failed to prevent or delay the onset of diabetes thus far, a growing body of evidence suggests that prevention of diabetic ketoacidosis and hospitalization in newly-diagnosed children is possible and should be a major goal of diabetes care systems. In the meanwhile, new studies have been initiated (oral insulin: ClinicalTrials.gov Identifier: NCT00419562; nasal insulin: ClinicalTrials.gov Identifier: NCT00336674; and GAD-alum: ClinicalTrials.gov Identifier: NCT01122446) and the field is eagerly awaiting the conclusions.

1.3.3 *Tertiary prevention and intervention*

In tertiary prevention trials, the main aim is the preservation of remaining beta cells and the induction of prolonged (partial) remission. Unfortunately, autopsy studies of beta-cell mass reported that diabetic patients, at diagnosis, have only ~10% of their beta cells left [174,175] and studies in new-onset patients revealed residual beta-cell function of approximately 40% of normal [176] while the autoimmune beta-cell destruction further progresses. A loss of beta-cell mass of ~50% in animals leads already to dysregulated insulin secretion and induction of insulin resistance. Residual beta-cell function can be retained for decades after the onset of diabetes in a subset of patients. However, for most patients very little normal beta-cell function is maintained, beta-cell apoptosis goes on, and there is negligible spontaneous beta-cell regeneration [177]. Pathological pathways covering disturbances in the expansion and development of either endocrine or exocrine pancreas are presumably part of the early immune pathogenesis of T1D. Interestingly, the group of P. Herrera found that in the pancreas new beta

cells can be generated throughout life after near total loss, although in the absence of autoimmunity, mainly by the spontaneous reprogramming of glucagon-producing alpha cells and somatostatin-producing delta cells [178,179]. Collectively, the major goal of tertiary prevention trials is a prolongation of residual beta-cell function, rather than complete disease remission. Benefits may include simpler insulin regimen, lower glycosylated hemoglobin (HbA1c), and reduced risk of hypoglycemia and microvascular complications. Cure-targeted interventions go one step further and have as goal to restore beta-cell function and halt further beta-cell loss. Here, several strategies can be envisaged: antigen-specific vaccines combined or not with (global) immunomodulators and beta-cell replacement or regeneration.

1.3.3.1 **Antigen-specific vaccines**

Diverse mechanisms have been proposed to trigger suppression of autoimmunity by antigen-specific approaches (i.e antigen- or peptide-based therapies, with or without adjuvants, through more exceptional strategies such as Treg therapy [180,181], tolerogenic DC vaccines [182–184], or DNA and peptide-MHC-based vaccines [185](Figure 1.3).

1.3.3.1.1 *Antigen- or peptide therapy*

The tolerance induction by antigen-specific vaccines is perhaps one of the most interesting options at stage 3 of the autoimmune T1D process. There are several routes to induce tolerance (e.g. oral [186], intranasal [187], intrathymic [188,189], subcutaneous [190,191], intradermal [192], intramuscular [193]). However, not one of the many antigen-specific therapies that have been initiated during the last 30 years in T1D patients has managed to achieve clinically significant benefit with a therapy that has an acceptable risk profile (reviewed in [169]).

Immunotherapies with insulin-related molecules are surely an interesting approach. However, although a modified peptide ligand of the immunodominant insulin B chain amino acids 9 to 23 peptide (insulin B:9-23)(NBI-6024; from Neurocrine Biosciences Inc.) demonstrated immunologic efficacy in a phase I study [194] it was ineffective in phase II B trial. Nevertheless, up to two years after the single injection, antigen-specific Tregs could be isolated from peripheral blood from subjects in the experimental arm, while not in the control arm.

The DiaPep277 peptide of heat shock protein 60 (HSP60) (from Hyperion Therapeutics Inc) was reported to preserve C-peptide in a small trial of LADA patients with relatively short follow-up [195]. However, Phase II trials in children showed no [196] or little [197] effect. Moreover, the company stopped developing the DiaPep277 vaccine for new-onset T1D patients

based on scientific misbehavior, including conspiracy with a third-party biostatistics company who obtained insights in the blinded data of the Phase III DIA-AID study and used these to manipulate the analyses to obtain satisfactory results.

The Diamyd vaccine (from Diamyd Medical AB) is based on the whole recombinant human GAD65 (rhGAD65) molecule formulated with aluminum hydroxide (GAD-alum) and has shown some efficacy. Clinical benefit was demonstrated in studies of LADA patients [198] and in children with new-onset T1D [191]. These studies showed that the maximum stimulated C-peptide decreased less in the GAD-alum group compared to the placebo group. However, more recent trials with GAD-alum in new-onset T1D patients were negative, despite GAD65-specific cellular and humoral immune responses after GAD-alum intervention [199,200]. Based on this knowledge, current research focusses on combinations in which the Diamyd vaccine is given together with anti-inflammatory and immunosuppressive agents including GABA (ClinicalTrials.gov Identifier: NCT02002130). Other combinations that are running in new-onset diabetic patients are the Diamyd vaccine combined with vitamin D (ClinicalTrials.gov Identifier: NCT02387164 and NCT02352974) alone or with ibuprofen (ClinicalTrials.gov Identifier: NCT01785108) or etanercept (ClinicalTrials.gov Identifier: NCT02464033).

While pre-clinical research is extremely important in instructing us on how T1D develops, making the jump from animal models of "cures" to remedies that actually work in human subjects has been challenging. There are multiple strategies that can prevent and a few that can reverse T1D in NOD mice. This has led to major criticism on the suitability of the NOD mouse. However, these disappointments in translation to the patient may prove useful in revealing both the similarities and dissimilarities between T1D in mouse and men. These observations also strengthen the necessity to standardize the disease stage, the assessment of immune and therapeutic targets, and therapeutic outcome in the NOD mouse. Timing and dosing thresholds for effective prevention or reversal are critical. The host lab published some studies on immune tolerance induction in the NOD mouse by intragastric delivery of islet antigens by genetically-modified *L. lactis*. We reached approximately 60% of new-onset diabetes remission in NOD mice when the *L. lactis*-based vaccine producing human proinsulin along with IL10 was combined with a short course of sub-therapeutic doses of anti-CD3 mAbs [74]. When using a similar approach consisting of a combination of *L. lactis* secreting $GAD65_{370–575}$ along with IL10 and an induction course with low doses of anti-CD3 the disease reversal rate was 67% [201]. We now find ourselves at a crucial point: the molecular understanding, the concept of oral tolerance induction, and the advantage of using recombinant *L. lactis* therein are present and could make the difference in the treatment of T1D (reviewed in [202]).

1.3.3.1.2 *Treg therapy*

The approach to replace or correct the Treg subsets in T1D patients and thereby restoring the immune imbalances holds huge promise. Several researchers endeavored to induce and expand Tregs with different agents (reviewed in [203]), and thus provide long term tolerance of beta cells in T1D [181] but few of them have led to trials in T1D subjects with varying success rates. The host lab found that 1,25-dihydroxyvitamin D_3, and its non-hypercalcemic structural analog TX527, promote a stable Treg phenotype in T cells from healthy controls and T1D patients [204]. These findings warrant further confirmation of vitamin D-induced Tregs in view of impending autologous adoptive immunotherapy in T1D. A Phase I trial in T1D patients receiving, in four dosing cohorts, *ex vivo*-expanded autologous $CD4^+CD25^+CD127^{lo/-}$ polyclonal Tregs reported no adverse effects. Immunological phenotypic studies demonstrated transient rises in recipient Tregs with broad $CD4^+CD25^{hi}CD127^{lo}Foxp3^+$ phenotype longterm [180] (ClinicalTrials.gov Identifier: NCT01210664). These observations are encouraging and justify further studies (ClinicalTrials.gov Identifier: NCT02772679) but especially reinforce the development of Phase II trials to test the efficacy of the Treg therapy.

1.3.3.1.3 *Tolerogenic DCs*

Under normal conditions, APCs, more specifically DCs, with tolerance-promoting (tolerogenic) properties can induce (antigen-specific) tolerance and this can be strengthened by other suppressor and immunomodulatory cells. As such, DC-based therapy has the potential to safely restore immune tolerance in T1D longterm (reviewed in [205]) The host lab and others have demonstrated that tolerogenic DCs generated by 1,25-dihydroxyvitamin D_3 with or without dexamethasone have a stable maturation-resistant phenotype with low expression of activating co-stimulatory molecules, skewed production of IL12 and high expression of inhibitory molecules and IL10. These immunomodulatory cells can increase apoptosis of Teff cells and induce antigen-specific Tregs, which work through linked suppression ensuring infectious tolerance [206–209]. Moreover, the host lab found that 1,25-dihydroxyvitamin D_3 induced human monocyte-derived tolerogenic DCs by metabolic reprogramming. Both glucose availability and glycolysis, under the control of the PI3K/Akt/mTOR pathway, determined the induction and maintenance of the tolerogenic DC phenotype and functionality [210]. Presently, several clinical trials are testing the safety of *ex vivo*-generated DC vaccines (ClinicalTrials.gov Identifier: NCT00445913 and NCT02354911).

1.3.3.2 **Global immunomodulators**

Several non-antigen-specific immunomodulators have been tried in new-onset T1D patients. Treatments with cyclosporine, azathioprine, and anti-thymocyte globulin (ATG) plus prednisolone had undesirable side effects, including the weakening of immunity to fight infections, toxicity (renal and pancreatic), and potential long-term risk of malignancies. The use of cyclosporine could prevent diabetes in the diabetes-prone BioBreeding rat and a pilot study in 41 newly diagnosed T1D patients showed its therapeutic potential in humans [211]. Of 30 patients treated within 6 weeks of diagnosis, 16 became insulin independent with concentrations of plasma C-peptide in the normal range and decreasing titers of ICAs. However, these beneficial effects were only stable for 1 year and the patients progressed to insulin dependency within 3 years. Renal and beta-cell toxicity, as well as the costs of the drug and the monitoring of its levels in the blood, led to a consensus that the risks outweighed the benefits. Although the short-term renal side effects of cyclosporine were completely reversible, long-term cyclosporine treatment may induce irreversible structural renal damage.

1.3.3.2.1 *ATG*

ATG has been used in organ transplantation to avoid acute rejection but has not yet demonstrated effectiveness in inducing immune tolerance. This polyclonal IgG directed against thymocytes targets several T-cell antigens and thus creates a unique therapy directed against T cells that could promote tolerogenic responses in autoimmunity. In new-onset diabetic NOD mice, a short course of rabbit anti-mouse ATG (mATG) induced durable remission [212]. Studies in the 80s and 90s suggested that short courses of prednisone and ATG plus prednisone in new-onset T1D patients were effective in reducing insulin requirements and lowering HbA1c levels [213,214]. A small Phase II, randomised, placebo-controlled, clinical trial (Study of Thymoglobulin to Arrest Type 1 Diabetes (START); ClinicalTrials.gov Identifier: NCT00515099) with a brief course of ATG (6.5 mg/kg polyclonal rabbit ATG (rATG) or placebo over a course of four days) did not result in proper preservation of beta-cell function 12 months after therapy stop in new-onset T1D patients [215]. Moreover, cytokine release syndrome and serum sickness were observed and generalized T-cell depletion with the absence of specific effector memory T-cell elimination could be noted. In a follow-up study 24 months after therapy stop, it was described that although ATG did not preserve beta-cell functionality, older patients had preserved C-peptide, which might warrant further study [216]. Perhaps low doses to avoid side effects could be of interest in combination regimens. Previously, low-dose ATG and granulocyte colony stimulating factor (GCSF) treatment led to a durable reversal of

diabetes in NOD mice. Therefore, the ATG-GCSF in New Onset Type 1 Diabetes (ATG-GCSF) trial will study whether low-dose ATG plus pegylated GCSF and low-dose ATG alone will have the ability to retain/enhance C-peptide production in new-onset T1D patients demonstrating residual beta cell function (ClinicalTrials.gov Identifier: NCT02215200).

1.3.3.2.2 *Monoclonal CD3 antibody*

The use of monoclonal CD3 antibody (anti-CD3 mAb) has been extensively studied in the context of avoiding acute graft rejection and autoimmune diseases. Kung *et al.* reported in 1979 the development of OKT3 (Ortho Kung T3), the first murine mAb recognizing CD3 surface antigen on human T cells [217]. However, the use of OKT3 in humans had several undesirable effects. OKT3 is a potent mitogen, promoting T-cell proliferation and cytokine secretion, triggering a wide spectrum of adverse effects including fever, chills, nausea, vomiting and headaches, summarized as 'flu-like,' 'cytokine-release' or 'first-dose' syndrome. Because of these unwanted toxic effects of OKT3, two humanized non-mitogenic anti-CD3 mAb variants were produced. hOKT3 γ1(Ala-Ala)(teplizumab; from MacroGenics and Eli Lilly) is a humanized version of the mouse OKT3 mAb which preserves the OKT3 binding but has mutations in the Fc region: amino acids 234 and 235 were both changed to alanines, and as such FcR binding decreased [218]. For the aglycosylated ChAglyCD3 (otelixizumab; from TolerX and GSK) amino acid 297 was replaced with an alanine resulting in the elimination of a glycosylation site necessary for FcR binding [219]. The use of anti-CD3 over the last 20 years in T1D intervention has been reviewed in [220]. In the 90s, the group of L. Chatenoud demonstrated in new-onset diabetic NOD mice that administration of a low-dose regimen of hamster anti-mouse CD3 mAb 145-2C11 (5 µg/day for 5 days) induced rapid, long-lasting and antigen-specific remission from disease but also prevented immune responses towards syngeneic pancreatic islet grafts and not against unrelated antigens as shown by normal rejection of skin allografts [221]. Such observations, that anti-CD3 mAbs were able to break active beta-cell autoimmunity, but were less efficient in preventing disease, led to an important discovery in the field of autoimmunity and transplantation. Although administration at the time of transplantation induced immunosuppression, a slightly delayed treatment induced long-lasting remission in pancreatic islet allografts [222] and heart transplantation [223], probably due to preferential depletion of effector memory T cells, the resistance of Tregs to anti-CD3 mAb-induced apoptosis and establishment of local immune privilege [224]. These observations initiated further successful studies on anti-CD3 mAb for tolerance induction in autoimmune diseases. Two randomized, placebo-controlled phase I/II trials with either teplizumab or

otelixizumab demonstrated a slower decline in stimulated C-peptide, lower HbA1c levels, and lower insulin requirements in new-onset T1D patients [225–227]. The C-peptide levels held for at least 1 year, especially in patients with higher baseline C-peptide levels, followed by a recurrence of progressive loss of C-peptide. Significant but smaller benefits in C-peptide levels persist up to 4 years after treatment with a single course of the mAb [228]. In the meanwhile, both mAbs have been tested in Phase III trials in new-onset T1D patients and each failed their primary endpoints [229,230]. However, post hoc analyses of the ABATE trial could demonstrate positive effects especially in subjects enrolled in the United States, younger than 18 years, enrolled within 6 weeks of diagnosis, and with higher levels of C-peptide at study entry [231,232]. To achieve better effects, this therapy will likely require repeated administration of the drug, which is being tested in several trials (ClinicalTrials.gov Identifier: NCT00378508; ClinicalTrials.gov Identifier: NCT02000817) or in combination with another therapeutic agent [233].

Several other immunomodulators have also been or are being tested in animal models of T1D as well as in new-onset patients and are targeting specific immune pathways (i.e. rituximab (B-cells), abatacept (costimulation), anakinra (IL1R), vitamin D (Tregs and tolerogenic DCs))(Figure 1.3).

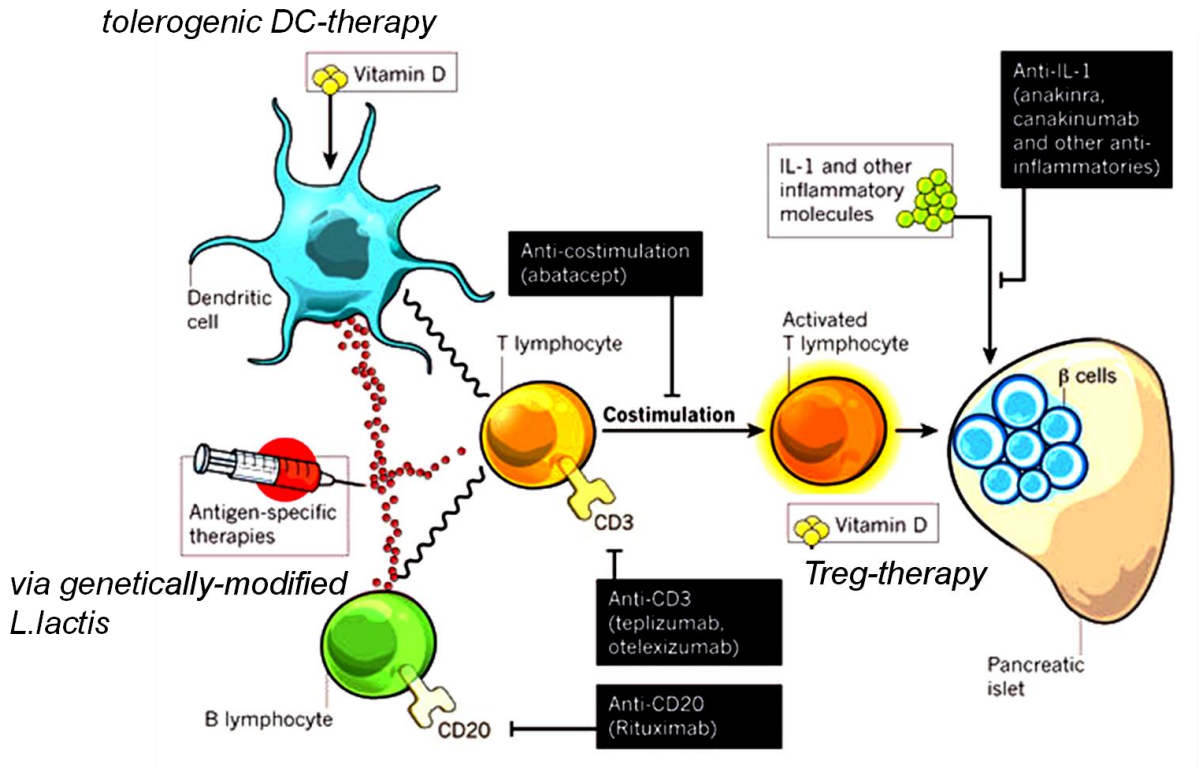

Figure 1.3. Therapeutic approaches in T1D. These strategies will prime or enhance 'natural' immune regulation by lymphocytes, in addition to medicinal immune suppression and depletion. Each contributor in the dialogue between beta cells and the immune system might be more or less specifically targeted by the immune intervention. Many of these approaches have been or are evaluated clinically (adapted from [234]).

1.3.3.3 ***Beta-cell replacement***

1.3.3.3.1 *Pancreas transplantation*

Beta-cell regeneration or replacement are alternative therapeutic approaches for a better blood glucose control and improved outcomes in terms of secondary complication rates in T1D patients. Among beta-cell replacement options both full pancreas transplantation and islets transplantation are clinically available. Over the last 50 years, whole pancreas transplantation has evolved from a pure experimental procedure to standard therapy for patients with brittle diabetes and established end-stage kidney failure or with overt diabetes complications and poor quality of life. On the other hand, whole pancreas transplantation remains an invasive surgical procedure and although improvements in surgical techniques, and better characterization of donor and recipient have tremendously increased the success rate of vascularized pancreas

transplantation (i.e. the estimated half-life of a pancreas transplant is now 14 years (simultaneous pancreas and kidney transplant; SPK), 7 years (pancreas after kidney transplant; PAK) and 7 years (pancreas transplant alone; PTA)), the number of pancreas transplants, especially in United States, has decreased by over 20% over the past decade, most particularly records of solitary pancreas transplantation (i.e. PTA or PAK) procedures [235,236]. Poor exploitation of deceased donor pancreases might be a leading cause of the declining graft volumes. Moreover, not only is there a decline in a number of patients allocated to the waiting lists, also the numbers of deceased donors, trained surgeons, centers that actively perform pancreas transplantation, and referrals from endocrinologists have decreased. On top of the surgery, life-long immunosuppression is needed, which in turn leads to morbidity and carries a non-negligible risk of mortality. But the most difficult barrier to widespread use of whole organ transplantation as a cure for people with T1D is the scarcity of donor organs.

1.3.3.3.2 *Islet cell transplantation*

In a pathology where the primary cell that is destroyed is the beta-cell, isolated islet cell transplantation should be sufficient to cure the disease, and this procedure does not require surgery, but can be performed on an ambulatory basis, via intraportal injections of islets, lodging them in the liver [237,238]. Additionally, the fact that islets can be cultured for at least several weeks offers a unique opportunity to immunologically modulate the graft, and optimize the host conditioning prior to transplantation. Islet cell transplantation is a rapidly evolving technology and impressive progress has been made since 2000 when a research team from the University of Alberta in Edmonton developed an innovative islet transplant protocol which was able to achieve insulin independence and near normal HbA1c levels in 80% of T1D patients [239]. Interestingly, after more than 10 years from the time of the first islet cell transplantation, all subjects had continued islet function [240]. In the meanwhile, islet cell transplantation is seen as the most effective strategy to treat T1D complicated by severe hypoglycemia [9]. However, some difficulties remain, such as the limited supply of donor pancreatic cells, the necessity of several donors for a single islet cell transplantation, and graft failure due to metabolic pressure, continued autoimmunity, allograft rejection, systemic immunosuppression, and oxidative stress caused by hypoxia or inflammatory cytokines. Especially, the permanent immunosuppressive drug treatment and the shortage of good-quality islets have led the islet cell transplantation benefits to less than 0.5% of patients with T1D [241]. There may be several ways to overcome some of these problems by e.g. encapsulating islets isolated from other sources such as pigs, by reprogramming exocrine pancreatic cells to beta cells or by deriving

beta cells from embryonic, fetal, or other stem-cell sources [242]. In addition, cell therapy, especially stem-cell therapy, has recently received a lot of attention to support islet cell transplantation. Several types of stem cells are given special interest for their capacity to self-renew, to differentiate into multiple lineages and for their paracrine (trophic) and immunomodulatory effects, making them interesting candidates in improving islet cell transplantation success.

1.3.3.3.3 *Multiple hurdles*

1.3.3.3.3.1 Limited supply of donor organs

As in other organ transplant programs that rely on organs from deceased donors, organ scarcity is an issue. Moreover, Eurotransplant (https://www.eurotransplant.org/cms/) reports that only 20-25% of the offered organs are being used for transplantation procedures. Acceptance and utilization might be increased by improving the quality of organ procurement, increasing the capacity of trained transplantation teams, and accepting organs of lesser quality (i.e. donors with advanced or very young age, obesity or with vascular and congenital abnormalities etc.) taking into account the risk of increasing post-transplant complications, cost, and number of organs per islet transplant. Indeed, the latter probably decreased rather than increased therapeutic success of islet cell transplantation [243–246].

To liberate the islets from the whole pancreas, this organ needs to be partially enzymatically digested, followed by density centrifugation separation of the exocrine tissue and stroma from the islets [247]. Despite improvements in the islet isolation and purification techniques which remain demanding, labor intensive, expensive procedures and are best carried out in expert centers, human islet cell transplantations from a single donor have not been very successful worldwide [248]. Moreover, less than half of the organs donated for pancreatic islet isolation have yielded a suitable preparation meeting the stringent criteria for human islet cell transplantation. The enzymatic digestion process disrupts the islet-to-exocrine tissue adhesive contact. Suboptimal collagenase composition leads to incomplete digestion of islets from exocrine tissue along with reduced yield, decreased purity, and increased duration of collagenase exposure adversely affects within-islet cell-to-cell adhesion, leading to loss of islet integrity and viability involving mainly the beta cells [249–252]. Several studies have shown that immediately after isolation, the peri-insular basement membrane is absent, giving rise to pyknotic nuclei and apoptotic bodies, accompanied by elevated pro-apoptotic p38 and c-Jun N-terminal kinase (JNK) activity relative to pro-survival extracellular signal-regulated kinase (ERK1/2) activity before transplantation has even occurred [253–255].

Because the supply of human donor pancreases is extremely limited, even the most successful method of the human pancreas or islet cell transplantation could only cure a very small fraction (< 1%) of all T1D patients. So other sources of cells are needed if we want to cure all T1D patients.

1.3.3.3.3.2 The necessity of several donors for a single islet cell transplantation

Since the Edmonton protocol was established in 2000 its success was attributed, in part, to the engraftment of an enormous islet cell volume (>11,000 islet equivalents per kilo body weight (IEQ))[239]. In this sense, the majority of islet graft recipients need islets from over 2 donors to become insulin independent, which reduces the amount of patients that can be treated [256]. Indeed, the ability to achieve routine single donor islet infusion will ensure availability of more islets for an ever-expanding T1D patient group with poor glycemic control worldwide and would allow islet cell transplantation to match the success of whole pancreas transplantation. In the meantime, several studies have identified donor- and recipient-related factors associated with single donor transplant success such as recipient age (age ≤40 years), insulin requirement at entry (<0.5 U/kg/day), donor weight (>90 kg), donor body mass index (BMI >30), islet graft mass (>5,646 IEQ/kg), and pre-transplant heparin and insulin administration [244,245,257].

Currently, the intrahepatic portal vein is the only site that has been routinely performed in the clinical setting and results in insulin independence. On the other hand, the rapid graft failure and the complexity in monitoring the viability and function of the islet transplant have started a search for alternative implantation sites that might offer better engraftment and long-term survival, together with diminished procedure-related problems. The pancreas [258], spleen [259], anterior eye chamber [260,261], testis [262], thymus [188], bone marrow [263], muscle [264], peritoneum, and omental pouch [265] have been explored but few of these sites have found their way into the clinic

1.3.3.3.3.3 Immediate graft failure

An observation common in islet cell transplantation is the early loss of functional beta-cell mass of around 60% in the first hours/days after transplantation [266,267]. Several causes have been quoted as underlying etiologies of this rapid cell loss [268]. Half of this loss happens within the first 72 hours after engraftment, before adequate vascular supply is reestablished. Part of the non-immune graft failure is most probably due to the implantation of a suboptimal islet quantity and quality. Additionally, islet implantation into T1D patients exposes the insulin-producing

beta cells to a number of harmful factors. These include hypoxia, instant blood-mediated inflammatory reaction (IBMIR), non-specific inflammation, chronic hyperglycemia, and eventually also immune-mediated damage.

Intrahepatic transplantation is a minimal-invasive portal vein infusion [269] that can result in islet entrapment in the portal branches surrounding the sinusoids [252,270]. Although this vascular microenvironment provides abundant physical and nutritional support to the injected islets which have been stripped from their dense vasculature and specialized extracellular matrix during the islet isolation procedure, the hepatic portal vasculature is also a hostile environment that limits islet engraftment and function. For example, oxygen tension in the liver parenchyma is well below that of the pancreas, which may lead to lower oxygen and nutrient supply to the inner core of islets, ultimately resulting in hypoxia and cell death [252,271]. The hypothesis that hypoxia, due to loss of the normal islet microvasculature, results in loss of beta-cell mass is further supported by the increased rates of apoptosis in dispersed islets at oxygen tensions <10 mmHg and by the described improvement in graft oxygenation with paralleled reduced apoptosis rates during the first weeks after transplantation (revascularization period) [272]. Another major cause of immediate graft failure is the non-specific inflammatory and thrombotic reaction termed IBMIR, which negatively influences islet engraftment through the expression of tissue factor, resulting in platelet adherence, activation, clot formation, and lymphocyte recruitment [252,273]. The direct effect of IBMIR on immediate loss of islet mass and function had yet to be fully characterized but given that platelet activation is one of the principal contributing elements in the initiation of an inflammatory response, IBMIR is most likely also one of the key processes that elicits an early immune-mediated response. Following islet cell transplantation, especially the proinflammatory cytokines (i.e. IL-1β, IFN-γ and TNF-α) are strongly amplified in and around the graft. Moreover, tissue-resident macrophages can facilitate damage to the newly infused islet cells [274]. Chemokines secreted by tissue-resident macrophages, ductal cells, and inflamed or destroyed endocrine cells may further attract recipients' macrophages to the transplantation site [275–277] Thus, the proinflammatory milieu in islet grafts probably promotes loss of beta-cell viability and function.

Implanted beta cells are placed under additional risk of damage and dysfunction by chronic metabolic stress [278] that can be intensified via continuous exposure to high levels of immune suppressive drugs [279], which are necessary to avoid immune-mediated graft rejection [258,278].

1.3.3.3.3.4 Alloimmunity

The ability of recipient T cells to recognize donor-derived antigens, so-called allorecognition, initiates allograft rejection. Once recipient T cells become activated, which is controlled by both signal 1 and 2, they undergo clonal expansion, differentiate into effector T cells, and migrate into the graft where they provoke tissue destruction. By their particular character, effector memory T cells are the most potent mediators of allograft rejection (reviewed in [280]). Their low threshold requirement for activation and their high expression levels of adhesion molecules allow rapid access to the grafted tissue [281]. Moreover, clinical data demonstrated that enlarged frequencies of donor-specific, cytokine-producing memory T cells correlate with high occurrences of graft rejection and poor long-term allograft survival [282]. Moreover, T cells can help B cells to produce alloantibodies. Yet, the exact mechanisms of islet allograft rejection and the distinction between effector and memory T-cell phenotypes in the context of islet allograft rejection remain incompletely understood. The full understanding of these immunological pathways and their unique properties will be an important tool to direct the use of immunomodulatory drugs and optimize the application of currently available therapies. Even with new and better drugs coming to the market there are still undesirable side effects. Besides beta-cell toxicity by the immunosuppressive drugs, also predisposition of patients to secondary infectious diseases needs to be avoided.

1.3.3.3.3.5 Autoimmunity recurrence

Autoimmunity recurrence, besides alloimmunity, is one of the major concerns in islet cell transplantation in T1D subjects. The development but also the continuity of autoreactive T cells with an antigen-experienced memory phenotype characterize the pathological basis for autoimmunity recurrence after islet transplantation. These autoreactive T cells can be reactivated upon antigen recall through grafting of new islet/beta cells. In the past, their expansion was seen as a consequence of immune system hyperstimulation in an inflammatory environment. However, more recent data put forward that homeostatic proliferation (alternatively called homeostatic expansion or lymphopenia-induced proliferation), mediated by IL7 and IL15 [283], can be the driving force of autoreactive T-cell expansion in islet transplantation, especially following lymphocyte depletion strategies (i.e. rATG and the anti-CD52 Ab alemtuzumab (Campath-1H))[284,285]. Recent publications suggest that IL7 is involved in the development of T cells with a stem cell-like memory phenotype [286] and in the bioenergetic phenotype of T cells via transcription of the glycolytic enzyme *hexokinase 2* [287]. Strategies that target IL7-mediated homeostatic proliferation of memory autoreactive T

cells in T1D patients after islet cell transplantation might have great promise. While rapamycin and FK506 have been shown to be ineffective on this pathway, mycophenolate mofetil (MMF) has the ability to significantly diminish the degree of homeostatic T-cell proliferation at therapeutic serum concentrations. IL15 seems mainly responsible for driving a basal proliferation of memory $CD8^+$ T cells [288]. Poor islet transplant outcome during the first year after grafting was associated with the presence of peritransplant autoreactive T cells [289–291] and peritransplant or *de novo* donor-specific cytotoxic and $CD4^+$ T cells [291,292]. In detail, Huurman *et al*. described that seven out of eight patients without pre-existent T-cell autoreactivity became insulin independent, versus none of the four patients reactive to both peritransplant islet autoantigens (i.e. GAD and IA-2)[290]. Demeester *et al*. reported that recipient HLA-A*24 status and rising autoantibody levels, mostly GADA, within 6 months after the first islet transplant associated with poor graft outcome [293]. Of interest, Piemonti *et al.* revealed that donor-specific alloantibodies or autoantibodies increase after islet transplantation and might serve as prognostic markers [294]. In this regard, van der Torren *et al.* identified serum cytokine signatures that can predict or associate with clinical outcome of islet cell transplantation [295]. Together, these results clearly indicate that control of beta-cell autoimmunity or re-establishment of self-tolerance is crucial for long-term clinical outcomes after islet transplantation. While the significance of the homeostatic T-cell proliferation pathway is increasing in T1D and islet cell transplantation, there are few drugs targeting this pathway in men and limited clinical studies to assess whether this could be a real approach to control T-cell-mediated beta-cell autoimmunity.

1.3.3.4 ***Stem-cell therapy to advance islet cell transplantation***

Many types of stem cells exist and their potential to be used as an infinite and renewable source of insulin-producing beta cells to solve the problem of limited donors in human islets transplantation has led to stimulating avenues of application. Stem cells have the ability to self-renew and possess multi-lineage differentiation. Both embryonic or pluripotent (which are derived from pre-implantation embryos and proven to generate cells of all tissues of the adult organism) and adult or multipotent stem cells (which can be found in a variety of tissues in the fetus and after birth)(http://stemcells.nih.gov/) demonstrated encouraging outcomes in producing insulin *in vitro* and correcting high blood glucose concentrations in pre-clinical models.

However, stem cells could also contribute to better outcomes in islet cell transplantation as 'companion cells' secreting trophic factors enhancing islet engraftment (revascularization), function and survival after transplantation (Figure 1.4).

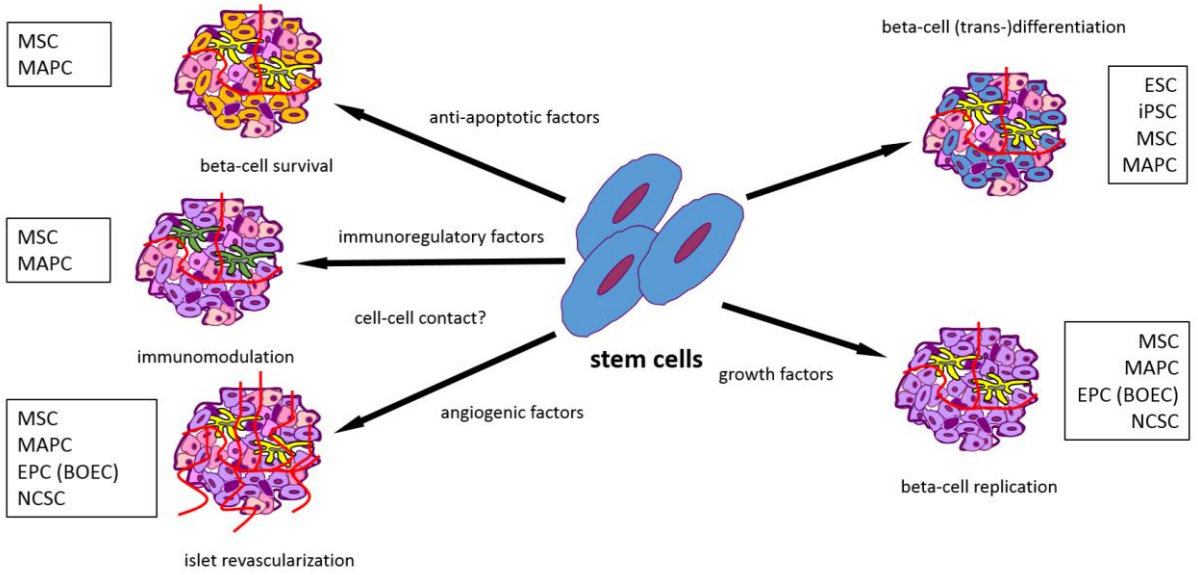

Figure 1.4. Modes of stem cell-based therapies in islet cell transplantation. In principle, new beta cells can be formed from adult stem cells residing in the pancreatic ducts or islets, through transdifferentiation of other pancreatic cells or via differentiation from pluripotent (ESC and iPSC) or multipotent (MSC and MAPC) stem cells under stringent in vitro culture conditions that try to recapitulate the embryological development of beta cells. Although strong insulin production in response to glucose has only been observed in hESC-derived insulin-positive cells, reprogramming of fibroblasts and umbilical cord cells into iPSCs have recently been successful in generating glucose-responsive insulin-producing cells. On the other hand, the formation of pancreatic progenitors from bone marrow-derived MSCs and MAPCs remains to be confirmed; however, these cells surely promote survival and regeneration of beta cells. Moreover, these cells can enhance islet engraftment and function by secreting trophic factors that can avoid apoptosis, modulate the innate and adaptive immune system, and promote revascularization. It is likely that MSC and MAPC effects on the immune system may be affected not only by cell-to-cell interactions, but also by environmental cues shaping their phenotype and functions.

1.3.3.4.1 *Embryonic stem cells*

Several protocols have been described to differentiate human embryonic stem cells (ESCs) into pancreatic progenitors [296–302]. Advancements in the understanding of embryonic beta-cell development have resulted in efficient directed differentiation protocols for converting pluripotent stem cells into pancreatic progenitors *in vitro* [301]. While initial studies were able to generate definitive endoderm from pluripotent stem cells by the introduction of the TGF-β family member, activin A [303], more recent studies combined retinoic acid, the sonic hedgehog (Shh) inhibitor cyclopamine, and fibroblast growth factor 10 (FGF10) to generate

pancreatic epithelium *in vitro* from definitive endoderm derived from pluripotent stem cells [299]. Meanwhile, several methods for the generation of human ESC-derived pancreatic epithelium are established.

When transplanted, the pancreatic precursors derived from human ESCs had the potential to further differentiate into glucose-sensitive insulin-producing cells and were able to reverse diabetes *in vivo* [304–308]. In fact, chemically-induced diabetic immunocompromised mice transplanted with the pancreatic progenitors derived from human ESCs sustained normoglycemia and had comparable human insulin and C-peptide concentrations to mice engrafted with substantial human beta-cell mass. Removal of the grafts in these mice induced recurrence of hyperglycemia [307,309]. These findings suggest an important *in vivo* contribution of the graft microenvironment to the terminal differentiation/maturation of pancreatic progenitors into true insulin-producing cells [298,309]. Despite impressive progress, knowledge on the *in vivo* factors or circumstances required for the final maturation steps is limited. Moreover, the amount and characteristics of the cells within the pancreatic precursor population that can further differentiate *in vivo* have not been distinguished. Several studies in this field are ongoing to attempt to fully understand these processes.

Recently, the group of T.J. Kieffer reported on a new seven-stage *in vitro* differentiation procedure that built upon protocols previously used to specify pancreatic progenitors. They used GDF8 (a TGF-β family member), a GSK3b inhibitor followed by FGF7, vitamin C, TPB (PKC activator), SANT-1 (a hh antagonist) and LDN (a small-molecule inhibitor of the BMP receptor) and molecules such as ALK5iII (TGF-β receptor), thyroid hormone (T3), N-acetyl cysteine, AXL inhibitor to generate cells that could secrete insulin after glucose stimulation in similar fashion as human islets *in vitro* and express markers of mature pancreatic beta cells such as MafA. Almost at the same time, the group led by D. Melton reported their own differentiation procedure with slight modifications, employing keratinocyte growth factor (KGF), SANT-1 and low concentration of retinoic acid, followed by molecules that affected numerous signaling pathways, comprising of wnt, activin, hh, epidermal growth factor (EGF), TGF-β, thyroid hormone, and retinoic acid, as well as γ-secretase inhibition, to generate glucose-responsive beta cells from human pluripotent stem cells *in vitro*. In contrast to previous protocols, these stem-cell-derived beta cells expressed mature beta-cell markers, fluxed Ca^{2+} in response to glucose, packaged insulin into secretory granules, and produced insulin as effectively as adult human beta cells in response to various nutritional challenges. Xenogeneic transplantation experiments demonstrated that the cells were capable of rescuing chemically-induced diabetes

in mice [310]. Clearly, various multi-step protocols have been described in different stages of pancreas development with different results but also many concerns [301].

One concern is the low yield and purity of the pancreatic progenitors in these *in vitro* cultures. However, new methods of expansion and banking and a suspension-based differentiation system may generate higher yields of cells expressing markers of pancreatic endoderm [297]. Additionally, the probability of forming teratomas developing from contaminating pluripotent stem cells may impede the widespread clinical utility of human ESC-derived islet progenitor cells, especially in patients that may be immunocompromised through the lifelong use of immunosuppressive drugs. As endogenous islets, these pancreatic progenitors will also be vulnerable to immediate graft failure and immune-mediated destruction. Thus, a robust and retrievable capsule may be used to safely administer the stem-cell product by limiting the cells to one place but also providing some immune protection. At the moment, creating this type of encapsulation system is being explored by several biotech companies such as TheraCyte Inc., Beta-O2 Technologies Ltd., and ViaCyte Inc. The latter used pancreatic progenitors derived from human ESC line (CyT49 line) in a two-dimensional planar drug delivery system (Encaptra™) which allowed the exchange of molecules but kept the cells contained and protected by the recipients' immune system [311]. Interestingly, the human ESC-derived pancreatic endoderm in the encapsulation system further matured to glucose-responsive insulin-producing cells and other endocrine cells without evidence of increased biomass or cell escape[300]. Further analyses of the composition and function of these subcutaneously implanted macro-encapsulated human ESC-derived grafts revealed higher glucose-responsive C-peptide levels and a lower proinsulin / C-peptide ratio in animals using the Encaptra™ device than human islet-cell implants under the kidney capsule of immunocompromised mice [312]. These interesting results with macro-encapsulation of human ESC-derived pancreatic endoderm steered the biotech ViaCyte, Inc.to start recruiting participants for a Phase I/II trial called STEP ONE, or Safety, Tolerability, and Efficacy of VC-01™ Combination Product in T1D patients (ClinicalTrials.gov Identifier: NCT02239354).

1.3.3.4.2 *Induced pluripotent stem cells*

Somatic cell reprogramming into pluripotent ESC-like cells, called induced pluripotent stem cells (iPSCs), is possible via the introduction of transcription factors, like Oct4, Klf4, Sox2, c-Myc, and Lin28, under ESC culture conditions [313]. These cells can be derived theoretically from any adult cell, being obtained from different origin, such as fibroblasts, stomach cells, liver cells, keratinocytes, amniotic cells, blood cells and adipose cells [314–316]. iPSCs can

have the same haplotype as the recipient, allowing powerful patient-specific autologous therapies. Consequently, immunosuppressive drug consumption to circumvent allorejection may not be necessary, albeit autoimmunity will still pose a challenge. These cells would also avoid ethical concerns of human ESC-derived products [317]. iPSCs could ideally be directed down a pancreatic endocrine developmental program and then be used to generate insulin-producing cells. This new approach has not yet progressed as far as the human ESC field, but several studies using directed differentiation protocols have demonstrated the production of pancreatic progenitors that express insulin and some other markers of mature beta cells [242,301,310,318,319].

1.3.3.4.3 *Adult stem cells in the pancreas*

For many years, the nature and even existence of adult pancreatic stem or progenitor cells have been a matter of debate in the area of beta-cell replacement for diabetes, while there was no doubt that the fetal pancreas is rich in endocrine progenitors [320,321]. Nowadays, it is established that multipotent stem cells do exist in the adult pancreas [322], residing in the epithelium of pancreatic ducts [323] and inside the islet clusters [324]. Interestingly, beta-cell precursors can be activated in injured adult mouse pancreas to increase functional beta-cell mass by proliferation and differentiation rather than by self-duplication of endogenous beta cells and are located in the ductal lining [325]. Next, it was further established that stem-cell populations in the adult mouse pancreas can be expanded *in vivo* in response to beta-cell or pancreas injury and can be induced to proliferate and differentiate *in vitro* to glucose-responsive insulin-producing endocrine cells [326]. Collectively, these studies revealed that the adult pancreas maintains its capacity to reactivate its embryonic model of beta-cell development. Recent studies demonstrated how stem cells meet these requirements by switching between functional states tuned to homeostasis or regeneration [327]. This plasticity extends to differentiating cells, which are able of reverting to stem cells after tissue damage.

1.3.3.4.4 *Adult stem cells outside the pancreas*

1.3.3.4.4.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are the most studied adult stem cells. These cells can be isolated from the stromal fraction of numerous adult tissues in multiple species [328]. The MSC is a non-hematopoietic multipotent self-renewing stem cell that is described to differentiate under standardized *in vitro* conditions into several cell types of mesenchymal origin, such as osteoblasts, adipocytes and chondrocytes [329], but they also have the capacity to differentiate

into cell types of endo- and ectodermal lineages, including endothelial cells [330], lung epithelial cells [331], renal tubular cells [332] neural cells [333], hepatocytes [329], and insulin-producing cells [334–337]. Nevertheless, such multiple differentiation competencies of MSCs are not universally recognized. Indeed, although MSCs can be expanded and differentiated into a wide diversity of cells *in vitro*, it remains controversial whether these cells differentiate to form functional tissues *in vivo* [338]. Hence, several studies relate the benefits of MSCs to their reparative effects via migration to sites of injury/ischemia and/or to their release of trophic factors that can influence cell migration, proliferation, and survival of surrounding cells. Besides, MSCs also possess potent immunoregulatory effects with the ability to modulate both innate and adaptive immune responses [339]. Still, as MSCs lack definitive homing mechanisms, these cells upon intravenous infusion often do not traffic to the damaged tissue to induce tissue regeneration, to treat inflammation, or to promote neoangiogenesis and instead get stuck mainly in the lungs [340].

The direct delivery of these multipotent stem cells at the site of injury will be crucial to fully benefit from their therapeutic effects [341]. At the site of injury, the major benefits of MSCs seem to be attributed to their capacity to enhance repair processes via the secretion of a variety of cytokines and growth factors with paracrine and autocrine activities. These secreted bioactive molecules can enhance neo-angiogenesis, inhibit fibrosis and apoptosis, suppress inflammation and even modulate the adaptive immune system by the induction of regulatory T cells and the recruitment of myeloid-derived suppressor cells, and stimulate differentiation of tissue-intrinsic (stem) cells. In the islet cell transplantation field, pre-culturing islets with MSCs using a direct contact co-culture system enhanced transplantation outcome in diabetic mice by improving islet quality, survival and function [342,343]. Here, possible explanations for the superior graft function of the cultured islets *in vivo* could be the anti-apoptotic and pro-angiogenic abilities of the MSCs. First, recent work identified ANXA1, also known as lipocortin-1, as a crucial MSC-secreted factor able to enhance insulin secretion *in vitro* [344]. Second, improved vascularization of islet transplant could only be detected when MSCs were physically present at the implantation site [345,346]. Several studies have shown that direct contact by co-transplanting islets with MSCs also better maintained islet cytoarchitecture which resembled more closely that of endogenous islets [346,347] and reduced islet death compared to the transplant with islets only [345]. Together, these studies highlight that the presence of MSCs during islet culture as well as transplantation might be required to benefit optimally from the multipotent cells. Finally, although allogeneic MSCs were believed to be immune privileged, recent evidence proposes that these cells can be recognized by the innate and adaptive immune

system [348]. Still, these cells have numerous immunomodulatory properties when exposed to an inflammatory microenvironment. To date, evidence shows that MSCs have immunoregulatory effects on T cells by inhibiting both T-cell proliferation, cytotoxic T-cell activity and decreasing IFN-γ production [349,350]. Furthermore, the immunomodulatory capacity of MSCs seems to involve the cleavage of CD25 from the surface of T cells by matrix metalloproteinase (MMP)-2 and -9, thereby attenuating T-cell responses to IL2 [351,352]. MSCs can also modulate several players of the innate immune system like a complement, toll-like receptor (TLR) cascades, macrophages, DCs, and NK cells [353]. In islet cell transplantation, MSCs can alleviate allorejection by exerting suppressive effects through various T-cell subsets and DCs [354]. In fact, MSCs can shift the Th and cytotoxic T-cell balance and reduce the numbers of naive and memory T cells in peripheral blood after islet cell transplantation [354]. MSCs can also promote long-term islet allograft survival in the presence of short-term immunosuppression across a full MHC-mismatch in chemically-induced diabetic rats by promoting the generation of IL10-secreting Tregs [355]. Based on these encouraging findings, MSCs have found their way to the patient and have been under investigation as adjunctive therapy in the transplantation setting of islets (ClinicalTrials.gov Identifier: NCT00646724, NCT02384018) or as immunomodulatory therapy with the intention to reset the autoimmune system in (new-onset) T1D patients (ClinicalTrials.gov Identifier: NCT00690066).

1.3.3.4.4.2 Multipotent adult progenitor cells

Multipotent adult progenitor cells (MAPCs), which can be derived specifically from adult bone marrow but also from muscle and brain from human and/or rodent donors, are non-hematopoietic, non-endothelial stem cells that have the potential to differentiate into cells with meso-, neuroecto- and endodermal (liver, lung and gut, respectively) characteristics *in vitro* and upon transplantation [356–358]. In this context, they are equivalent to ESCs and distinctive from MSCs, which can also be isolated from bone marrow. It has been postulated that MAPCs could be more primitive cells than MSCs; however, the potential association between MSCs and MAPC has yet to be recognized. MAPCs can be positive for telomerase, Oct3A (Oct3/4) or a combination thereof. As such, these cells can expand long term under stringent culture conditions without genetic instability, and can be administered without tissue matching.

As ESCs and iPSCs, MAPCs can be sequentially committed to the formation of definitive endoderm, pancreatic endoderm, and beta-cell-like cells *in vitro*. The differentiation protocol made use of high concentrations of Activin A, a shh inhibitor, and optionally bone

morphogenetic protein 4 (BMP4), subsequently EGF or hepatocyte growth factor (HGF) or both to produce cells with increased neurogenin 3 (Ngn3) expression which after final exposure to nicotinamide and/or exendin-4 developed into insulin-positive cells [359]. When the mixed progeny containing a small fraction of glucose-responsive insulin-producing cells was implanted *in vivo* under the kidney capsule of chemically-induced diabetic immunocompromised mice it further matured and permanently reversed hyperglycemia [359].

Like MSCs, MAPCs can promote tissue repair and healing and induce neo-angiogenesis, possibly by delivering pro-angiogenic factors that activate/recruit endogenous vascular cells and that seem to be specifically tailored to the immediate needs of the damaged tissue [360–363].

Although not yet established in the condition of islet cell transplantation, MAPCs behave non-immunogenic and possess potent immunomodulatory effects on allogeneic T-cell responses [364]. Likewise, these cells can suppress T-cell proliferation and Th1 and Th17 cytokine secretion while improving antiinflammatory IL10 production and favoring Treg proliferation. Interestingly, MAPCs can downregulate autoreactive effector T-cell function from T1D patients [365,366]. Currently, clinical-grade large scale-expanded MAPCs, MultiStem®, are being evaluated in a number of phase 1/2 clinical trials in patients with ischemic stroke, ulcerative colitis, acute myocardial infarction, and for the prevention of graft versus host disease (GVHD) in patients undergoing allogeneic hematopoietic stem-cell transplantation (HSCT). In prevention of GVHD, both single- and multiple-dose infusions of MultiStem® were well-tolerated, and a low incidence of severe acute GVHD was observed [367]. The fine-tuning of stem-cell products in order to achieve consistent and safe products for off-the-shelf use is currently a major effort of process development.

1.3.3.4.4.3 Endothelial progenitor cells

Human endothelial progenitor cells (EPCs) have been defined as circulating cells that express a number of cell surface markers similar to those expressed by vascular endothelial cells (ECs), adhere to the endothelium at sites of damaged tissue or ischemia, and take part in vascular formation, repair and remodeling, especially after transplantation [368,369]. This cell type has enormous potential in the treatment of ischemic diseases such as myocardial and hindlimb ischemia [370,371].Although these progenitor cells have the ability to integrate into new developing blood vessels, they can also deliver humoral factors that may recruit accessory cells to impact beneficially on blood vessel remodeling, and elicit pro-survival mechanisms. Several studies have examined the potential of EPCs in improving outcomes in islet cell transplantation.

Co-transplantation of pre-cultured islets with EPCs accelerated the engraftment process in a marginal mass of islets in a syngeneic murine transplant model. The rapid revascularization by EPC co-transplantation was suggested to induce better graft perfusion and recovery from hypoxia. Moreover, soluble factors released by EPCs in islet culture were shown to modulate the expression of the beta-cell gap junction protein connexin 36 (Cx36), thereby favorably affecting glucose-stimulated insulin release *in vitro* [372]. Indeed, Cx36-dependent signaling seems necessary for proper beta-cell function, particularly for the pulsatility of intracellular Ca^{2+} and glucose-stimulated insulin secretion [373]. Other studies have demonstrated very similar results in improving islet revascularization after co-transplanting with EPCs [374–377]. In the transplant setting, it is common knowledge that matures donor-derived ECs contribute to the blood vessel formation in the graft [378,379] along with the ingrowth of recipient-derived ECs and bone marrow-derived cells [380,381].When EPCs from green fluorescent protein (GFP) transgenic rats were co-transplanted with syngeneic islets into the portal vein, they triggered neo-angiogenesis from host origin in infused islets. Moreover, the EPC-secreted pro-angiogenic factors (e.g. VEGF-A) prompted higher endothelial thickness and vessel density, leading to long-lasting normal blood glucose control in chemically-induced diabetic rats [374]. Another study, however, demonstrated that EPCs derived from bone marrow improved islet graft organization and promoted graft revascularization not only through paracrine effects but also via incorporation into the host vascular system [375]. Just now, co-transplantation of human blood outgrowth endothelial cells (BOECs) with islets in a mouse model of diabetes resulted in a significant and specific enhancement of glycemic control with superior C-peptide responses. Moreover, these cells reduced beta-cell death and increased beta-cell proliferation as well as graft-vessel and beta-cell volume, irrespective of the surgical techniques using a blood clot-dependent or -independent tubing system [376,382]. Interestingly, EPCs not only have the capacity to improve islet graft revascularization but these cells seem to be crucial for the activation of endogenous pancreatic stem cells during islet regeneration [383].These data further support the use of EPCs in clinical studies that aim to improve current islet cell transplantation protocols.

1.3.3.4.4.4 Neural crest stem cells

The neural crest is a transient embryonic structure in vertebrates that initially generates neural crest stem cells (NCSCs) which then migrate throughout the body to produce a diverse array of mature tissue types. Due to the rarity of adult NCSCs as well as several ethical and technical issues surrounding the isolation of early embryonic tissues, biologic studies of human NCSCs

are extremely challenging [384]. However, this stem-cell population can also be isolated from hair follicles [385,386] and the oral mucosa of human origin [387]. These cells can proliferate *in vitro* and can be directed towards peripheral nervous system lineages (e.g. peripheral neurons, Schwann cells) and mesenchymal lineages (e.g. smooth muscle, adipogenic, osteogenic and chondrogenic cells)[388]. Recent work demonstrated that NCSCs are also capable of considerably improving islet function by promoting beta-cell proliferation [389]. In addition, when mouse islet cells were cultured in intimate contact with NCSCs, more than 35% of the islet beta cells showed increased cell cycle activity compared to control islets cultured without NCSCs [390]. On the other hand, exposure of human islets to NCSCs induced higher proliferation *in vitro* and *in vivo*. Moreover, NCSC-exposed human islet grafts *in vivo* had much higher neural and vascular densities with substantially higher blood perfusion and oxygen tension in the newly formed blood vessels 4 weeks after transplantation [391]. These results were explained by a combination of improved recipient blood vessel in-growth and expansion of donor blood vessels. In summary, exposure of islets to NCSCs was shown to stimulate beta-cell proliferation and improve both neural and vascular engraftment of transplanted islets.

CHAPTER 2

OBJECTIVE AND AIMS

2.1 **OVERALL OBJECTIVE**

In T1D patients the pancreatic insulin-producing beta cells are selectively destroyed by the immune system, leading to an impaired glucose metabolism. The steep increase in the incidence of this chronic autoimmune disease especially in young children in the last decades raises serious concerns. To date, insulin therapy is considered the gold standard for the treatment of T1D. Nevertheless, limitations persist, such as the frequent episodes of hypoglycemia, and the chronic micro- and macrovascular complications. Islet cell transplantation offers an alternative treatment for T1D patients, specifically for those with hypoglycemic unawareness following insulin administration. Despite the improved outcome of islet cell transplantation over the last few years, drawbacks remain, such as a limited supply of cadaveric donors, the necessity of several donors for a single transplantation, and (immediate) graft failure through metabolic pressure, continued autoimmunity, alloimmunity, high concentrations of immunosuppressive drugs, and oxidative stress caused by hypoxia or due to cytokine exposure

In this thesis, we propose that in the long-term, tolerogenic antigen-based and beta-cell replacement/regenerative approaches could be promising in both blocking beta-cell autoimmunity to prevent continued beta-cell destruction and boosting beta-cell function and revascularization to restore sustainable insulin regulation.

2.2 **SPECIFIC AIMS**

2.2.1 ***Restoration of self-tolerance after islet cell transplantation***

A cure for longstanding T1D patients, which have hardly any functional beta-cell mass left, implies replacement of the destroyed beta cells and reinstatement of tolerance to islet antigens. Our group has demonstrated oral tolerance induction to islet antigens delivered by genetically-modified *L. lactis*. We reached approximately 60% of new-onset diabetes remission in NOD mice when the *L. lactis*-based vaccine producing human proinsulin along with pro-tolerogenic IL10 was combined with a short course of sub-therapeutic doses of anti-CD3 mAbs. Here, we want to test whether our therapeutic intervention also works in longstanding autoimmune diabetic mice after islet supplementation.

2.2.2 *Improvement of beta-cell engraftment*

MAPC have been shown to promote tissue repair and healing and induce neo-angiogenesis, possibly by delivering angiogenic growth factors that activate/recruit endogenous vascular cells and that seem to be specifically tailored to the immediate needs of the injured tissue. *In vivo*, these cells are short-lived as they experience only a minimally prolonged residence time. Moreover, in contrast to other cell types, MAPC can be expanded long-term (for >80 population doublings) without genetic instability, and administered without tissue matching, making them into an optimal stem cell product for routine clinical use (MultiStem®, Athersys Inc., Cleveland). Therefore, we aim in this thesis to assess the therapeutic efficacy of co-transplantation of undifferentiated human MAPC with mouse islets as separate or composite pellets in a syngeneic marginal mass islet cell transplantation model.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 RESTORATION OF SELF-TOLERANCE AFTER ISLET TRANSPLANTATION

3.1.1 *Animals*

NOD mice, originally obtained from Professor Wu (Department of Endocrinology, Peking Union Medical College hospital, Beijing, China), were inbred in our animal facility since 1989 and kept under semi-barrier conditions. At regular time intervals, mice were screened for glucosuria and consequently considered diabetic if nonfasting blood glucose concentrations exceeded 200 mg/dl for 2 consecutive days (AccuCheck, Roche Diagnostics Belgium, Vilvoorde, Belgium). Animals were kept under insulin pellets (Linbit™; LinShin Canada, Inc., Ontario, Canada) until the day of islet implantation designated as day 0. Animals were handled in accordance with the Guide for the care and use of laboratory animals, Eighth edition (2011) as well as Katholieke Universiteit Leuven (KULEUVEN, Leuven, Belgium) Animal Care and Use Guidelines.

3.1.2 *Islet isolation and transplantation*

Islets were isolated from 2-3-week-old prediabetic male NOD mice as described [392]. Density gradient centrifugation followed by handpicking of islets certified that preparations were highly enriched in islets and had an accurate determination of islet number. For transplantation, 500 freshly isolated islets were grafted beneath the left renal capsule of each diabetic NOD recipient (between 0 and 4 weeks after diabetes diagnosis). Transplantation was considered successful if the nonfasting blood glucose concentration returned to normal (< 200 mg/dl) within 24 hours after surgery. Weight and blood glucose concentrations from tail were monitored three times a week after transplantation with an AccuCheck glucometer. Graft survival was calculated as the number of days before diabetes recurrence. The day of diabetes recurrence was defined as the first of 2 consecutive days of nonfasting blood glucose concentrations >250 mg/ml. Mice were killed after confirmation of diabetes recurrence for metabolic, histological and flow cytometric analysis 6 weeks after initiation of treatment.

3.1.3 *L. lactis culture*

Genetically-modified *L. lactis* strain secreting human proinsulin with human IL10 were generated by transformation of the IL10-secreting sAGX0037 strain with proinsulin-encoding plasmid [74]. The amino acid sequence for proinsulin was retrieved from the National Center for Biotechnology Information (NC_00011). This strain was grown in GM17TE medium (i.e. M17 broth supplemented with 0.5% glucose, 200 µM thymidine (Sigma) and 5 µg/ml

erythromycin (Sigma, St. Louis, MO)). Stock suspensions were stored in -80°C in 50% glycerol (Merck) in cryovials. For intragastric inoculations, stock suspensions were diluted 1000-fold in growth medium and incubated for 16 hrs at 30°C, reaching a saturation density of 2×10^9 colony forming units (CFU)/ml. Bacteria were harvested by centrifugation and concentrated 10-fold in BM9 medium. Treatment dose consisted of 100 µl of this suspension.

3.1.4 *Treatment groups*

Two different treatment regimens were used. In the first regimen (early), therapy started prior to islet implantation (d–5 to d–1) and continued for a total of 6 weeks. In the second regimen (delayed), therapy started at the time of islet implantation (d0). Hamster anti-mouse CD3 mAbs (clone 145-2C11, Bio X Cell, West Lebanon, NH) were administered iv (2.5 µg/d; total of 12.5 µg) for 5 consecutive days. Genetically-modified *L. lactis* bacteria were given by intragastric inoculation (2×10^9 CFU/d) 5 times per week for a period of 6 weeks.

3.1.5 *Metabolic β-cell function*

Random C-peptide concentrations in sera were measured by ELISA (Merck Millipore, Massachusetts, MA). Pancreata were harvested for histological analyses (see 3.1.6) and/or for insulin content determination by ELISA (Mercodia, Uppsala, Sweden) after acid/ethanol extraction.

3.1.6 *Histology and confocal immunofluorescence*

Graft-bearing kidneys were fixed in 10% buffered formalin and embedded in paraffin at the time of diabetes recurrence or at selected time points after islet implantation. Sections were stained with hematoxylin and eosin. Insulin staining was performed using a guinea pig anti-insulin primary antibody (#A0564; Dako Belgium nv/sa, Heverlee, Belgium) and an AF88-conjugated goat anti-guinea pig IgG (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) secondary antibody. Beta-cell mass of the islet grafts was done by Z-stack confocal microscopy analysis, capturing five different focal planes from each graft and then quantifying the insulin+ volume (μm^3) at Volocity software.

Immunofluorescence imaging on graft-bearing kidneys snap-frozen in Tissue-Tek OCT compound (Sakura Fineteck, Torrance, CA) was used to determine immune populations in the graft. Using cryomicrotome and Superfrost Plus slides (Fisher Scientific, Thermo Fischer Scientific), 6-µm tissue sections were cut. Sections were stained by the use of antibodies

directed to CD4 (clone RM4-5; #550280, BD Biosciences, Erembodegem, Belgium) or CD8a (clone 53-6.7; #550281, BD Biosciences) followed by AF488-conjugated goat anti-rat IgG as secondary antibody. Slides were imaged using a Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA). Obtained images were adjusted linearly for presentation using ImageJ (free software that can be downloaded at http://rsbweb.nih.gov/ij/).

3.1.7 *Flow cytometry*

Single cell suspensions were prepared from lymphoid organs (i.e. spleen, pancreatic draining lymph nodes, and kidney draining lymph nodes) by mechanical disruption. Cell suspensions were rendered free of red blood cells by exposure to a solution containing 0.83% NH_4Cl. Cells were stained with directly conjugated Abs against CD4, CD8α, CD25, CD44, CD62L, CD69 and matching isotype controls (eBioscience, San Diego, SA). Intracellular stainings for Foxp3 and CTLA4 were also performed according to eBioscience's instructions. Samples (>10,000 cells) were acquired on a Gallios™ (Beckman Coulter, Suarlée, Belgium) or BD Canto II (BD Biosciences) flow cytometer and data were analyzed with either Kaluza™ (Beckman Coulter) or FlowJo (Tree Star Inc., Ashland, OR) software.

3.1.8 *Statistics*

Statistical analyses were performed with GraphPad Prism software (La Jolla, San Diego, CA). Kaplan-Meier analysis was performed for diabetes-free survival determination, and differences were assessed with the Mantel-Cox log-rank test. FACS, ELISA and insulin content data were analyzed for statistical significance by either Mann-Whitney U-test or Kruskal-Wallis test with Dunn's correction, unless indicated otherwise; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$

3.2 **IMPROVEMENT OF BETA-CELL ENGRAFTMENT**

3.2.1 *Animals*

C57BL/6 mice (purchased from Charles River, L' Arbresle, France) were used as islet donors and transplant recipients in all procedures. The mice were handled in accordance with the *Guide for the care and use of laboratory animals*, Eighth edition (2011) as well as Katholieke Universiteit Leuven (KULEUVEN, Leuven, Belgium) Animal Care and Use Guidelines.

3.2.2 *Preparation and characterization of human MAPC*

Human MAPC (n=2) used in this study were isolated by ReGenesys BVBA (Athersys Inc. affiliate in Heverlee, Belgium) from bone marrow of a 30-year-old female and a 45-year-old male volunteer, with informed consent and ethical approval. Isolation and culture of the cells were carried out as described [393]. Human MAPC were expanded on the Quantum cell expansion system (Terumo BCT, Lakewood, CO). This closed automated culture system is comprised of a synthetic hollow-fiber bioreactor connected to sterile closed-loop, computer-controlled media and gas exchangers. The bioreactor contains ~11,000 fibers generating an expansion surface area of 2.1 m^2. After coating the bioreactor with fibronectin, cells were seeded on the inside of the hollow fibers and expanded in MAPC culture medium. Cells were harvested after 5-6 days using trypsin/EDTA.

Phenotypic analysis of the human MAPC was performed using fluorochrome-conjugated antibodies recognizing a cluster of differentiation 3 (CD3), CD31, CD34, CD40, CD44, CD86, CD105, Flk1, HLA-ABC, and HLA-DR (eBioscience). The acquisition was done by using a Gallios™ multicolor flow cytometer (Beckman Coulter). For analysis of the samples, FlowJo (Tree Star Inc.) software was used.

Cell-free supernatants were assayed for human basic fibroblast growth factor (bFGF), C-reactive protein (CRP), eotaxin, eotaxin-3, soluble fms-like tyrosine kinase 1 (sFlt1), granulocyte-macrophage colony-stimulating factor (GM-CSF), soluble intracellular adhesion molecule-1 (sICAM-1), IFN-γ, IL1α, IL1β, IL10, IL12 p70, IL12/IL23p40, IL13, IL15, IL16, IL17A, IL2, IL4, IL5, IL6, IL7, IL8, IP-10, monocyte chemoattractant protein-1 (MCP-), MCP4, macrophage-derived chemokine (MDC), macrophage inflammatory protein-1α (MIP-α), MIP-β, placental growth factor (PlGF), serum amyloid A (SAA), thymus- and activation-regulated chemokine (TARC), Tie2, TNF-α, TNF-β, soluble vascular cell adhesion molecule-1 (sVCAM-1), VEGF-A, VEGF-C, and VEGF-D by multiplex electrochemiluminescence (Meso Scale Discovery, Rockville, MD) as per manufacturer's protocol.

The angiogenic potential of human MAPC was examined in the chick chorioallantoic membrane (CAM) as described [394].

3.2.3 *Marginal mass syngeneic islet cell transplantation diabetes model*

To induce diabetes in recipients, a single intravenous injection of alloxan (90 mg/kg; Sigma) was administered to male C57BL/6 mice, and animals were considered to be diabetic after two consecutive non-fasting tail vein blood glucose concentrations of ≥200 mg/dl, measured by an

AccuCheck Glucometer (Roche Diagnostics). Before transplantation, islets from 2-3 week-old C57BL/6 mice isolated by collagenase digestion were washed, counted, and in some cases mixed with human MAPC [392]. Thereafter, the cellular pellets were transferred to silicon microtubing (BD Biosciences), centrifuged for 5 minutes at 1500 rpm. During transplantation, the mice were anesthetized, and the left kidney was exposed by a lumbar incision. Diabetic recipient mice were given 150 islets alone, 150 islets and 2.5×10^5 human MAPC as separate pellets (SEP) or 150 islets and 2.5×10^5 human MAPC as a composite pellet (MIX) under the renal capsule. Non-fasting blood glucose levels from the tail vein of each recipient were measured daily during the first week post-transplantation and thereafter three times weekly. Mice were considered cured when having blood glucose levels <200 mg/dl after 3 consecutive measurements. All islet cell transplantations were performed at random in all experimental groups. On week 2 and 5 after islet cell transplantation, graft-bearing kidneys were removed and fixed in 4% formaldehyde followed by paraffin embedding or were used for RNA isolation.

3.2.4 *Physiological studies*

Glucose tolerance tests were performed after a 16-hour fast. Mice were injected IP with D-glucose (2 g/kg body weight), and blood glucose levels were measured at the indicated times.

For serum insulin and C-peptide determination, blood was collected from the saphenous vein. Serum was isolated by centrifugation, levels of pancreatic hormones were determined by ultrasensitive enzyme-linked immunosorbent assay (ELISA) kits (Mercodia or Merck Millipore).

3.2.5 *Morphometry and immunohistochemistry*

Graft-bearing kidneys were embedded in paraffin, and 6 µm sections were obtained from the total graft area. Insulin (guinea-pig, #A0564, Dako Belgium nv/sa), glucagon (mouse, #G2654, Sigma), somatostatin (rat, #ab30788, Abcam, Cambridge, UK), endomucin (rat, #sc-65495, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and α-smooth muscle actin (SMA) (goat, #EB06450, Everest Biotech Ltd, Oxfordshire, UK) stainings were used to evaluate beta-cell mass and blood vessel density respectively with the aid of the Ventana system (Ventana Medical Systems Inc., Tucson, AZ). The endomucin as well as α-SMA antibody is recommended for detection of endomucin or α-SMA respectively of mouse and not human origin.

For quantification of the beta-cell and blood vessel volumes, all images were captured using a Nikon Eclipse TE2000-E microscope using a 40× magnification objective and the large image-capture feature so that the entire graft area of each section could be pictured at once. Insulin+, glucagon+ and somatostatin+ areas as well as endomucin+ areas within the endocrine compartment of the islet graft were measured semi-automatically by ImageJ/Fiji software on approximately 15% (i.e. every fifth section) of the total graft as described [376]. The vessel/beta cells ratio was calculated as (blood vessel area/insulin+ area) × 100%. Vessel density was calculated as the number of intra-islet vessels per mm^2.

3.2.6 *Quantitative PCR*

Islet graft RNA was isolated using the PureYield™ RNA Midiprep System (Promega, Madison, WI) as described [394], and a 1-µg aliquot was reverse transcribed into cDNA (Superscript II; Life Technologies, Carlsbad, CA). cDNA was then subjected to quantitative PCR using gene-specific forward and reverse primers (own design or purchased from Integrated DNA Technologies, Coralville, IA (CD31/PECAM-1) or Thermo Fisher Scientific, Waltham, MA (α-SMA)) using either Fast SYBR® Green Master Mix or a gene-specific TaqMan® probe in combination with TaqMan® Fast Universal Master Mix (Life Technologies). Primer and probes sequences are listed in table 1. Each quantitative reaction was carried out in duplicate or triplicate, and islet grafts from 6-11 mice of each experimental group were independently tested. Relative mRNA expression value is calculated using the ΔΔCt method. All samples were normalized to the average of Actine, HPRT and RPL27 as housekeeping genes. Background amounts of each target gene were calculated from the non-grafted kidney. Results are expressed as the mean ± SEM.

Table 1: Primer and probe characteristics

Target	Sequence
Insulin	F 5'-CCGGGAGCAGGTGACCTT-3' R 5'-GATCTACAATGCCACGCTTCTG-3' P 5'-AGACCTTGGCACTGGAGGTGGCC-3'
Glucagon	F 5'-AACAACATTGCCAAACGTCA-3' R 5'-TGGTGCTCATCTCGTCAGAG-3' SYBR
Somatostatin	F 5'-GGAAACAGGAACTGGCCAAGT-3' R 5'-GGGTTCGAGTTGGCAGACC-3' SYBR
CD31/PECAM-1	F 5'-GCATGTCTTTTATGATCTCAGAC-3' R 5'-CATCGGCAAAGTGGTCAAGA-3' SYBR

F: forward; R: reverse; P; probe; SYBR: Fast SYBR® Green

3.2.7 *Statistical analysis*

Statistics were calculated with Prism software 5.0 (GraphPad Software Inc.). The chi-square test was applied to identify the significance of the difference between diabetes reversal rates between different groups. All numerical values were presented as the mean ± SEM. Significance was determined using Mann-Whitney U-test or Kruskal-Wallis test, and a value of $P<0.05$ was considered significant.

CHAPTER 4

RESTORATION OF SELF-TOLERANCE AFTER ISLET TRANSPLANTATION[2]

[2]based on João Paulo Monteiro Carvalho Mori Cunha, Dana Cook, Giuliana Ventriglia, Guido Sebastiani, Francesca Mancarella, John Kappler, Philippa Marrack, Lothar Steidler, Pieter Rottiers, Francesco Dotta, Conny Gysemans, Chantal Mathieu. Induction of active tolerance after islet cell transplantation in longstanding diabetic NOD mice after *L.lactis*-based vaccine combined with low-dose anti-CD3: a matter of timing. Manuscript in preparation

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4.1 INTRODUCTION

T1D is a chronic autoimmune pathology characterized by selective destruction of the insulin-producing beta cells in the pancreas [2]. Until today exogenous insulin administration remains the standard treatment, however, with often undesirable recurrent episodes of hypoglycemia and long-term macro- and microvascular complications. Especially for T1D patients with hypoglycemia unawareness, beta-cell replacement in the modality of intraportal allogeneic islet transplantation has been shown to provide a significant improvement in glycemic control with the eradication of severe hypoglycemic episodes [9,395]. Most islet transplant recipients at qualified centers reach insulin independence 1 year after islet implantation. Although the long-term outcome of clinical islet transplantation improved substantially since the introduction of the Edmonton protocol [239,396], adverse events are often related to the immunosuppressive drugs used to avoid immune responses to alloantigens and recurrence of autoimmunity.

Currently, most islet transplant recipients receive an induction immunosuppression therapy with biologicals like ATG or anti-CD25, followed by glucocorticoid-free maintenance schedules consisting of tacrolimus and MMF or sirolimus [239,397]. Another approach to induction therapy includes the use of either anti-CD3 mAbs [398] or a mixture of rATG or alemtuzumab combined with etanercept (anti-TNF-α) [399,400]. These novel regimens with anti-CD25 or ATG preferentially deplete lymphocytes, but maintain or increase Treg frequencies and functionality, and diminish cytokine toxicity on the implanted islets. Unfortunately, lymphodepletion strategies cause "cytokine storm" in the initial days after administration, including the release of IL7 and IL15 which can induce homeostatic expansion of islet-specific autoreactive T cells with effector memory phenotype [401]. Also, preclinical data demonstrated that recurrent autoimmune diabetes is mainly driven by $IGRP_{206–214}/H2K^{d}$ (NRP-V7)-reactive T cells from the memory pool [402]. This also implies that local islet antigen expression, in addition to MHC class I expression is indispensable for the increase of autoreactive $CD8^{+}$ T cells into islet grafts [403]. These findings underscore the importance of tackling autoreactive T-cell memory after beta-cell replacement therapy. The implementation of induction therapy prior to and at the moment of islet implantation clearly improved 5-year insulin independence when combined with maintenance regimens [399] but also emphasized the need for immunomodulatory regimens which aim at inducing tolerance to both foreign and beta-cell antigens in islet transplant recipients.

Several preclinical studies have documented that combining lymphodepletion with other modalities may induce peripheral tolerance [404–407], although this has yet to be fully accomplished in men. Emphasis is put on peptide-specific strategies, concentrating on the tolerogenic delivery of beta-cell antigens and non-mitogenic anti-CD3, which specifically targets the TCR in the absence of costimulatory signals. In this context, we reached approximately 60% of new-onset diabetes remission in NOD mice when genetically-modified *L. lactis*-based vaccines producing either human proinsulin or a GAD65 peptide along with IL10 was combined with a short course of sub-therapeutic doses of anti-CD3 mAbs [74,201]. In this current study, we tested whether our antigen-specific intervention could also work in longstanding autoimmune diabetic mice after islet supplementation.

4.2 **EXPERIMENTAL DESIGN**

Recipients for the islet cell transplantation experiments were severely diabetic male NOD mice, usually over 20 weeks of age, which presented blood glucose concentrations exceeding 200 mg/dl and had diabetes duration between 0 and 4 weeks after diagnosis. These mice received sc implantation with insulin pellets (LinBit™) to prevent death. Transplants (500 insulitis-free islets isolated from 2-3-week-old NOD mice) were placed unilaterally under the left kidney capsule to facilitate histological examination. Mice were treated either prior to islet implantation (early regimen from d−5 to −1: Figure 4.1A) or at the time of transplantation (delayed regimen from d0: Figure 4.1B). Treatment regimens consisted out of hamster anti-mouse anti-CD3 mAbs (clone 145-2C11) for 5 consecutive days at low doses of 2.5 µg per day and were given simultaneously with genetically-modified *L. lactis* bacteria secreting human proinsulin and IL10 (LL-PINS+IL10; 2×10^9 CFU/d) 5 times weekly for a total period of 6 weeks.

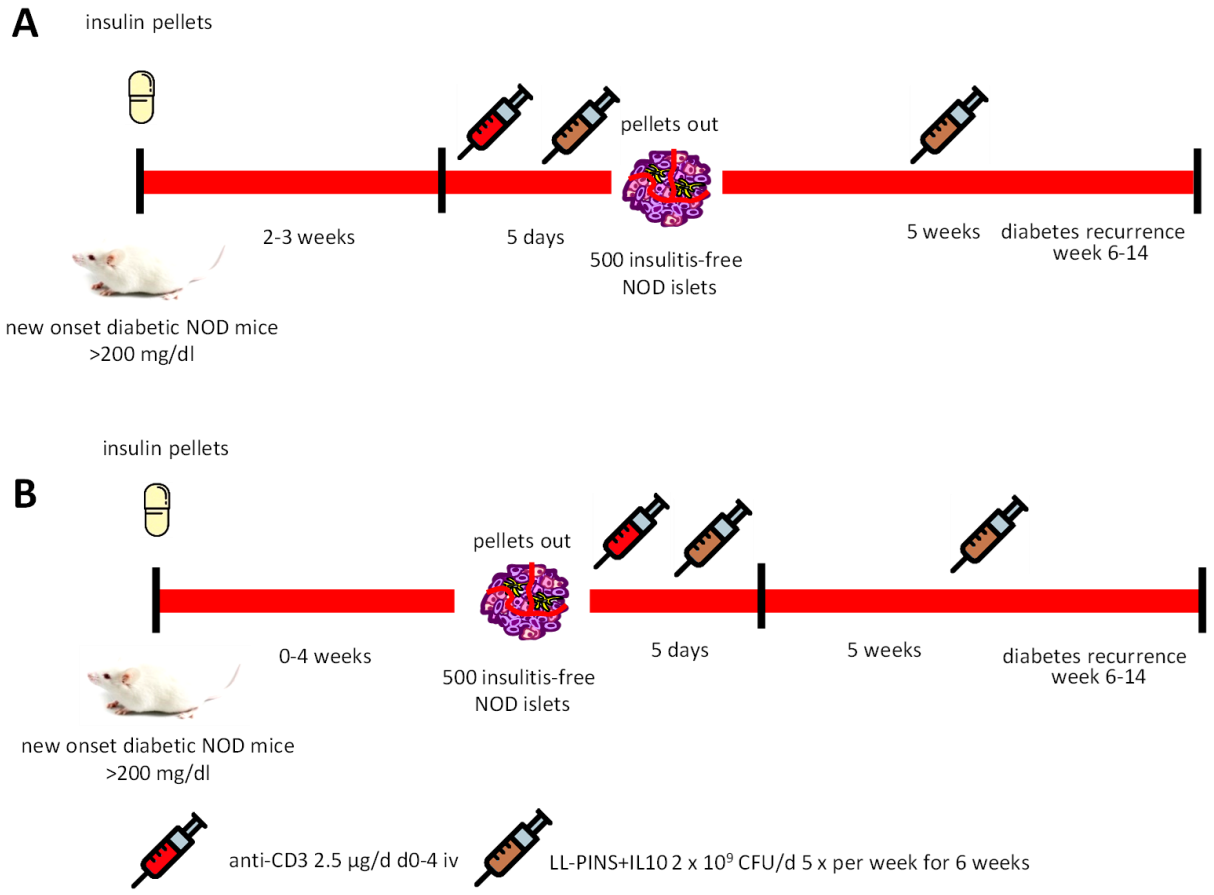


Figure 4.1. Treatment regimens in NOD islet recipient mice. New-onset diabetic NOD mice were sc implanted with insulin pellets and kept for an additional 1 – 3 weeks after diagnosis. Animals were injected with low-dose CD3 mAbs (clone 145-2C11; 2.5 µg/d iv) for 5 consecutive days prior to (**A**; early regimen) or at the time of islet cell transplantation (**B**; delayed regimen). Intragastric administration of genetically-modified *L. lactis* bacteria secreting proinsulin and IL10 (LL-PINS+IL10; 2×10^9 CFU/d 5 times weekly) started together with anti-CD3 induction therapy and persisted for a total period of 6 weeks. Weight and blood glucose concentrations were measured three times weekly.

4.3 RESULTS

4.3.1 *Pharmacodynamic profile of anti-CD3*

We first examined the pharmacokinetics of anti-CD3 (clone 145-2C11) on different lymphocyte frequencies ($CD3^+$, $CD4^+$ and $CD8^+$ T cells) in new-onset diabetic NOD mice (without islet supplementation) by multicolor flow cytometry. We assessed the splenic T-cell proportions prior to (day 0) and weekly after dosing (day 7, 14, 21 and 28). Mice receiving total of 12.5 µg of anti-CD3 mAbs (clone 145-2C11; 2.5 µg/d iv for 5 consecutive days) had 7 days after anti-CD3 injection significantly lower proportions of $CD3^+$ T cells in the spleen, due to selective depletion of $CD4^+$ T cells but not of $CD8^+$ T cells which remained fairly stable after anti-CD3 injection (Figure 4.2A).

Earlier studies established that anti-CD3 mAbs are particularly effective at controlling recently activated effector T cells [408]. In our study, we found that although bulk $CD4^+$ and $CD8^+$ T cells recovered by day 21 after anti-CD3 injection, but the distribution of $CD4^+$ and $CD8^+$ T-cell populations continued to be modified (Figure 4.2B and C). In detail, anti-CD3 augmented the T-cell fraction with a putative effector/memory phenotype ($CD69^+CD44^-$ and $CD69^-CD44^+$) in especially $CD4^+$ but also in $CD8^+$ T cells in the spleen.

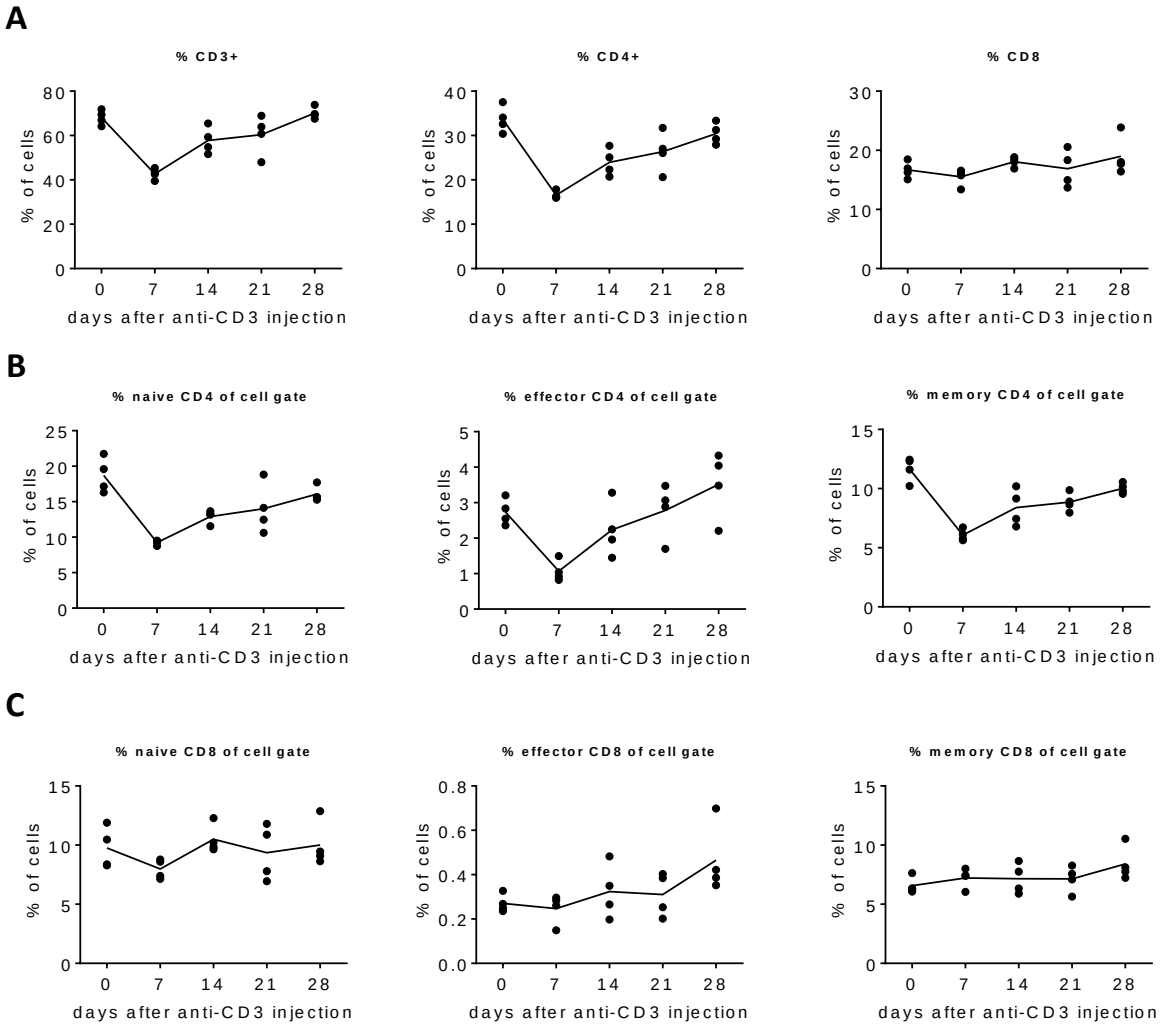


Figure 4.2. Low-dose anti-CD3 mAb treatment transiently reduces splenic T-cell frequencies. NOD mice were treated with anti-CD3 mAb (2.5 µg/d i.v., day 0 – 4). (**A**) T-cell subset frequencies in the spleen were determined by flow cytometry and displayed as a percentage of live cells. The frequencies of naive ($CD44^-CD69^-$), recently activated ($CD44^+CD69^+$) and memory ($CD44^+CD69^-$) T cells were determined and plotted as percentage of $CD4^+$ (**B**) or $CD8^+$ (**C**) T cells. Symbols represent values of individual mice (n=4), the line connects the mean per time point.

4.3.2 *Prevention of autoimmune diabetes recurrence by L. lactis-based therapy combined with low-dose anti-CD3 depends on the timing of drug administration and diabetes duration*

Based on the time kinetics of anti-CD3 mAbs, we hypothesized that induction therapy with low-dose anti-CD3 alone or combined with genetically-modified *L. lactis* secreting proinsulin and IL10 initiated prior to islet implantation (d−5 to −1: Figure 4.1A) could prevent autoimmune diabetes recurrence as lymphodepletion after anti-CD3 injection was then almost at its highest level (Figure 4.2). NOD islet recipient mice (0 to 4 weeks after diabetes diagnosis), left untreated, demonstrated autoimmune disease recurrence in all animals (mean survival time = 8 days). When anti-CD3 either alone or combined with the *L. lactis*-based vaccine was administered prior to islet implantation only 17 and 25% of mice respectively evaded disease recurrence (Figure 4.3A).

We reasoned that early induction immunosuppression might prevent graft destruction in the immediate post-transplantation period but might not be able to inhibit specifically the strong memory autoimmune responses directed against the newly implanted islets (antigens). Therefore, we designed a regimen in which therapy started at the time of islet implantation (Figure 4.2B). With this delayed initiation of therapy, 34% of anti-CD3-treated NOD islet recipient mice and 56% of combination therapy-treated animals remained normoglycemic during therapy and even 14 weeks after transplantation (Figure 4.3B). These data put forward that the crucial period for the therapeutic efficacy of anti-CD3 therapy either alone or combined with *L. lactis*-based vaccine is not prior to islet implantation.

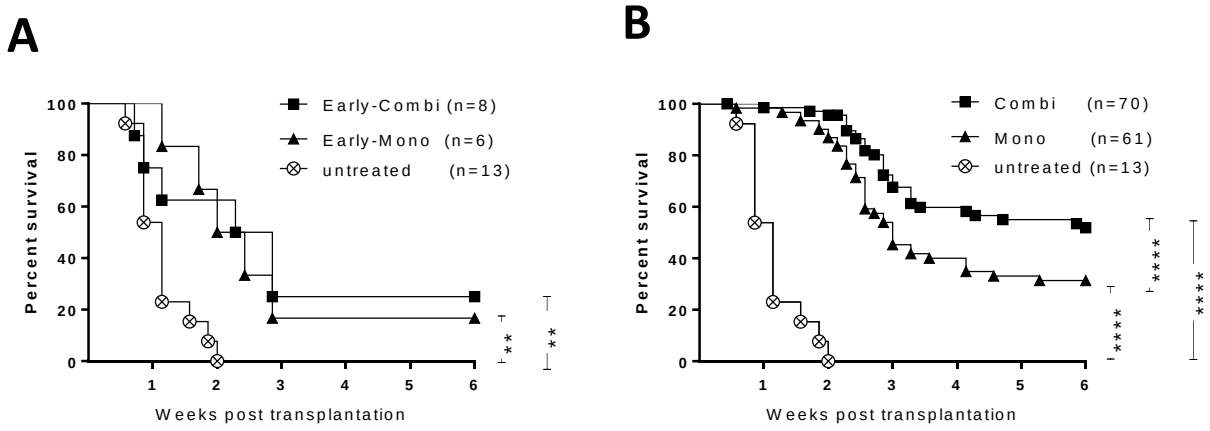


Figure 4.3. The start of *L. lactis*-based therapy in diabetic NOD mice determines the therapeutic outcome. NOD islet recipient mice were treated with anti-CD3 alone (mono) or combined with *L. lactis*-based vaccine (combi) 5 consecutive days prior to (early) (**A**) or at the time of islet implantation (**B**). Kaplan-Meyer curves where the endpoint was sacrifice were compared with the log-rank test. **P < 0.01; ****P < 0.0001 vs. untreated controls.

As NOD islet recipient mice had different duration of diabetes (between 0 to 4 weeks after diabetes diagnosis) at the moment of islet implantation, we also studied whether this parameter would have an effect on the therapeutic outcome. Interestingly, the efficiency to prevent autoimmune diabetes recurrence diminished in anti-CD3-treated mice with diabetes duration (50, 60, 37, 20 and 14% in mice 0, 1, 2, 3 and 4 weeks post-diagnosis).

In contrast, combination therapy-treated mice were significantly better at avoiding autoimmune disease recurrence and much less influenced by diabetes duration (86, 67, 53, 42 and 60% in mice 0, 1, 2, 3 and 4 weeks post diagnosis)(Figure 4.4).

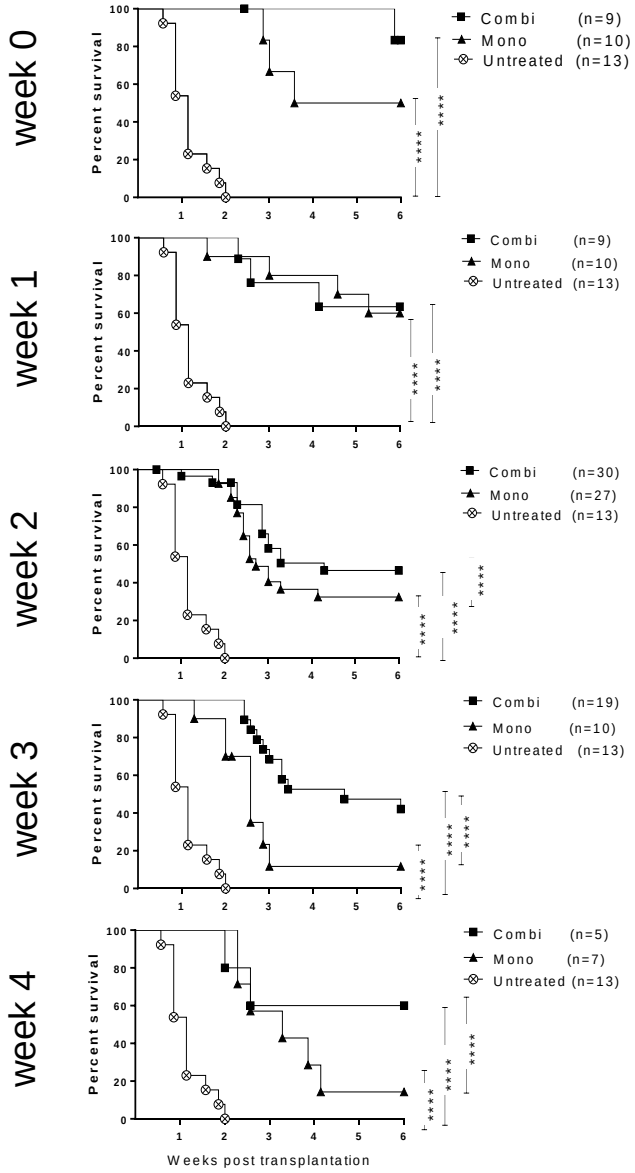


Figure 4.4. Time after diabetes diagnosis determines therapeutic success of the *L.lactis*-based immunomodulatory therapy given at the time of islet implantation. Diabetic NOD mice (diabetes duration between 0 – 4 weeks) were transplanted with islets from NOD donors (syngeneic) and treated with anti-CD3 alone (2.5 µg/d iv for 5 consecutive days) with LL-PINS+IL10 (2×10^9 CFU/d 5 days per week for 6 weeks). Kaplan-Meyer curves where the endpoint was sacrifice were compared with the log-rank test. ****P < 0.0001 vs. untreated controls.

4.3.3 *L. lactis-based combination therapy preserves beta-cell function of the islet grafts and remaining endogenous islets*

The mechanisms by which our *L. lactis*-based therapy regimen may exert benefit might be two-fold. First, the immunomodulatory regimen may offer better protection of the implanted islets from innate immune responses in the perioperative period, thereby maintaining islet graft function. Second, these regimens may alleviate recurrent autoimmunity directed at transplant islets by eliminating at least temporarily, the autoreactive T-cell compartment and by promoting antigen-specific Treg generation.

In our study, we first observed that the random serum C-peptide concentrations 6 weeks after transplantation were higher at all time point after diabetes diagnosis in the 'cured' NOD islet recipient mice given either anti-CD3 or *L. lactis*-based combination therapy together with islet implantation compared to untreated animals (Figure 4.5A). This functional profile was mirrored by the beta-cell mass of the islet grafts as measured by confocal microscopy (data not shown).

We also determined the endogenous beta-cell mass by analyzing the insulin content of the native pancreas. As expected, the pancreas from untreated and 'non-cured' animals given either anti-CD3 or *L. lactis*-based combination therapy had negligible concentrations of insulin (Figure 5.5B). Unexpectedly, long-term preservation of endogenous beta-cell mass was observed in the pancreatic islets of previously hyperglycemic recipients. Both anti-CD3 alone and *L. lactis*-based combination therapy protected the endogenous beta cells, although this decreased with diabetes duration (Figure 4.5B). These observations imply tht 'cured' treated NOD islet recipients with long-term diabetes duration rely exclusively on their newly implanted islets for proper glucose control.

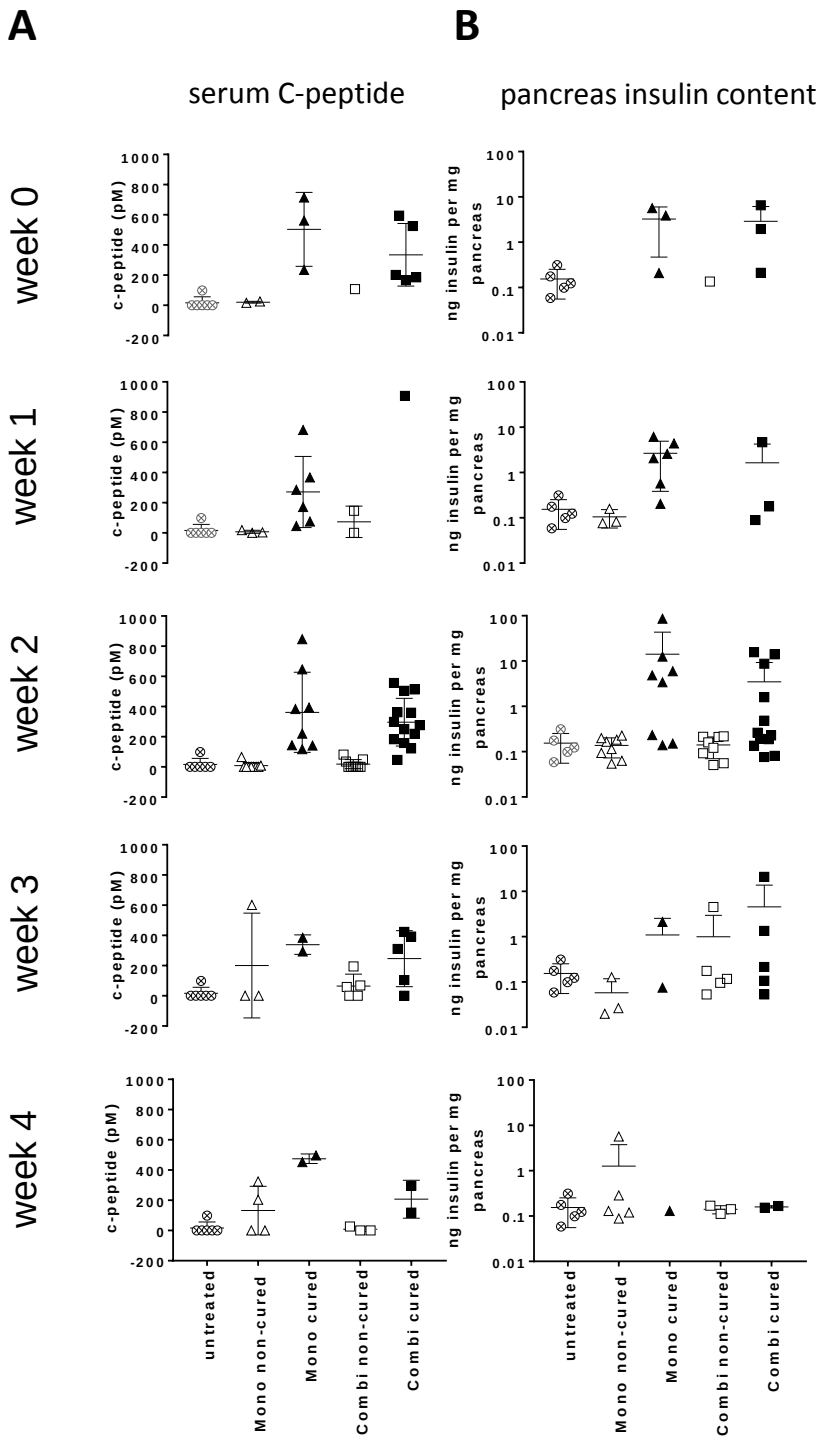


Figure 4.5. Serum C-peptide concentrations and insulin content of the native pancreas 6 weeks after islet implantation in NOD recipient mice. Diabetic NOD mice (diabetes duration between 0 – 4 weeks) were transplanted with syngeneic islets and treated with anti-CD3 alone (mono; 2.5 µg/d iv for 5 consecutive days) with or without LL-PINS+IL10 (combi; 2×10^9 CFU/d 5 days per week for 6 weeks). (**A**) C-peptide concentrations were measured in serum from random-fed NOD islet recipients. (**B**) Total pancreatic insulin content in random-fed NOD islet recipients. Crossed circles: untreated controls; white symbols: non-cured; black symbols: cured; triangles: monotherapy; squares: combination therapy.

4.3.4 *Graft acceptance despite massive immune infiltration*

The kidney containing islet grafts from NOD recipient mice (2 weeks after diabetes diagnosis) that had been treated with anti-CD3 alone (mono) or combined with *L. lactis*-based antigen-specific therapy (combi) was examined histologically 6 weeks after islet implantation. Untreated animals had almost completely destroyed islet grafts with striking lymphocytic infiltration, a characteristic of autoimmune diabetes recurrence (data not shown). Few intact islets were detected in the grafts. Similar histological characteristics were apparent in islet grafts retrieved from non-cured treated NOD recipient mice 6 weeks after islet implantation (data not shown). Despite mononuclear infiltration in both groups (untreated and treated), islet architecture was preserved in NOD recipient mice cured by anti-CD3 mono and combination therapy (Figure 4.6). Moreover, immunofluorescent analysis of the graft CD4 and CD8 staining showed analogous mononuclear accumulation around the grafted tissue in both treatment groups. Comparable histological observations were also found in NOD islet recipient mice with different diabetes duration at islet implantation (data not shown).

Next, we examined at therapy discontinuation the distribution of insulin-producing beta cells and Tregs by histology in islet grafts of NOD recipient mice (2 weeks after diabetes diagnosis) treated with anti-CD3 alone (mono) or combined with *L. lactis*-based therapy (combi). Densities of $Foxp3^+$ cells were markedly increased around islets in NOD recipient mice with accepted grafts compared to untreated controls, irrespective of therapy (Figure 4.6). However, also in the islet grafts of non-cured treated NOD recipient mice $Foxp3^+$ cells were present at the site of extensive tissue damage (data not shown). We hypothesize that these Tregs may be largely outnumbered by antigen-experienced effector T cells. We speculate that regardless of success or failure in protecting the islet transplant, Tregs may persistently interact with effector T cells, but an imbalance in the numbers of Tregs versus activated effectors might determine the result. This hypothesis needs further investigation.

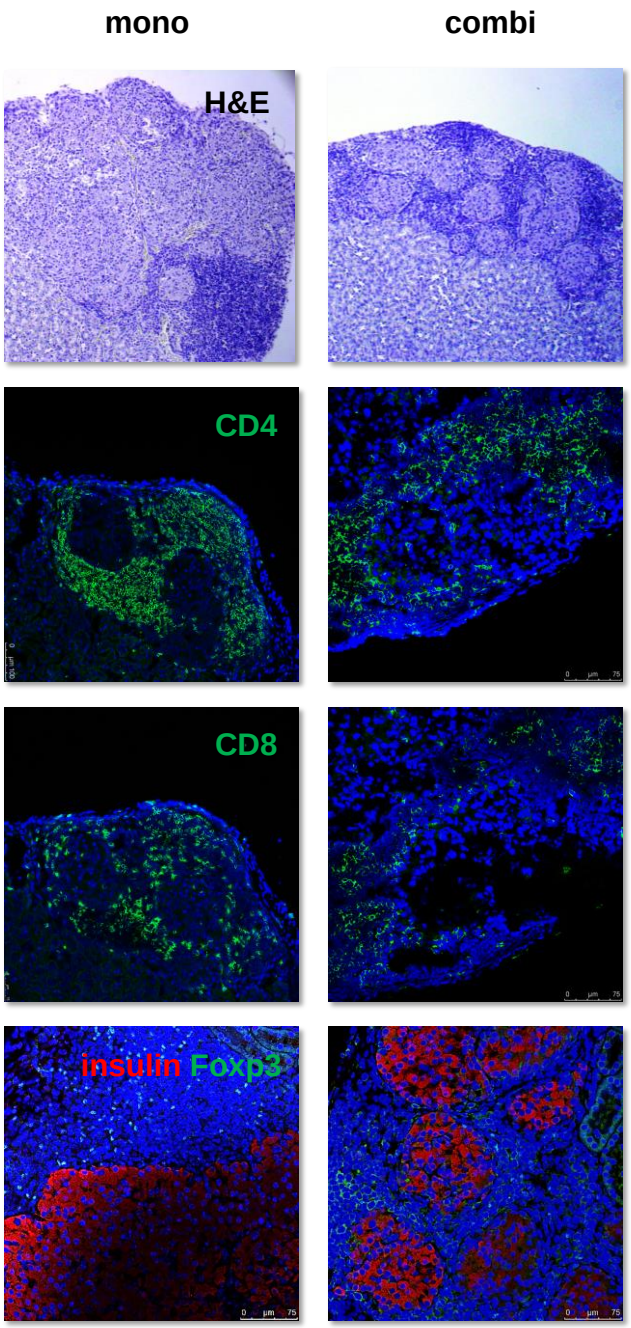


Figure 4.6. Islet graft histology and immunofluorescence for $CD4^+$, $CD8^+$, and $Foxp3^+$ T cells. Sections from necroscopy islet grafts were stained with hematoxylin and eosin and the indicated immune cells (green) to reveal mononuclear infiltration in and around the islet grafts of NOD recipient mice (2 weeks after diagnosis) 6 weeks after therapy start (i.e. anti-CD3 alone (mono) or *L. lactis*-based combination therapy (combi)). DAPI was used as a nuclear stain (blue). At the time of normoglycemia at week 6 after transplantation, graft samples revealed well-granulated islets with strong insulin staining (red) and massive infiltration of $CD4^+$ or $CD8^+$ T cells around the islets. Most notably, accepted grafts revealed the presence of $Foxp3^+$ T cells around the islets. Photomicrographs (scale bar 75 µm) are representative of ~10 sections per recipient. The patterns of cell infiltration were consistent across animals studied. Stains: hematoxylin and eosin (H&E, top row).

4.3.5 *L. lactis-based protection is associated with T-cell subset alterations*

To determine whether the therapeutic effect obtained by the treatment with anti-CD3 with or without *L. lactis*-based vaccine was related to a measurable alteration in the relative percentages of the main T-cell subsets, $CD4^+$ and $CD8^+$ T-cell populations were analyzed in spleen, pancreatic draining (PLN) and kidney draining lymph nodes (KLN) of cured and non-cured treated NOD islet recipient mice (2 weeks after diagnosis) 6 weeks after transplantation. Phenotypic characterization of T cells isolated from spleens and any of the lymph node compartments studied did not show significant differences in the percentages of $CD4^+$ and $CD8^+$ T cells (data not shown), and the $CD4^+/CD8^+$ ratios (Figure 4.7) remained comparable in all cured NOD islet recipient mice, regardless of therapy. However, the $CD4^+/CD8^+$ ratios were markedly higher in the spleen and PLN of cured treated NOD islet recipients compared to untreated controls and their non-cured treated counterparts. We did not observe differences in these T-cell frequencies related to diabetes duration at the time of islet implantation (data not shown).

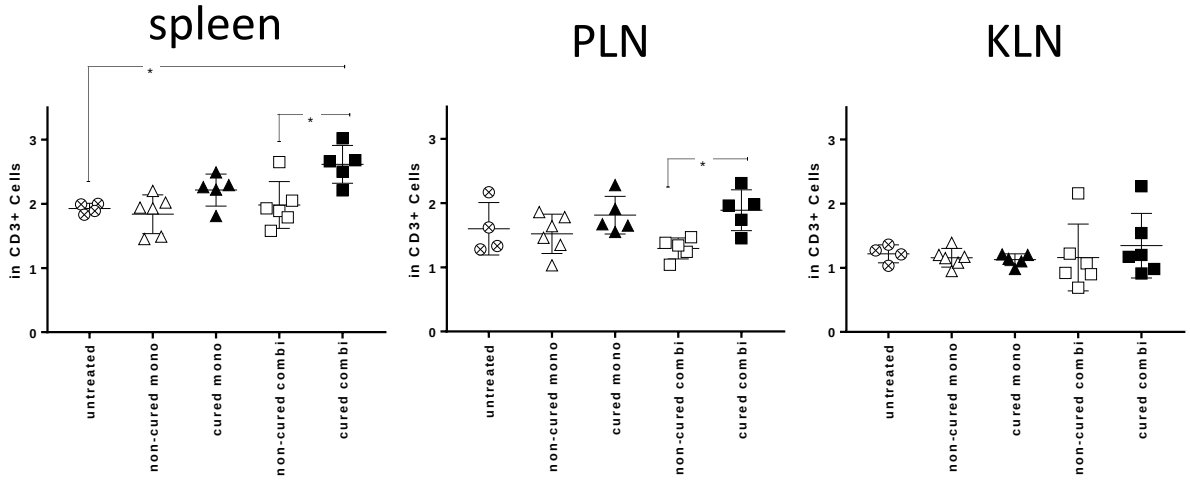


Figure 4.7. The ratio of $CD4^+$ over $CD8^+$ T cells in spleen, pancreatic and kidney draining lymph nodes of NOD islet recipients (2 weeks after diagnosis) 6 weeks after transplantation and treated with anti-CD3 alone (mono) or combined with *L. lactis*-based vaccine (combi). Each symbol represents an individual mouse, and horizontal bars indicate the median value. Crossed circles: untreated controls; white symbols: non-cured; black symbols: cured; triangles: monotherapy; squares: combination therapy. Statistical significance was calculated using Mann-Whitney t-test; *P < 0.05, **P < 0.01. PLN, pancreatic lymph node. KLN, kidney draining lymph node

Immune effector function at the target tissue can be controlled by various mechanisms coordinated by Tregs [409]. Unexpectedly, we observed lower $CD4^+Foxp3^+$ Treg frequencies in PLN and KLN of cured NOD islet recipients, irrespective of therapy, compared to untreated controls and non-cured treated counterparts (Figure 4.8). The percentages of $CD4^+Foxp3^+$ Tregs in different organs studied was not influenced by the time of diabetes diagnosis at the moment of islet implantation (data not shown).

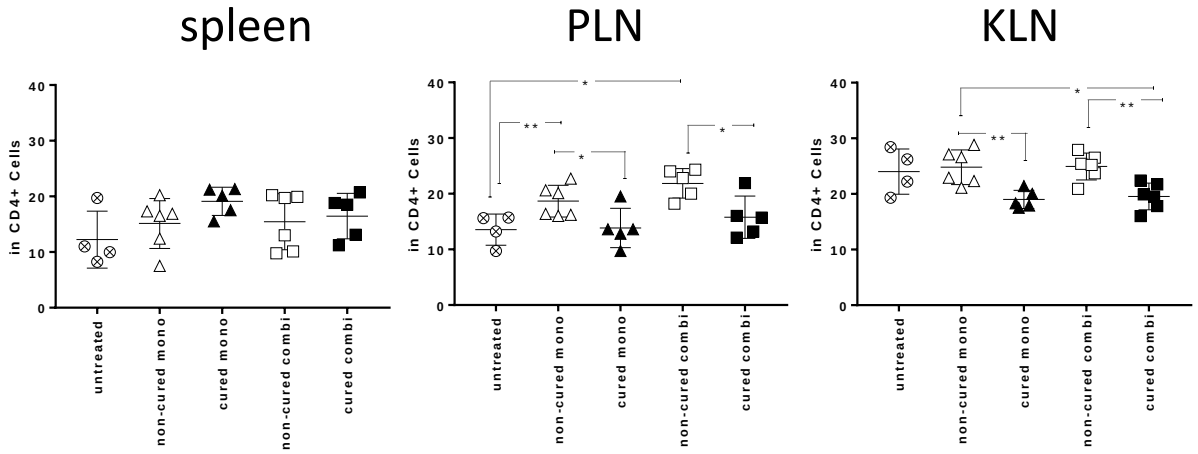


Figure 4.8. Flow cytometry of cells from spleen, pancreatic and kidney draining lymph nodes of NOD islet recipient mice (2 weeks after diagnosis; n=5 – 6), analyzing the frequency of $Foxp3^+CD4^+$ Treg cells in those tissues 6 weeks after transplantation and treated with anti-CD3 alone (mono) or combined with *L. lactis*-based vaccine (combi). Each symbol represents an individual mouse, and horizontal bars indicate the median value. Crossed circles: untreated controls; white symbols: non-cured; black symbols: cured; triangles: monotherapy; squares: combination therapy .PLN, pancreatic lymph node. KLN, kidney draining lymph node. Statistical significance was calculated using Mann-Whitney t-test; *P < 0.05, **P < 0.01.

How exactly Tregs suppress the immune response *in vivo* is still debated. Whether in accepted islet grafts antigen-specific Tregs control the majority of antigen-experienced T cells at the site of inflammation/transplantation via direct contact, by modulating APCs and dampening the expression of the co-stimulatory molecules CD80 and CD80, or via other mechanisms is a matter of further studies.

4.4 DISCUSSION

Contrasting with alloimmune responses, autoimmune disease recurrence is difficult to control with standard immunosuppression and therefore poses a challenging obstacle for a successful long-term islet cell transplantation outcome. Currently used T-cell-targeting strategies (i.e. ATG, alemtuzumab or anti-CD3) administered prior to the time of islet implantation are specifically designed to tackle alloimmunity and reduce the necessity for maintenance immunosuppression [399] but unexpectedly might predispose the graft to destruction by promoting the development and expansion of effector memory T cells [401]. An interesting approach that could diminish homeostatic expansion of autoreactive T cells is combining mild lymphodepletion with antigen-specific approaches that have the potential to reinstate immunological tolerance by the induction of islet antigen-specific Tregs and that obviate the need for maintenance immunosuppression [404–407].

In this study, we used a combination therapy consisting of a short induction therapy of low doses of anti-CD3 mAbs combined with the intragastric delivery of a genetically-modified *L. lactis*-based vaccine producing human proinsulin along with pro-tolerogenic IL10 to reestablish tolerance to islet antigens in longstanding diabetic NOD islet recipients. This therapeutic approach has been shown by our group to reach approximately 60% of new-onset diabetes remission in NOD mice [74,201]. We postulate that the remaining 40% of the non-cured treated mice had possibly insufficient beta-cell mass at therapy entry to reach proper glycemic control, especially since mice with mild starting blood glucose concentrations (<350 mg/dl) had a superior (74%) diabetes reversal outcome [74]. Moreover, also the non-cured treated mice responded immunologically to the *L. lactis*-based combination therapy as demonstrated by the dramatic accumulation of $Foxp3^+$ Tregs locally in the pancreas (Takiishi *et al.* personal communication). Although some T1D patients with disease duration of over 30 years still have detectable beta-cell function [410], it is clear that duration of diabetes leads to a progressive loss of beta-cell mass and function over many years [411]. As such, we transplanted, in the current study, 500 insulitis-free syngeneic NOD islets in either new-onset or longstanding diabetic NOD mice to immediately restore insulin secretion and promote normalization of hyperglycemia.

Chatenoud *et al.* established in beta-cell autoimmunity that anti-CD3 mAb therapy can shift the balance of activated effector T cells and Tregs toward a more tolerant phenotype but only when

applied in the context of a primed immune system [408,412]. Based on these observations and the pharmacokinetic profile of anti-CD3, we designed a first therapeutic regimen starting day –5 of islet implantation to specifically tackle the activated effector T-cell pool, which is a prerequisite for the generation of a therapeutic window allowing Tregs to induce and maintain graft tolerance. Although this early regimen with low-dose anti-CD3 combined or not with *L. lactis*-based antigen-specific therapy induced graft acceptance in around 20% of NOD islet recipients compared to 0% in untreated controls, most islet grafts were destroyed [221,413]. We speculate that since effector memory $CD8^+$ T cells are eventually resistant to lymphodepletion [414,415], repopulate faster than their $CD4^+$ counterparts in a lymphopenic environment [416], especially after an antigen recall [417], without homing to secondary lymphoid organs and contact with MHC class I molecules [418,419], islet implantation (i.e. antigenic stimulation) during the period of lymphopenia-induced homeostatic proliferation of remaining T cells seems not the best therapeutic option. Our data further support the theory that resistance to tolerance induction after homeostatic proliferation is most likely the result of the generation of antigen-experienced T cells. Importantly, others already demonstrated that reconstitution of memory T cells in the presence of an allograft accelerates graft rejection [416].

In a second therapeutic regimen in which therapy started at the time of islet implantation, the overall graft acceptance was around 34 and 56% in anti-CD3- and combination therapy-treated NOD islet recipients, respectively. Although antigen-experienced T cells can mount strong (auto)immune responses in the absence of secondary lymphoid organs [420], these unique properties are not shared by either naïve or memory-like T cells generated in the absence of an antigenic (re)encounter [418], implying that islet implantation (i.e. antigen stimulation) before lymphodepletion would not only limit the homeostatic proliferation of the antigen-experienced T-cell pool, but would preferentially trigger the expansion of $Foxp3^+$ Tregs and promote long-term tolerance induction. These hypotheses need further investigation.

An import observation of our study is that not only the timing of islet implantation in relation to the T-cell-depletion strategy influences transplant outcome but also that diabetes duration before islet implantation impacts on the rate of success. Interestingly, the efficiency to prevent autoimmune diabetes recurrence clearly diminished in anti-CD3-treated mice with diabetes duration. In contrast, *L. lactis*-based combination therapy-treated mice were significantly better at avoiding disease recurrence and much less influenced by duration of diabetes. Here, we put

forward that the rate of normoglycemia restoration and the rate of graft acceptance in relation to the timing of diabetes diagnosis is in part related to the protection of residual endogenous islets in the pancreas as reflected by the insulin content of the native pancreas at different time points after diabetes diagnosis. Although we established in previous studies that our *L. lactis*-based combination therapy does not stimulate beta-cell regeneration in the pancreas, it can preserve the functional beta-cell mass at therapy entry, which is directly related to a higher degree of diabetes reversal [74]. Likewise, some patients can maintain endogenous insulin secretion, as estimated from their C-peptide concentrations, for over 30 years after diagnosis [410], implying that C-peptide positive patients might have better islet transplant outcomes compared to T1D patients with established disease (i.e. C-peptide negative).

Next, we suggest that the *L. lactis*-based combination therapy is superior in preventing autoimmune diabetes recurrence in NOD islet recipients compared to anti-CD3 monotherapy due to the delivery of an islet antigen (i.e. proinsulin) in a tolerogenic manner. We demonstrated in the past that this approach can reliable and safely reestablish long-term tolerance to autoantigens by generating antigen-specific $CD4^+Foxp3^+CTLA4^+$ Tregs without elimination of the autoreactive T-cell compartment, which implies some resetting of the balance between autoreactive and tolerogenic cells [74]. Using a humanized mouse reconstituted with human hematopoietic stem cells, Waldron-Lynch *et al.* reported that anti-CD3 induced only a modest and transient T-cell depletion, but alternatively triggered the generation of a "gut-homing" $CD4^+CD25^{hi}CCR6^+Foxp3^+$ Treg population which produced IL10 on migration to the gut and subsequently returned to the circulation [421]. Nishio *et al.* further demonstrated that Treg expansion after anti-CD3 injection occurred not through conversion from $Foxp3^-$ conventional T cells, but via expansion of a monoclonal Treg population, normally selected and sustained at low frequency [224]. Interestingly, they also proposed that a minor cytokine storm elicited by anti-CD3 mAbs may actually be crucial contributing elements in the therapeutic effect, via resetting of the Treg niches. We suggest that when combined with islet antigen delivered via the tolerogenic route, anti-CD3 mAbs may encourage, rather than a general Treg expansion, specific modifications in the homeostatic control of their islet antigen-specific repertoire or a phenotypic redistribution. The *L. lactis*-based combination therapy would preferentially generate and expand islet antigen-specific Tregs which are known to be more suitable for controlling autoimmunity *in situ*, especially when the islet antigen is still present, than polyclonal Tregs [422]. In our study, we found that NOD islet recipients treated with anti-CD3 alone or combined with the *L. lactis*-based vaccine had $Foxp3^+$ Tregs present in the lymph

nodes draining the islet implantation site (i.e. kidney) but this percentage was not different between mono- and combination therapy and independent of diabetes duration. In addition, we observed that non-cured treated counterparts had even higher Treg frequencies in their kidney and pancreas-draining lymph nodes compared to lymph nodes from NOD islet recipients with accepted grafts. We propose that Tregs may accumulate at the implantation site, where they suppress the effector function of islet antigen-specific T cells. Much more insights are needed on whether the intragraft Tregs are indeed enriched with islet antigen-specific Tregs and how these Tregs can control the diverse repertoire of antigen-experienced T cells present in the islets [423]. Tregs can function through numerous mechanisms at the site of antigen stimulation; both in draining lymph nodes and at the site of inflammation/transplantation, to create a tolerogenic milieu that promotes bystander suppression and infectious tolerance. Also, the kinetics of peripheral islet antigen-specific Treg memory cells into tissues (other than the lymphoid tissue) remain unknown.

In summary, a mild lymphodepletion with low-dose anti-CD3 mAbs in combination with the gut delivery of proinsulin along with pro-tolerogenic IL10 by genetically-modified *L. lactis* can by-pass autoimmune diabetes recurrence in 56% of syngeneic islets transplanted in longstanding diabetic NOD mice. Islet transplantation outcome will surely depend on the time of islet implantation in relation to T-cell-targeting strategy and on the time of islet implantation after diabetes diagnosis. Integration of these antigen-specific technologies to enhance engraftment and combat graft destruction may help to advance the therapeutic efficacy and availability of islet cell transplantation in selected patient populations.

CHAPTER 5

IMPROVEMENT OF BETA-CELL ENGRAFTMENT[3]

[3]based on João Paulo Monteiro Carvalho Mori Cunha, Gunter Leuckx, Peter Sterkendries, Hannelie Korf, Gabriela Bomfim-Ferreira, Lutgart Overbergh, Bart Vaes, Harry Heimberg, Conny Gysemans, Chantal Mathieu. Human multipotent adult progenitor cells enhance islet function and revascularization when co-transplanted as a composite pellet in a mouse model of diabetes. Diabetologia 2017, 60(1), 134-142

Acknowledgements

Gunter Leuckx and Harry Heimberg performed histological quantifications (Figure 5.9 and 5.10); Gabriela Bomfim-Ferreira performed flow cytometry on human MAPC (Figure 5.2), Hannelie Korf performed multiplex analyses on human MAPC (Figure 5.3), Peter Sterkendries and Bart Vaes provided human MAPC (ReGenesys BVBA (Athersys Inc. affiliate in Heverlee, Belgium)); Lut Overbergh provided scientific support for real-time PCR analyses (Figure 5.8; 5.9 and 5.11); Conny Gysemans performed CAM assay (Figure 5.3) and shares senior authorship with Chantal Mathieu.

5.1 **INTRODUCTION**

To date, insulin therapy is considered the gold standard for the treatment of T1D. Nevertheless, limitations persist, such as frequent episodes of hypoglycemia, and chronic micro- and macrovascular complications [5,8]. Islet cell transplantation offers an alternative treatment for T1D patients, specifically for those with hypoglycemic unawareness following insulin administration. Despite the improved outcome of islet transplantation over the last few years, drawbacks remain, such as a limited supply of cadaveric donors, the necessity of several donors for a single transplantation, and (immediate) graft failure through metabolic pressure, continued autoimmunity, alloimmunity, high concentrations of immunosuppressive drugs, and oxidative stress caused by hypoxia or due to cytokine exposure [424]. Rapid revascularization of the islets seems crucial to avoid these last two complications. Several studies aiming to increase engraftment with cell therapy are currently ongoing (www.clinicaltrials.gov; NCT00646724, NCT02384018).

MSC (30-35 µm in diameter), the major stem cells used for cell therapy, are self-renewing cells that can be isolated from the bone marrow but possibly also from many if not all tissues and have shown for over 10 years benefits in the treatment of several clinical diseases, mainly tissue injury and immune disorders [425–427]. When co-transplanted with islets, MSC improves islet cell transplantation outcome in preclinical animal models through immunomodulation, increasing islet revascularization and/or preserving islet morphology [345,346,355,428]. In spite of the progress in the understanding of MSC biology, there is little knowledge on the nature of their local microenvironment (probably hypoxic) and their functions *in vivo*. Moreover, long population doubling time, early senescence, and DNA damage during *in vitro* expansion as well as poor engraftment after transplantation are considered to be among the key disadvantages of MSC therapy [429]. Furthermore, with long-term culture expansion, MSC can become karyotypically abnormal which may pose a risk of tumor formation.

MAPC® (15-20 µm in diameter) are adult stem cells isolated from postnatal bone marrow, muscle or brain from rodent and human tissue [356,357]. These non-hematopoietic, non-endothelial stem cells most probably use similar immunosuppressive and angiogenic mechanisms as MSC although displaying unique features (e.g. secretome, transcriptome, and miRNA profiles) distinctive from most adult stem cells [364,430–432]. Interestingly, MAPC have been shown to promote tissue repair and healing and induce neo-angiogenesis, possibly

by delivering angiogenic growth factors that activate/recruit endogenous vascular cells and that seem to be specifically tailored to the immediate needs of the injured tissue [360–363]. *In vivo*, these cells are short-lived as they experience only a minimally prolonged residence time. Moreover, in contrast to other cell types, MAPC can be expanded long-term (for >80 population doublings) without genetic instability, and administered without tissue matching, making them into an optimal stem cell product for routine clinical use (MultiStem®, Athersys Inc., Cleveland).

5.2 EXPERIMENTAL DESIGN

The therapeutic potential of co-transplantation of undifferentiated human MAPC with mouse islets as separate or composite pellets was validated in a syngeneic marginal mass islet cell transplantation model. For this, alloxan-induced diabetic C57BL/6 mice were transplanted with syngeneic islets either alone (control) or co-transplanted as separate pellets (SEP) with human MAPC or as composite pellets (MIX) with human MAPC (Figure 5.1).

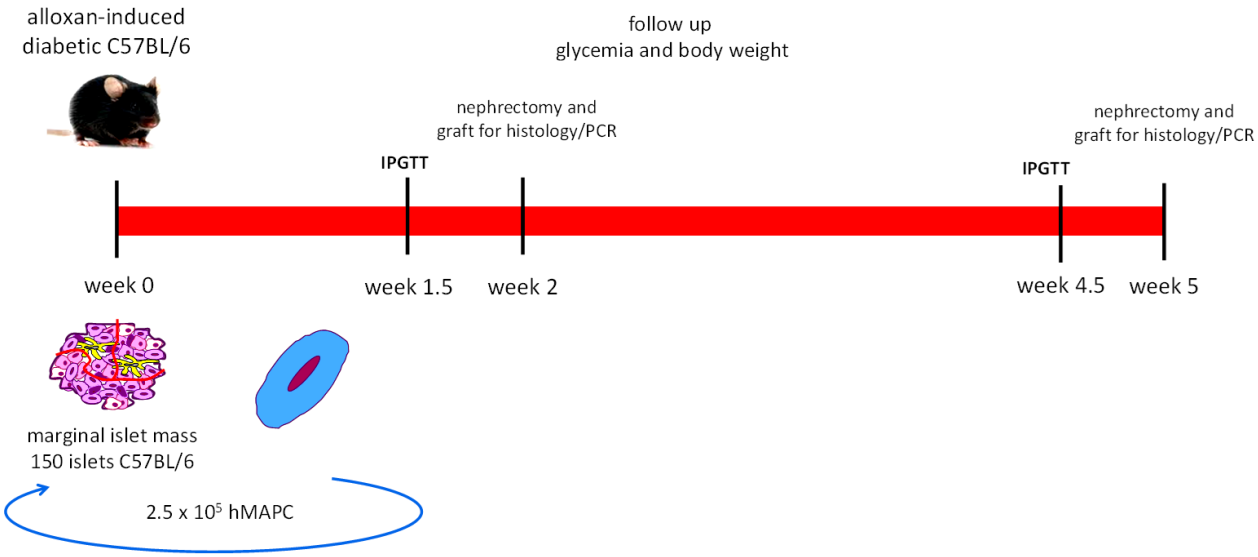


Figure 5.1. Experimental setup. C57BL/6 mice were rendered diabetic by iv injection of alloxan (90 mg/kg BW). After 2 days, diabetic mice were transplanted with 150 C57BL/6 islets under left kidney capsule either alone (control) or together as separate pellets with human MAPC (2.5×10^5) or as composite pellets with human MAPC (2.5×10^5). Weight and glycemia were measured three times weekly. IPGTTs were performed two days before sacrifice. Animals were killed for graft histology and gene expression analyses 2 and 5 weeks post-transplantation.

5.3 **RESULTS**

5.3.1 ***Human MAPC secrete angiogenic growth factors and have neo-angiogenic potential in the in vivo CAM***

Human MAPC presented a low expression of HLA-ABC (<25%) and lacked expression of HLA-DR, CD40, CD86, CD3, Flk1/VEGFR2/KDR, CD31/PECAM-1, and CD34 (< 1%), which are typical cell surface markers for MHC class II and co-stimulation molecules, T cells and endothelial cells, respectively (Figure 5.2). Human MAPC were positive for CD44 and CD105 (>95%)[365]. Their surface marker signature defines a unique phenotype that distinguishes them from any other known class of stem cells [365].

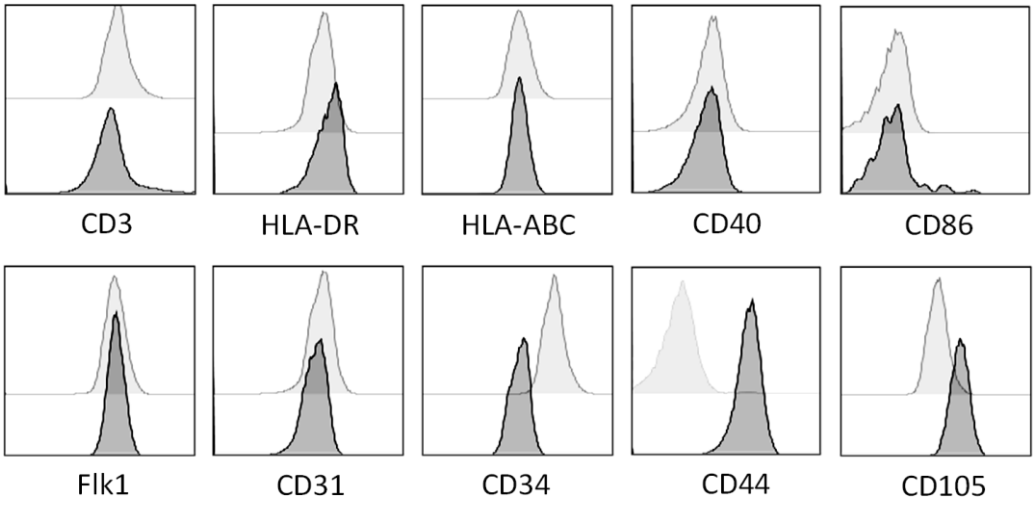


Figure 5.2. Characterization of human MAPC. Cell surface marker expression of human bone marrow-derived MAPC. Flow cytometry histograms show the expression levels (shaded dark gray peaks) of selected markers associated with the characterization of human MAPC [CD44 and CD105] compared with negative isotype controls (shaded light gray peaks).

The culture supernatant of human MAPC was analyzed with a human biomarker 40-Plex kit containing a proinflammatory panel, cytokine panel, chemokine panel, angiogenesis panel and vascular inflammation panel (Figure 5.3A). The cells produced numerous angiogenic growth factors, including VEGF (VEGF-A, -C and -D), PlGF, sFlt-1, bFGF, and IL8. On the other hand, the cells had negligible secretion of various cytokines (i.e. IFN-γ, IL1α, IL1β, IL2, IL4, IL5, IL6, IL7, IL10, IL12p70, IL12/IL23p40, IL13, IL15, IL16, IL17A, TNF-α, and TNF-β), and chemokines (Eotaxin, Eotaxin-3, IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, and TARC)(data not shown).

The neo-angiogenic potential of human MAPC was tested using the CAM angiogenesis model. Inoculation with 5 µg recombinant human VEGF markedly increased the number of blood vessels directed toward the implant (Figure 5.3B). Human MAPC (2.5×10^5) significantly increased vessel formation by 2.5- and 4.6-fold compared to control implants containing either 150 mouse C57BL/6 or 50 µg BSA respectively (Figure 5.3B).

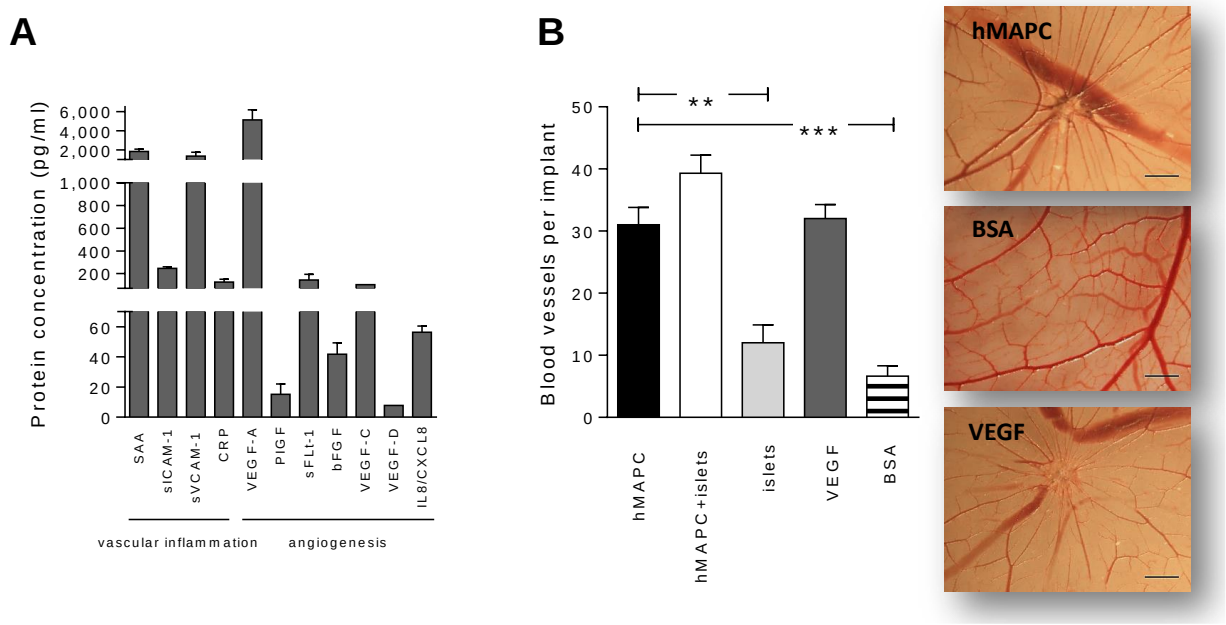


Figure 5.3. Angiogenic potential of human MAPC. (**A**) Culture medium of human MAPC was analyzed with human biomarker 40-Plex kit containing a proinflammatory panel, cytokine panel, chemokine panel, angiogenesis panel and vascular inflammation panel (protein secretion in pg/mL); (**B**) Pro-angiogenic properties of 2.5×10^5 human MAPC with or without 150 C57BL/6 mouse islets in a chorioallantoic membrane (CAM) assay, with 150 C57BL/6 mouse islets alone and BSA as negative control and VEGF-A as positive control (mean ± SEM, n=3-9 per group). **P < 0.01; ***P < 0.001.

5.3.2 *Co-transplantation of islet-human MAPC as a composite pellet improves the outcome of marginal mass islet cell transplantation*

First, we titrated the number of pancreatic islets transplanted to determine 'a marginal islet mass' that would be just at the edge of achieving normoglycemia in around 50% of recipients. Transplantation of 50 syngeneic C57BL/6 islets did not reverse hyperglycemia (0 out of 7 mice), whereas 100% (4 out of 4 mice) achieved normoglycemia when 300 islets were transplanted under the kidney capsule. We assessed that the marginal islet number was approximately 150 islets (25 out of 45 mice, 56% achieving normal blood glucose concentrations 5 weeks post-transplantation). This number of islets was selected for further experiments.

Next, we investigated the outcome of the co-transplanted marginal islet mass with 2.5×10^5 human MAPC as separate or composite pellets and monitored blood glucose levels of transplanted animals up to 5 weeks. Co-transplantation of pancreatic islets with human MAPC as separate pellets (SEP) slightly improved the average blood glucose concentrations compared to mice transplanted with islets alone. Interestingly, mice receiving islet-human MAPC composites (MIX) had better glycemic control at all measured time points from 2 weeks onwards (Figure 5.4A). Three weeks after transplantation, 81% of the mice transplanted with islet-human MAPC composites (13 out of 16 mice in the MIX group) were normoglycemic compared to 50% of the mice transplanted with islet-human MAPC as separate pellets (13 out of 26 mice in the SEP group; $P < 0.05$) and 47% in the mice transplanted with islets alone (21 out of 45 mice in the control group; $P < 0.05$)(Figure 5.4B). By the end of the observation period (week 5 post-transplantation), even a greater proportion of mice co-transplanted with islet-human MAPC reversed diabetes compared to mice transplanted with islets alone (94% in the MIX group, $p<0.01$ and 85% in the SEP group, $P < 0.001$ versus 56% in the control group).

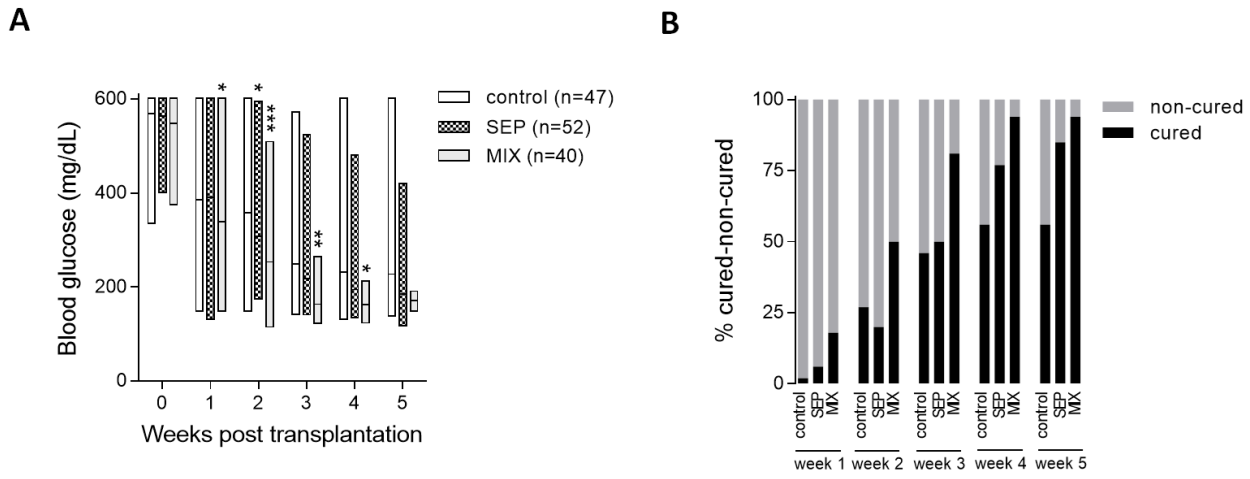


Figure 5.4. *In vivo* function of a marginal islet mass co-transplanted with human MAPC. (**A**) Blood glucose measurements of alloxan-induced diabetic C57BL/6 mice transplanted with 150 islets alone (control; white bars) or 150 islets co-transplanted as separate (SEP, dark gray bars) or composite pellets (MIX, light gray bars) with 2.5×10^5 human MAPC. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus islet-alone group (control), (**B**) Percentage of cured (back bars) and non-cured (gray bars) mice after islet cell transplantation.

After nephrectomy, the blood glucose concentrations of normoglycemic islet recipients rapidly progressed to severe hyperglycemia, indicating that the improvement in metabolic glucose control was resulting from the transplanted syngeneic islets and not from the regeneration of remnant islets in the alloxan-treated pancreas of the islet recipients (Figure 5.5A). Moreover, there was no significant difference in body weight between transplant recipients from different

experimental groups on day 0 (22.8 ± 0.27, 23.5 ± 0.2 and 22.9 ± 0.21 g for the control, SEP, and MIX groups, respectively, n=40-52) or at week 5 post-transplantation (25.6 ± 0.33, 26.2 ± 0.32 and 25.4 ± 0.27 g, for the control, SEP, and MIX groups, respectively, n=40-52)(Figure 5.5B).

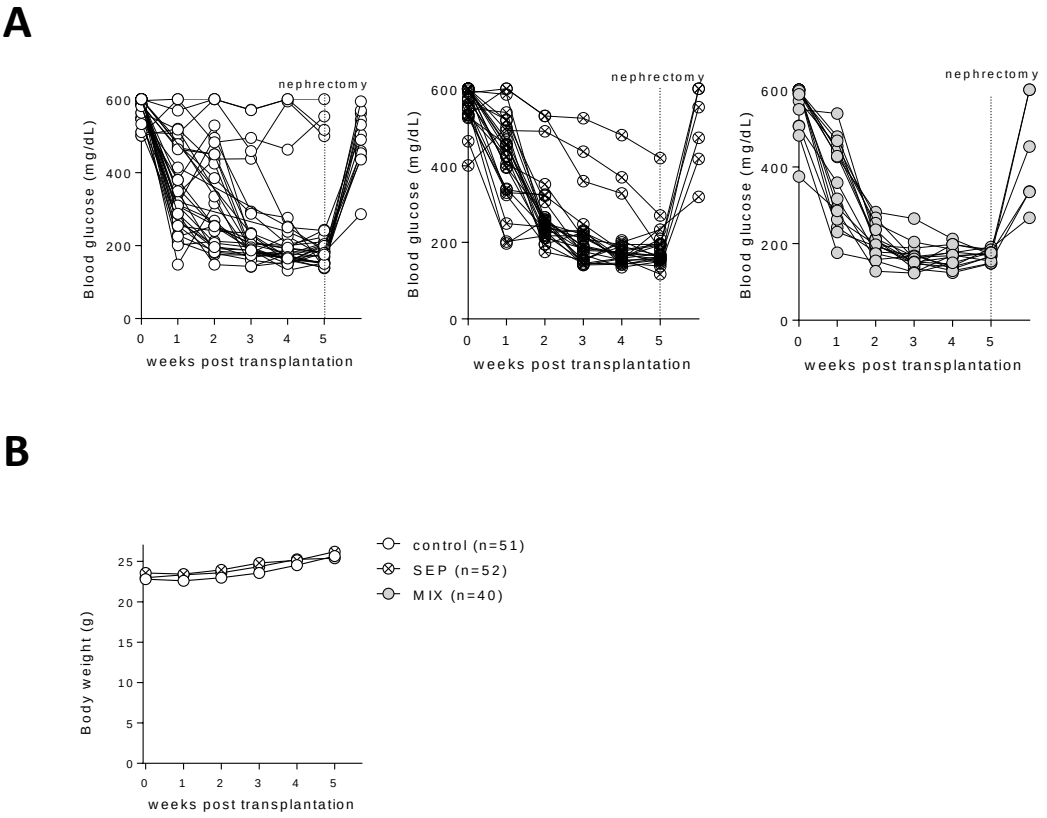


Figure 5.5. Non-fasting glycemia and body weight of transplant recipients. (**A**) Blood glucose concentrations were monitored in alloxan-induced diabetic C57BL/6 mice transplanted with 150 syngeneic islets either alone (white circles) or co-transplanted with 2.5×10^5 human MAPC (as separate (crossed circles) or composite (gray circles) pellets) for over 5 weeks. Recovery nephrectomies performed in randomly selected animals of each group at 5 weeks post-transplant resulted in 100% return to hyperglycemia. (**B**) Body weight changes did not significantly differ between the various groups throughout the study period. Each value represents the mean ± SEM.

Serum mouse insulin and C-peptide levels were measured 2 and 5 weeks after transplantation, as an index of islet graft function. At week 2 post-transplantation, insulin and C-peptide concentrations were not significantly different between the various experimental groups (Figure 5.6A). However, at week 5 post-transplantation, C-peptide values were significantly better in mice co-transplanted with islet-human MAPC as separate pellets (304 ± 80 pmol/l in the SEP group; n=10, $P < 0.01$) as well as composite pellets (282 ± 77 pmol/l in the MIX group; n=10,

P = 0.05) compared to values from the islet-alone mice (232 ± 52 pmol/l in the control group; n=10) (Figure 5.6B).

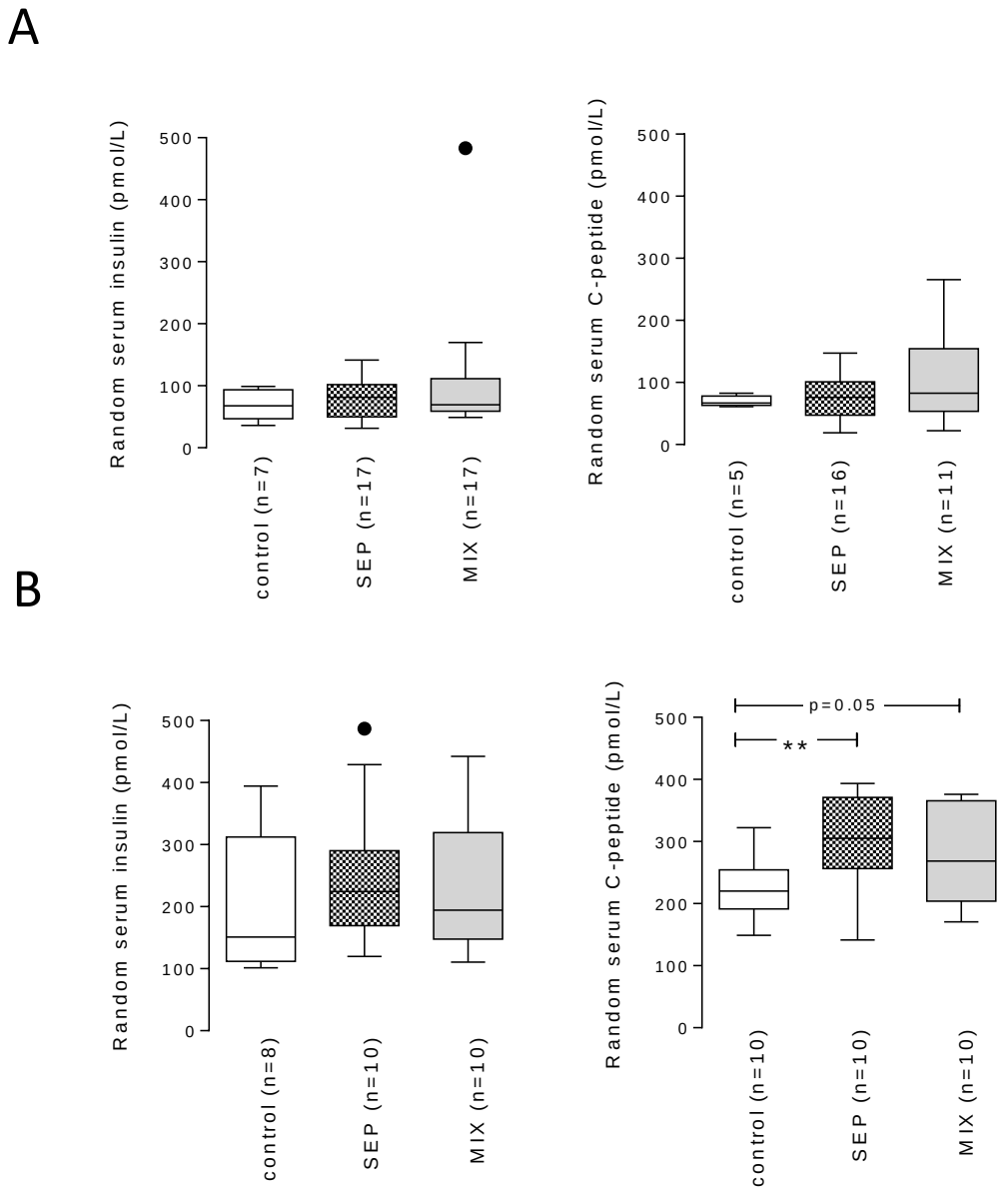


Figure 5.6. Serum insulin and C-peptide concentrations 2 (**A**) and 5 weeks (**B**) post-transplantation. Mice were transplanted with 150 islets alone (white bars) or with 150 islets together with human MAPC as separate pellets (SEP, dark gray bars) or as a composite pellet (MIX, light gray bars). **P < 0.01 versus islet-alone group (control).

To investigate the insulin secretory capacity of the islet transplant, a series of intraperitoneal glucose tolerance tests (IP-GTT) were performed week 2 and 5 post-transplantation. At week 2 post-transplantation, there were no significant differences in glucose clearance among the studied groups. On the other hand, at week 5 post-transplantation, mice co-transplanted with islet-human MAPC composites (MIX) cleared glucose more efficiently than mice transplanted

with islets and human MAPC as separate pellets (SEP) or with islets alone (control)(Figure 5.7A-B). To further support the observations of the IP-GTT, area under the curve (AUC) was calculated and found to be significantly different between the group transplanted with islet-human MAPC composites (MIX) compared to the group transplanted with islets and human MAPC as separate pellets (SEP) ($P < 0.01$) or transplanted with islets alone (control)($P < 0.01$). (Figure 5.7A-B).

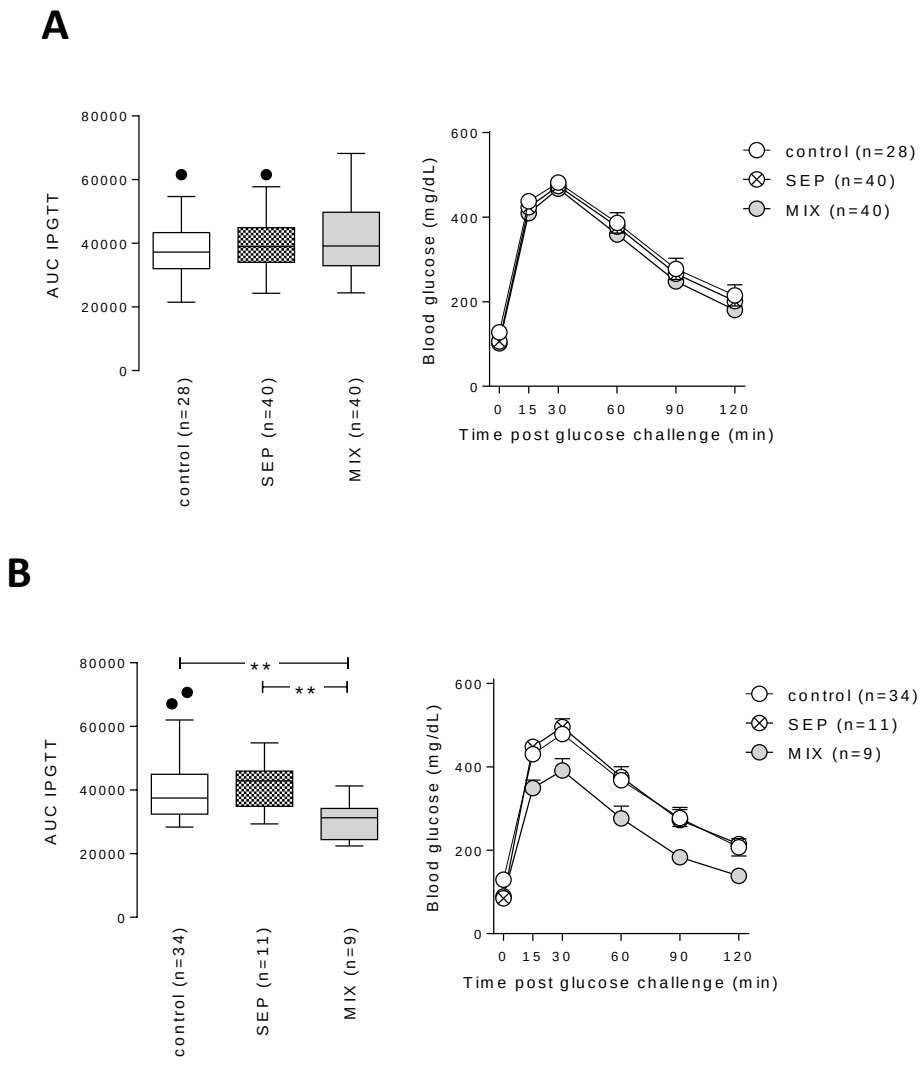


Figure 5.7. *In vivo* function of a marginal islet mass co-transplanted with human MAPC. (**A, B**) Area under the curve (AUC) and blood glucose measurements of IPGTTs in all mice 2 (**A**) and 5 (**B**) weeks after transplantation, **$P < 0.01$ versus islet-alone group (control).

5.3.3 ***Increased beta- and alpha-cell volume and blood vessel formation in mice transplanted with islet-human MAPC composites***

Grafts from the co-transplant and islet-alone groups were evaluated for their gene profile, cytoarchitecture and revascularization process. Insulin and glucagon mRNA expression levels

were significantly higher in mice co-transplanted with islet-human MAPC composites (MIX) compared to those of the islet-alone group (control) 2 weeks after transplantation. There was no difference in somatostatin mRNA expression levels at this time point. At week 5 post-transplantation, the intragraft mRNA levels of the studied endocrine hormones were comparable in all groups (Figure 5.8A-B).

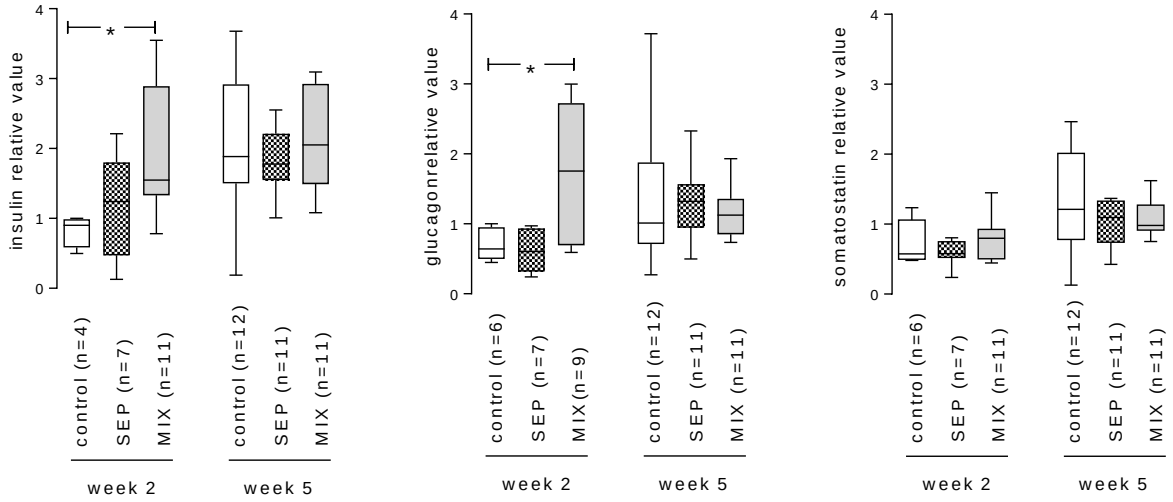


Figure 5.8. Gene expression in islets co-transplanted with human MAPC 2 and 5 weeks post-transplantation. Box and whiskers plots of mRNA levels of mouse insulin, glucagon, and somatostatin in isolated islet grafts. Data are expressed as a relative value compared to house-keeping genes. Statistical analysis was calculated using Mann-Whitney t-tests. *P < 0.05.

These measures were corroborated by histology of the grafts, showing significantly higher insulin-, glucagon-, and somatostatin-positive areas in the grafts of mice transplanted with islet-human MAPC composites (MIX) 2 weeks after transplantation, compared to mice transplanted with islets only (control) (Figure 5.9A-B).

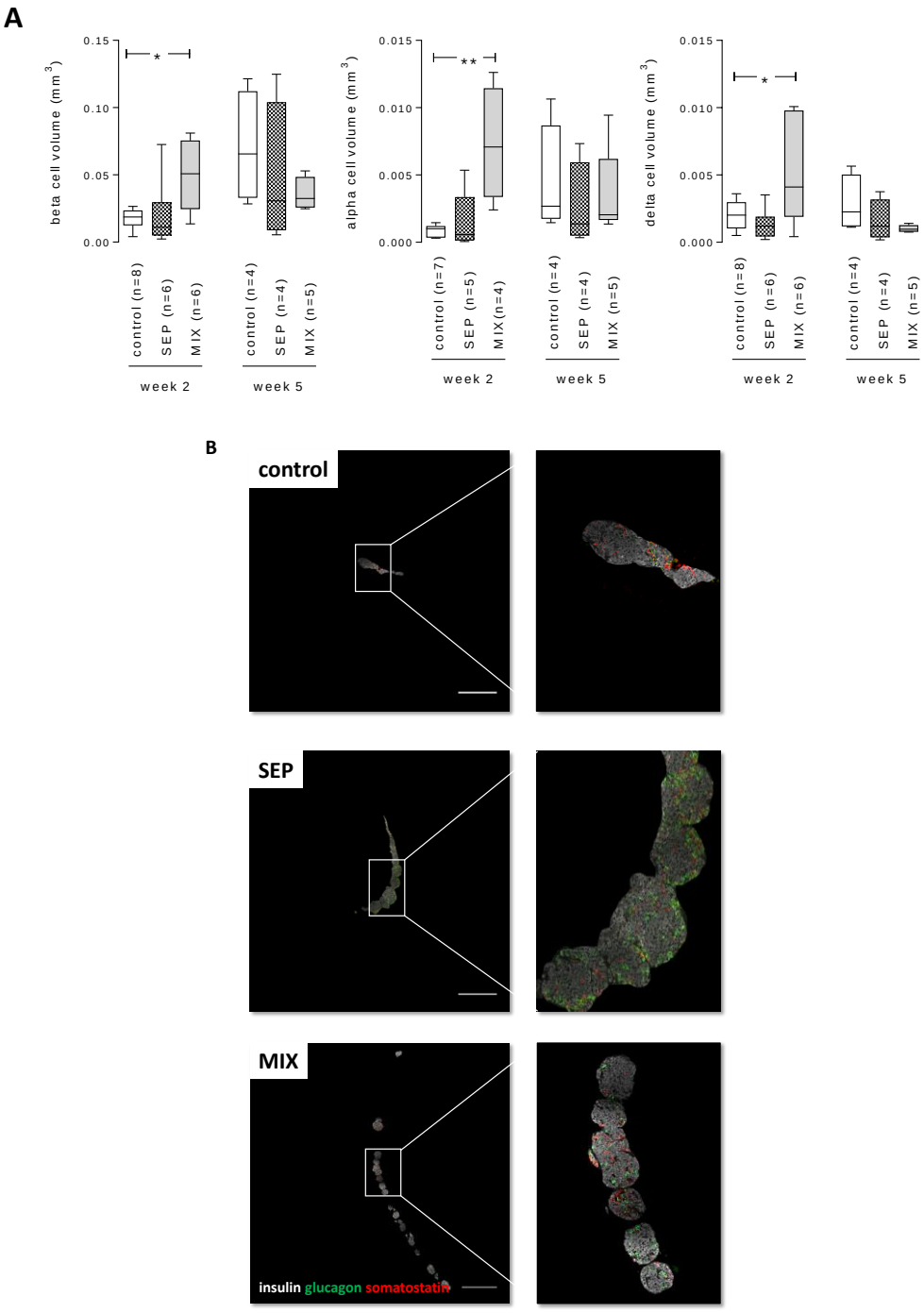


Figure 5.9. Morphology and composition of islets co-transplanted with human MAPC 2 and 5 weeks post-transplantation. (**A**) Box and whiskers plots of volumes of beta-, alpha- and delta-cells of grafts derived from mice transplanted with a marginal islet mass alone or combined with human MAPC as a separate or composite pellet. Statistical analysis was calculated using Mann-Whitney t-tests. *P < 0.05; **P < 0.01. (**B**) Distribution of mouse insulin- (white), glucagon- (red), and somatostatin (green)-positive cells in islet grafts composed of islet-human MAPC as separate (SEP) or composite (MIX) pellets or of islets alone (control) at 2 weeks post-transplantation. Stitched composite images are representative of sections from 4-8 different animals. Scale bar is 100 µm. Higher magnification of the boxed area in control demonstrates a compact graft with a normal distribution of insulin, glucagon and somatostatin positivity. Higher magnification of the boxed area in SEP and MIX group shows spread-out grafts with high insulin and glucagon positivity.

Blood vessel formation was measured by endomucin expression, a marker for vascular endothelial cells. At week 2 post-transplantation, graft vessel density and area as well as ratio of the vessel area over insulin-positive area did not differ between the studied groups. At week 5 post-transplantation, enhanced graft revascularization was observed in mice co-transplanted with islets-human MAPC composites (MIX) compared to mice transplanted with islet-human MAPC as separate pellets (SEP) or with islets alone (control). In the MIX grafts, 1256 ± 203 vessels per mm2 were detected, compared to 702 ± 106 per mm2 in the SEP grafts and 515 ± 52 per mm2 in the islet-alone grafts (both $P < 0.05$; Figure 5.10A-B). Another index of neo-angiogenesis, graft vessel area, was significantly higher in the MIX grafts (4.85 ± 1.32%, n=5), than in the SEP grafts (2.04 ± 0.57%, n=5) or the grafts of islets only (1.26 ± 0.25%; n=4, $P < 0.05$, Figure 5.10B). Additionally, mice transplanted with the MIX grafts had a higher ratio of vessel per insulin-positive area than the SEP grafts and islet-alone grafts (0.079 ± 0.027 versus 0.019 ± 0.006 and 0.014 ± 0.003 vessels per islet, both $P < 0.01$)(Figure 5.10A-B).

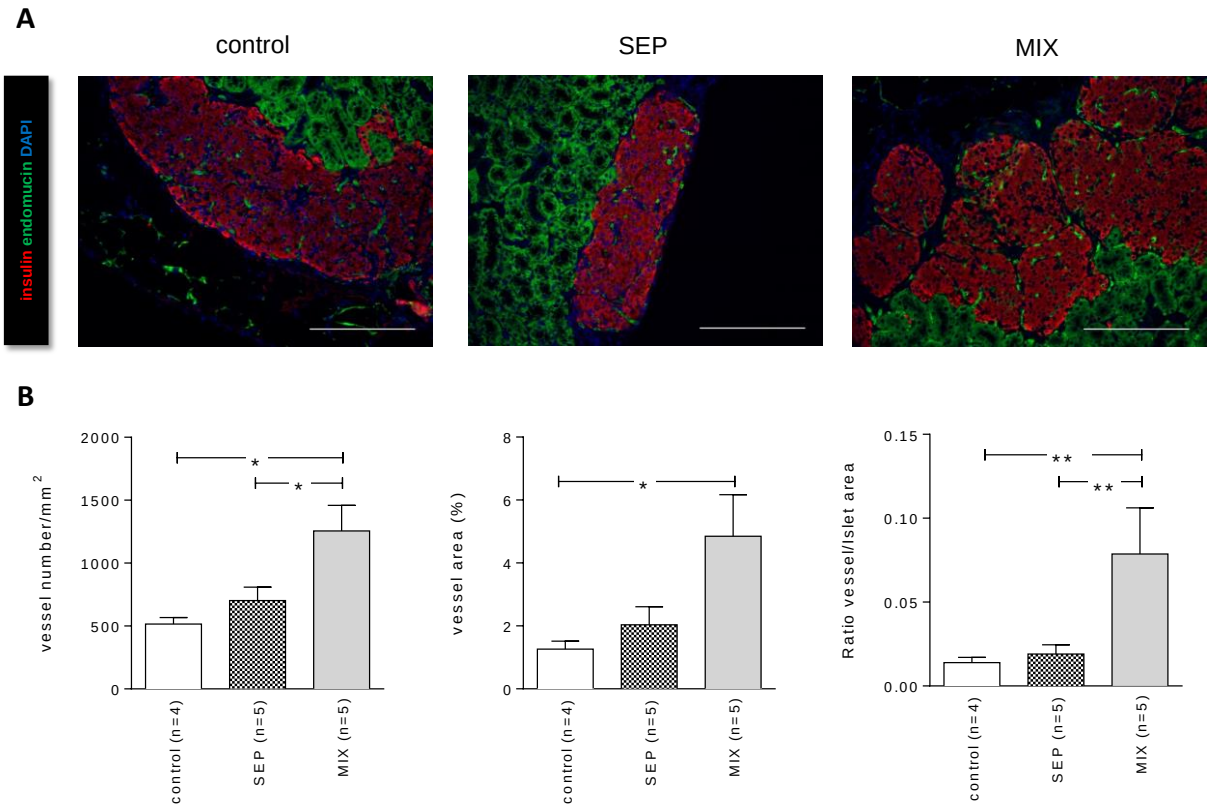
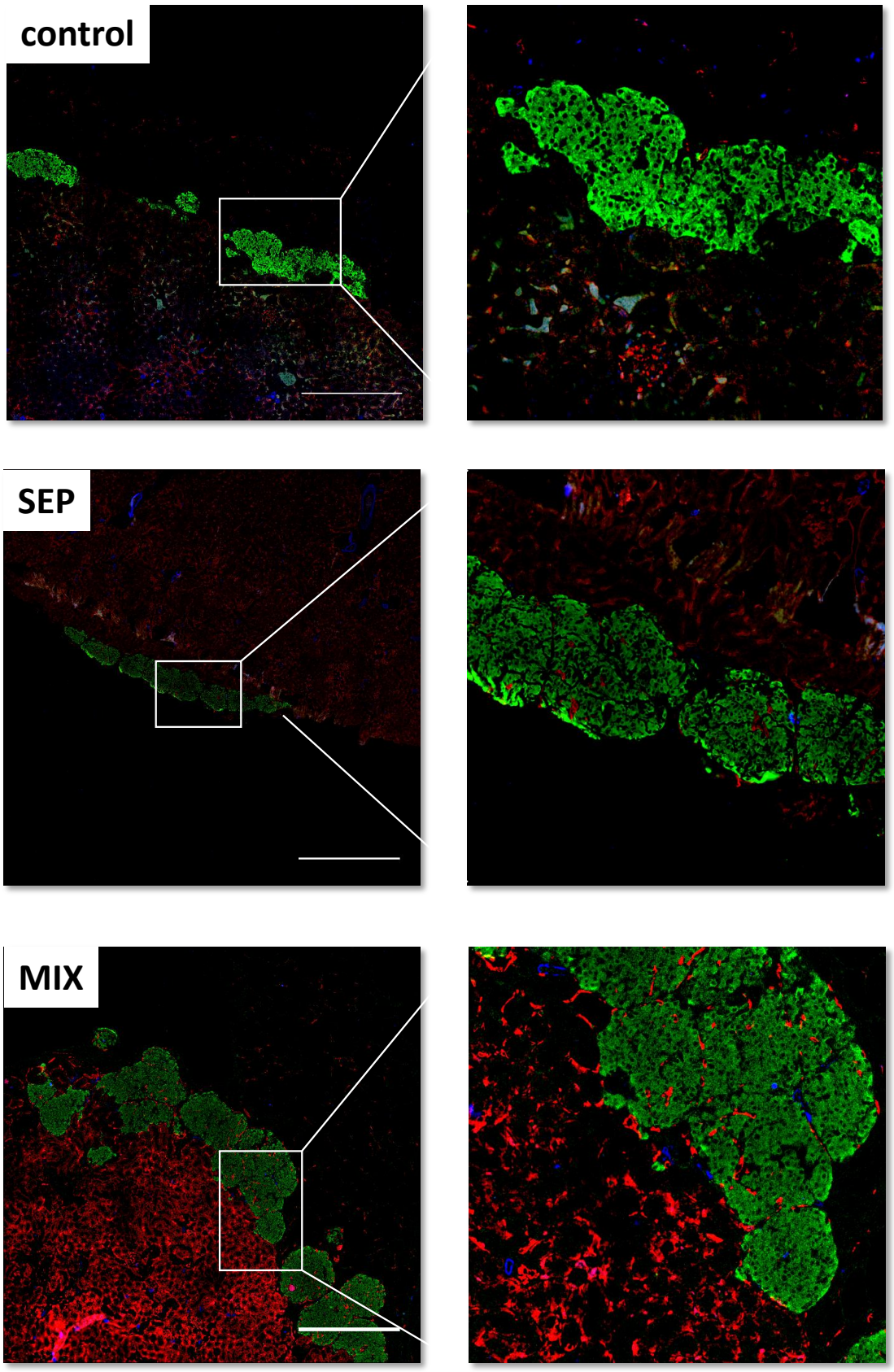


Figure 5.10. Co-transplantation of islets with human MAPC as composites promotes graft revascularization in a marginal islet mass diabetic mouse model. (**A**) Representative sections of 5-week grafts consisting of mouse islets transplanted alone or with human MAPC as separate (SEP) or composite (MIX) pellet. Images are representative of insulin and endomucin (vessel) staining for 3-4 animals in each transplant group. Scale bar is 100 µm. (**B**) Vessel morphologic parameters assessment was determined as described in material and methods section. Data are means ± SEM. Statistical analysis was calculated using Mann-Whitney t-tests. *P < 0.05, **P < 0.01.

Collectively, grafts composed of islets mixed with human MAPC retrieved 5 weeks after transplantation contained high numbers of endomucin-positive endothelial cells (Figure 4.10A) and only a few α-SMA-positive cells (Figure 5.11A), indicative of the presence of new capillary-like structures. Moreover, PCR analyses demonstrated that both mouse CD31/PECAM-1 in addition to α-SMA mRNA were more abundantly present in the MIX grafts compared to the islet-alone group (Figure 5.11B), while expression of the basement membrane marker Col4a1 was not different among the experimental groups (data not shown).

A



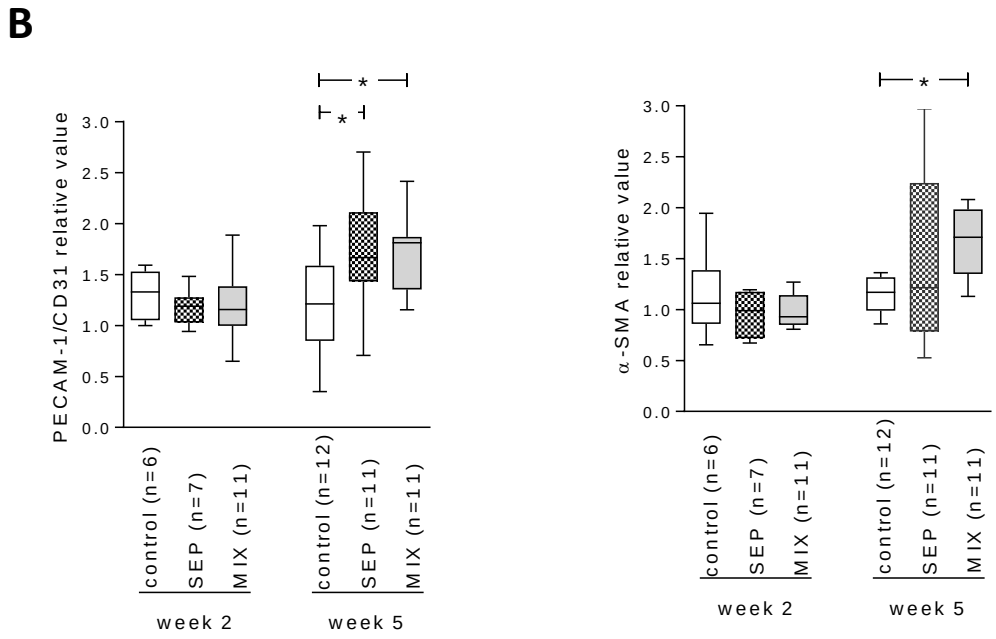


Figure 5.11. Human MAPC influence islet graft revascularization under kidney capsule 5 weeks post-transplantation. (**A**) Distribution of mouse insulin- (green), endomucin- (red), and α-SMA (blue)-positive cells in islet grafts composed of islet-human MAPC as separate (SEP) or composite (MIX) pellets or of islets alone (control) at 5 weeks post-transplantation. Stitched composite images are representative of sections from 4-8 different animals. Scale bar is 500 μm. Higher magnification of the boxed area in the MIX group shows a high number of endomucin-positive endothelial cells with very few α-SMA-positive cells that encircle a well-defined lumen with fenestrations towards the adjacent endocrine cells, indicative of a new capillary-like network. This aspect may support the advanced functionality of the graft. (**B**) Box and whiskers plots of mRNA levels of mouse CD31/PECAM-1 and α-SMA in isolated islet grafts. Data are expressed as a relative value compared to house-keeping genes. Statistical analysis was calculated using Mann-Whitney t-tests. *$P < 0.05$.

5.4 **DISCUSSION**

Several hurdles still prevent the progression of clinical islet transplantation, including early graft failure and the loss of transplanted islet mass due to nonimmunological reasons [433]. In the first days following transplantation, islets lack an adequate vascular network, leading to severe hypoxia and cell death. It is believed that this is one of the major causes of the poor performance of islet grafts longterm. Therefore, there is a need for methods to improve the early survival, function and engraftment of transplanted islets. A variety of (stem) cell populations (i.e. endothelial progenitor cells, MSC) have been described to enhance transplanted beta cell survival and function after co-transplantation, however there are still hurdles to their wide application in the clinic, such as inconsistent stem cell potency, poor cell engraftment and survival, and age/disease-related host tissue impairment [434].

A novel class of progenitor cells is the MAPC® which can be derived from the postnatal bone marrow stromal compartment but also from muscle and brain of several species, including rodents and humans [357]. These cells have demonstrated extensive in vitro expansion capacity, durable cytogenetic stability, and higher plasticity compared to MSC [435]. MAPC are also non-immunogenic cells with a strong immunomodulatory profile [364–366,436–439], permitting safe non-HLA-matched allo- and even xenogeneic use without the need for immunosuppression [440,441]. Interestingly, MAPC seemed to exhibit an unusual capacity to evade the immune system and can regulate homeostatic T-cell proliferation, prevent the expansion of Th1, Th17, and Th22 effector T cells, and block the generation of pathogenic allogeneic responses [366]. Moreover, MAPC has been described to secrete angiogenic growth factors and to improve vascular remodeling in different ischemic models, such as those for cardiac infarction and severe limb ischemia [362,441]. Based on these phenotypic and functional characteristics, we wanted to study the localized effect of human MAPC on islet graft function in a murine syngeneic marginal mass transplant model.

Here we show for the first time that human MAPC co-transplanted as composite pellets with mouse islets can improve islet graft function as measured by the initial glycemic control, diabetes reversal rate, glucose tolerance, and serum C-peptide concentration compared with transplantation of islets alone. Moreover, we found that grafts composed of islet-human MAPC composites had an improved revascularization process. The human MAPC actively participated

in the revascularization process mainly by producing angiogenic growth factors, soluble adhesion molecules and IL8.

Several studies have shown that MAPC from both allo- and xenogenic sources exert positive effects in ischemia models, mainly through the secretion of trophic factors such as VEGF-A, platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1) [441,442]. Our present data corroborated the angiogenic potency of human MAPC as supernatants of cultured cells were shown to contain high concentrations of vascular inflammation and angiogenic factors such as VEGF-A, PlGF, bFGF, all major regulators of islet vascularization. The neo-angiogenic potential of human MAPC was further validated in vivo in a chicken CAM assay, where implanted human MAPC formed new blood vessels at a rate comparable to recombinant VEGF-A. VEGF-A is described to be one of the major regulators of islet vascularization, innervation and function, as beta cell-specific deletion of VEGF-A leads to diminished and abnormal islet vascularity, impaired postnatal nerve fiber ingrowth, and a pre-diabetic phenotype [443–445]. Moreover, VEGF-A seems to be required for revascularization of transplanted islets. Based on these data, several investigators have tried to enhance VEGF production locally in the pancreatic islets although not continuously as this might trigger vascular tumor formation [446–448]. Just now, it has been reported that islets co-transplanted with human embryonic stem cell-derived MSC that conditionally overexpress VEGF allow a 50% reduction in the islet mass required to reverse diabetes in mice. These cells significantly improved islet metabolic function and revascularization [428].

Islet engraftment is a slow process, and while vascular sprouting, angiogenesis, and revascularization occur within 1-2 weeks after transplantation, the maturation of the blood vessels is likely to take several weeks to even months. Although VEGF can boost the process of islet revascularization, this protein is also critical to maintain the intraislet endothelial cell pool [444]. We found a significant improvement in the development of a new islet capillary network in mice where islets were co-transplanted with human MAPC. Indeed, higher numbers of capillary-like structures with a lining of endothelial cells (detected with mouse-specific endomucin antibody), were found on the periphery and in the intra-islet space of the islet-human MAPC composites at week 5 post-transplantation, suggesting that host-derived vessels are directly feeding transplanted islets and that close proximity and even direct contact between the transplanted pancreatic islets and human MAPC is of critical importance for the improved

glucose control, diabetes reversal rate and increased revascularization. The absence of human CD31/PECAM-1 mRNA expression in the islet grafts further supports the idea that human MAPC do not incorporate into new capillaries but possibly secrete growth factors to initiate angiogenesis and to support functional tissue survival (data not shown). These observations are in full agreement with previous observations that the major role of human MAPC is to provide angiogenic growth factors in the first days after implantation, after which they are cleared rapidly from the body, without leading to immune activation [360,441].

The present data encouraged the use of human MAPC in islet cell transplantation protocols as our results demonstrate the improvement of islet graft morphology and function by transplantation of islets-human MAPC composites, possibly via the promotion of graft revascularization mediated by human MAPC.

CHAPTER 6

GENERAL CONCLUSIONS AND PERSPECTIVES

Type 1 diabetes (T1D) is a debilitating disease characterized by a chronic incapability to normalize hyperglycemia. Transplanting cadaveric pancreases or isolated islets can restore glucose homeostasis during the first year and return hypoglycemia awareness, thus alleviating severe hypoglycemia. Despite the improved outcome of islet transplantation over the last few years, it is clear that the organ demand outnumbers the supply. Therefore, all islet transplant programs need to be highly selective in which patients to admit. Immunosuppression is also challenging [424]. Research into more focused, less harmful immunosuppressive regimens is urgently needed to tackle allorejection, recurrence of autoimmune diabetes and immediate graft failure.

Several studies described that anti-CD3 mAbs can induce long-term tolerance in allografts and in autoimmune settings [222,223]. Unfortunately, lymphodepletion regimens like induction therapy with anti-CD3 mAbs have been shown to be accompanied by a homeostatic expansion of islet-specific autoreactive T cells with effector memory phenotype [401] and seem to predispose the graft to destruction. Therefore, therapeutic approaches that could block both allo- and autoimmunity while circumventing homeostatic expansion of autoreactive T cells would be the medicinal Holy Grail. Ongoing efforts are addressing this need by combining mild lymphodepletion with antigen-specific approaches that have the potential to reinstate immunological tolerance by the induction of islet antigen-specific Tregs and that could obviate the need for maintenance immunosuppression [404–407].

In **chapter 4**, we used a combination therapy consisting of a short induction therapy of low doses of anti-CD3 mAbs combined with the intragastric delivery of a genetically-modified *L. lactis*-based vaccine producing human proinsulin along with pro-tolerogenic IL10 to reestablish tolerance to islet antigens in longstanding diabetic NOD islet recipients. We demonstrated that the success of the therapy depended not only on the time of islet implantation in relation to diabetes duration but also to T-cell-targeting strategy as already demonstrated by others [222,223]. We speculate that islet implantation before, instead of, after lymphodepletion would not only limit the homeostatic proliferation of the antigen-experienced T-cell pool but would also allow the expansion of Foxp3$^+$ Tregs and promote long-term tolerance induction. These hypotheses need further investigation. *Le Campion et al. demonstrated that IL2 and IL7 control the homeostatic balance between the regulatory and conventional CD4$^+$ T-cell compartments during peripheral T-cell reconstitution* [449]. *In addition, IL15 could play a role together with IL7 in the generation of memory T cells* [283,288]. *Thus, we believe that analyses of these cytokines in the serum at differents time points after islet implantation would help to understand*

the homeostatic proliferation in our work. Moreover, FACS analyses on peripheral blood can be used to establish the balance between the different T-cell populations.

We also found that NOD islet recipients treated with anti-CD3 alone or combined with the *L. lactis*-based antigen-specific vaccine had lower frequencies of Foxp3$^+$ Tregs in the lymph nodes draining the islet implantation site (i.e. kidney) compared to untreated controls. This percentage was not different between mono- and combination therapy and independent of diabetes duration. The fact that the frequency of Foxp3$^+$ Tregs did not increase after therapy in draining lymph nodes does not exclude that Tregs mediated syngeneic graft tolerance in NOD islet recipient mice. More insights are needed on whether intragraft Tregs are enriched with islet antigen-specific Tregs and how these islet antigen-specific Tregs can control the diverse repertoire of antigen-experienced T cells present in the islets [423]. Also, the kinetics of peripheral islet antigen-specific Treg memory cells into tissues (other than the lymphoid tissue) remain unknown. *The use of specific class I and II HLA tetramers with peptides from diabetes-associated antigens could offer a better understanding of these processes [111,450]. Analyses on peripheral blood with such tetramers at different time points after islet implantation would allow insights into the kinetics of autoantigen-specific T cells. In addition, it would aid our understanding of the beta-cell protective processes and would establish whether graft and endogenous islets are enriched with autoantigen-specific Tregs as we hypothesize.*

The *L. lactis* bacteria can be genetically modified to secrete virtually any protein or peptides [451]. *We propose that the use of L. lactis bacteria secreting human proinsulin along with other immunomodulatory cytokines like TGF-β instead of IL10 could expand the islet-resident antigen-specific Foxp3$^+$ Treg pool and consequently suppress ongoing T1D after islet transplantation. Perhaps, future work could test this concept.*

Finally, we describe specific events in the autoimmune responses after islet implantation and lymphodepletion which represent therapeutic opportunities to restore long-term tolerance. *More work is needed to fully understand the process of reestablishing tolerance in our model. We also need to further investigate how diabetes duration interferes with the efficacy of the treatment. These insights might be implemented in the clinic and could improve islet transplant outcomes in T1D patients after islet transplantation.*

Significant improvements in islet purification techniques and original immunomodulatory agents have renewed interest in islet transplantation. However, the field is still confronted by a limited supply of islet cells, insufficient engraftment, immediate graft failure and loss of

transplanted islet mass due to nonimmunological reasons [433]. In the first days following implantation, islets lack an adequate vascular network, leading to severe hypoxia and cell death. Another hypoxia-associated threat is that a microenvironment of hypoxia triggers the innate immune system, resulting in the release of inflammatory cytokines. It is believed that this is one of the major causes of the poor performance of islet grafts longterm. In **chapter 5,** we co-transplanted MAPC® with mouse islets to improve engraftment. In addition to the beneficial role of stem cells in the immunomodulatory response and in encouraging beta-cell regeneration following transplantation, they may also play an important role in promoting islet revascularization. We showed for the first time that human MAPC co-transplanted as composite pellets with a marginal mass of syngeneic islets under the kidney capsule of alloxan-induced diabetic mice can improve islet graft function as compared with transplantation of islets alone. Moreover, we found that grafts composed of islet-human MAPC composites had an improved revascularization process. We found a significant improvement in the development of a new islet capillary network in mice where islets were co-transplanted with human MAPC. These observations are in full agreement with previous observations that the major role of human MAPC is to provide angiogenic growth factors in the first days after implantation, after which they are cleared rapidly from the body, without leading to immune activation [360,441].

Several studies are ongoing in the (stem) cell therapy field. The potential of progenitor cells to be used as an infinite and renewable source of insulin-producing beta cells to solve the problem of limited donors and support engraftment in human islets transplantation has led to stimulating avenues of application. In our work, we found that the physical contact of MAPC with islets impacted positively on the engraftment and function of the graft. *Thus, devices or new technologies that could maintain the close interaction between the implanted islets and MAPC would be an interesting approach. Islets can be transplanted together with extracellular matrix (ECM) molecules under the skin or in the peritoneal cavity in 3-dimensional (3D) structures. Moreover, the new technologies with 3D printers which can make scaffolds for tissue engineering and 3D cell culture systems may hopefully advance the islet transplantation field* [452]. *These biopolymeric scaffolds provide a 3D structure for the islets, mimic the pancreatic microenvironment and keep the close interaction needed when MAPC are co-transplanted with islets. Furthermore, islets engraft more efficiently in 3D than in 2D structures, and the scaffold may increase viability by encouraging cell adherence and nutrient diffusion* [453] *which could further potentiate the favorable MAPC effects.*

In addition, scaffolding may also allow for local administration of immunomodulatory drugs to suppress the specific allo- and autoimmune responses against the islets. *Here, the exploitation of the immunomodulatory properties of MAPC can be used in allogeneic transplantation settings decreasing the use of immunosuppressive drugs that often have unwanted effects. Interestingly, MAPC seems to exhibit an unusual capacity to evade the immune system and can regulate homeostatic T-cell proliferation, prevent the expansion of Th1, Th17, and Th22 effector T cells, and block the generation of pathogenic alloimmune responses* [366]. *Future experiments need to be performed using allogeneic islets in the autoimmune diabetes setting to assess the full immunomodulatory potential of MAPC. As MAPC can regulate homeostatic T-cell proliferation, they could be exploited as maintenance therapy in islet transplantation after lymphodepletion regimens.*

As a **general conclusion**, the data presented in this PhD thesis explored promising therapeutic approaches to improve islet transplant outcomes. Our work indicates that these approaches should act on different levels to overcome the many hurdles of beta-cell replacement in the modality of islet cell transplantation (Figure 6.1). We showed that genetically-modified *L. lactis*-based antigen-specific vaccines combined with a short course of low doses of anti-CD3 mAbs can avoid autoimmune diabetes recurrence depending on the time of islet implantation in relation to T-cell-targeting strategy and on the time of islet implantation after diabetes diagnosis. We also showed that the use of human MAPC in islet cell transplantation can improve islet graft morphology and function by transplantation of islets-human MAPC composites, possibly via the promotion of graft revascularization mediated by human MAPC. In the near future, we hope it will be possible to reach long-term islet transplant outcomes by implementing and combining our approaches avoiding not only the immunological issues of islet cell transplantation but also improving islet engraftment.

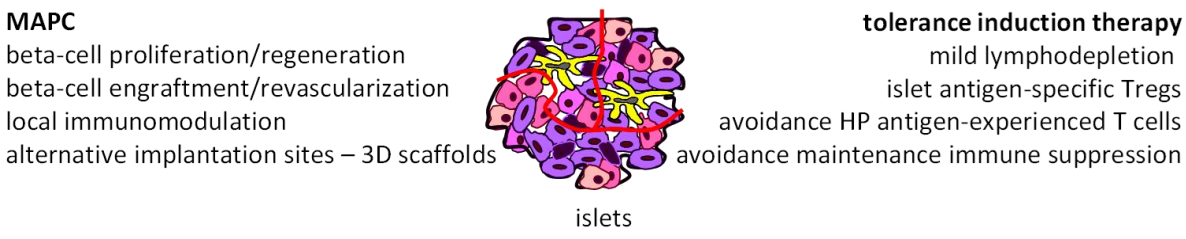


Figure 6.1. Potential benefits of MAPC® and tolerance induction therapy in islet cell transplantation in T1D patients.

We believe that a combination of approaches will be needed to improve islet transplant outcomes, as beta-cell replacement by islet transplantations is confronted with multiples hurdles. The use of anti-inflammatory agents, immune modulation without induction of homeostatic proliferation, antigen-specific and stem cell therapies need to be further investigated. In addition, some questions remain: When to transplant? Where to transplant? Which patient population to select?

CHAPTER 7

SUMMARY

In type 1 diabetic (T1D) patients, the pancreatic insulin-producing beta cells are selectively destroyed by the immune system, leading to an impaired glucose metabolism. The steep increase in the incidence of this chronic autoimmune pathology especially in young children in the last decades raises serious concerns. To date, insulin therapy is considered the gold standard for the treatment of T1D. Nevertheless, limitations persist, such as the frequent episodes of hypoglycemia, and the chronic micro- and macrovascular complications. Islet cell transplantation offers an alternative treatment for T1D patients, specifically for those with hypoglycemic unawareness following insulin administration. Despite the improved outcome of islet cell transplantation over the last few years, drawbacks remain, such as a limited supply of cadaveric donors, the necessity of several donors for a single transplantation, and (immediate) graft failure through metabolic pressure, continued autoimmunity, alloimmunity, high concentrations of immunosuppressive drugs, and oxidative stress caused by hypoxia or due to cytokine exposure. In this PhD thesis, we propose that in the long term, tolerogenic antigen-based and beta-cell replacement/regenerative approaches could be promising in both blocking the autoimmunity to prevent continued beta-cell destruction and boosting beta-cell function and revascularization to restore sustainable insulin regulation.

Contrasting with alloimmune responses, autoimmune disease recurrence is difficult to control with standard immunosuppression and therefore poses a challenging obstacle for a successful long-term islet cell transplantation outcome. In **chapter 4**, we used a combination therapy consisting of a short induction therapy of low doses of anti-CD3 mAbs combined with the intragastric delivery of genetically-modified *L. lactis*-based vaccines producing human proinsulin along with pro-tolerogenic IL10 to reestablish tolerance to islet antigens in longstanding diabetic NOD islet recipients. We first demonstrate that an early regimen (starting 5 days before islet implantation) with low-dose anti-CD3 combined or not with *L. lactis*-based antigen-specific therapy induced poor graft acceptance in around 20% of NOD islet recipients. However, when therapeutic regimen started at the time of islet implantation, the overall graft acceptance was around 56% in *L. lactis*-based therapy-treated NOD islet recipients. Next, we observed that diabetes duration before islet implantation impacts on the rate of success. The efficiency to prevent autoimmune diabetes recurrence markedly decreased in anti-CD3-treated mice with diabetes duration, while *L. lactis*-based combination therapy-treated mice were much less influenced by this parameter. The efficiency of glycemic control and the rate of graft acceptance in relation to the timing of diabetes diagnosis is in part related to the protection of residual endogenous islets in the pancreas as reflected by the insulin content of the native

pancreas at different time points after diabetes diagnosis. Further, we found that NOD islet recipients treated with anti-CD3 alone or combined with the *L. lactis*-based vaccine had $Foxp3^+$ Tregs present in the lymph nodes draining the islet implantation site (i.e. kidney), but this was not different between mono- and combination therapy and independent of diabetes duration. Based on these observations, we concluded that a mild lymphodepletion with low-dose anti-CD3 mAbs in combination with an antigen-specific approach using genetically-modified *L. lactis* can by-pass autoimmune diabetes recurrence in 56% of syngeneic islets transplanted in longstanding diabetic NOD mice. Moreover, we found that islet cell transplantation outcome will certainly depend on the time of islet implantation in relation to T-cell-targeting strategy and on the time of islet implantation after diabetes diagnosis. Integration of these antigen-specific technologies to enhance engraftment and combat graft destruction may help to advance the therapeutic efficacy and availability of islet cell transplantation in selected patient populations.

In the first days following transplantation, islets lack an adequate vascular network, leading to severe hypoxia and cell death. It is believed that this is one of the major causes of the poor performance of islet grafts longterm. Thus, in **chapter 5** we used a novel class of progenitor cells MAPC® and co-transplanted these with mouse islets to improve islet engraftment. First, we demonstrated that human MAPC produce high amounts of angiogenic growth factors, including VEGF, *in vitro* and *in vivo*, as demonstrated by the induction of neoangiogenesis in the CAM assay. Next, we showed for the first time that human MAPC co-transplanted as composite pellets with mouse islets can improve islet graft function in a syngeneic marginal mass islet cell transplantation model as measured by the initial glycemic control, diabetes reversal rate, glucose tolerance, and serum C-peptide concentration compared with transplantation of islets alone. Moreover, we found that grafts composed of islet-human MAPC composites had an improved revascularization process. We found a significant improvement in the development of a new islet capillary network in mice where islets were co-transplanted with human MAPC. Indeed, higher numbers of capillary-like structures with a lining of endothelial cells were found on the periphery and in the intra-islet space of the islet-human MAPC composites at week 5 post-transplantation, suggesting that host-derived vessels are directly feeding transplanted islets and that close proximity and even direct contact between the transplanted pancreatic islets and human MAPC is of critical importance for the improved glucose control, diabetes reversal rate and increased revascularization. The present data propose

that co-transplantation of mouse pancreatic islets with human MAPC, which secrete high amounts of angiogenic growth factors, enhance islet graft revascularization and subsequently improve islet graft function.

As a **general conclusion**, the data presented in this PhD thesis show promising therapeutic approaches to improve islet transplant outcomes. Our work also indicates that these approaches should act on different levels to overcome the many hurdles of beta-cell replacement in the modality of islet cell transplantation. We showed that genetically-modified *L. lactis*-based antigen-specific vaccines combined with a short course of low doses of anti-CD3 mAbs can avoid autoimmune diabetes recurrence depending on the time of islet implantation in relation to T-cell-targeting strategy and on the time of islet implantation after diabetes diagnosis. We also showed that the use of human MAPC in islet cell transplantation can improve islet graft morphology and function by transplantation of islets-human MAPC composites, possibly via the promotion of graft revascularization mediated by human MAPC. In the near future, we hope it will be possible to reach long-term islet transplant outcomes by implementing and combining our approaches avoiding not only the immunological issues of islet cell transplantation but also improving islet engraftment.

CHAPTER 8

SAMENVATTING

In type 1 diabetes (T1D) worden de insulineproducerende beta cellen in de pancreas selectief vernietigd door het immuunsysteem, wat leidt tot een gebrekkig glucosemetabolisme. De sterke toename in de incidentie van deze chronische autopathologie vooral tijdens de laatste decennia en in jonge kinderen, is aanleiding tot ernstige bezorgdheid. Tot op heden wordt insulinetherapie beschouwd als de gouden standaard in de behandeling van T1D. Toch zijn er nog altijd beperkingen zoals de frequente episoden van hypoglycemie en de chronische micro- en macrovasculaire complicaties. Eilandjestransplantatie biedt een alternatieve behandeling voor T1D patiënten, in het bijzonder voor mensen met hypo-onbewustheid na toediening van insuline. Ondanks dat de resultaten van eilandjestransplantatie de afgelopen jaren sterk verbeterd zijn blijven er nadelen, zoals een beperkt aanbod aan postmortale donoren, de noodzaak van verschillende donoren voor één transplantatie, en (direct) transplantfalen door metabolische druk, aanhoudende alloimmuniteit, noodzaak om hoge concentraties immunosuppressieve geneesmiddelen te gebruiken en oxidatieve stress veroorzaakt door hypoxie of door blootstelling aan proinflammatoire cytokinen. In dit proefschrift willen we onderzoeken of een autoantigeen-gebaseerde en beta-celvervangende/regeneratieve aanpak de resultaten van eilandjestransplantatie in T1D kan verbeteren. We vermoeden dat onze aanpak kan leiden tot het blokkeren van de autoimmuniteit en dus beta-celvernietiging kan voorkomen, alsook de functie en revascularisatie van eilandjestransplant kan stimuleren waardoor de insulinewerking blijvend kan herstellen.

In tegenstelling tot alloimmuniteit is het heroptreden van autoimmune diabetes na eilandjestransplantatie moeilijk te controleren met standaard immunosuppressie, en vormt dit een uitdagende hindernis voor een succesvolle eilandjestransplantatie. In **hoofdstuk 4** hebben we gebruik gemaakt van een combinatietherapie bestaande uit een korte inductietherapie met lage dosis anti-CD3 mAb in combinatie met toediening van tolerogene genetisch gemodificeerde *L. lactis*-gebaseerde vaccins die menselijke pro-insuline produceren, tesamen met IL10, om tolerantie voor eilandjesantigenen te herstellen in langdurig diabete NOD eilandjesrecipiënten. Ten eerste tonen we aan dat een vroege behandeling (vanaf 5 dagen vóór eilandjesimplantatie) met lage dosis anti-CD3, al dan niet gecombineerd met *L. lactis*-gebaseerde antigeenspecifieke behandeling, een transplant-acceptatie induceerde in amper 20% van NOD eilandjesrecipiënten. Wanneer echter de *L. lactis*-gebaseerde combinatietherapie gestart werd op het moment van de eilandjesimplantatie zagen we eilandjes acceptatie in 56% van de NOD recipiënten. Vervolgens stelden we vast dat de duur van diabetes voor het moment

van transplantatie gevolgen had voor het acceptatie percentage. De efficiëntie om heroptreden van autoimmune diabetes te voorkomen verminderde zichtbaar met diabetesduur in anti-CD3-behandelde muizen, terwijl muizen behandeld met *L. lactis*-gebaseerde combinatietherapie veel minder beïnvloed werden door deze parameter. De efficiëntie om diabetes onder controle te krijgen en de snelheid van transplant-acceptatie bleek in verhouding te zijn met de diabetesduur. In muizen met een korte diabetesduur was er een duidelijk therapeutic effect waar te nemen op de overblijvende endogene eilandjes in de pancreas, terwijl in muizen met langdurige diabetes er meestal geen endogene beta-celmassa overbleef om nog te beschermen. Verder vonden we dat NOD eilandjesrecipiënten behandeld met anti-CD3 alleen of gecombineerd met het *L. lactis*-gebaseerde vaccin Foxp3^{+}, regulatoire T cellen (Treg) hadden in de lymfeknopen die de eilandjesimplantatieplaats (de nier) draineren maar dit was niet verschillend tussen de mono- en combinatietherapie en onafhankelijk van de diabetesduur. Gebaseerd op deze waarnemingen concluderen we dat een milde lymfodepletie met een lage dosis anti-CD3 mAb in combinatie met de antigeen-specifieke aanpak van genetisch gemodificeerde *L. lactis* bacteriën, het heroptreden van autoimmune diabetes kan omzeilen in 56% van langdurige diabetische NOD muizen met syngene eilandjes getransplanteerd. Bovendien toonden we aan dat het resultaat van de eilandjestransplantatie sterk afhankelijk is van het tijdspunt van eilandjesimplantatie met betrekking tot T-cel-targetingstrategie en tot de diabetesduur. De integratie van deze tolerogene antigeen-specifieke technologie om het transplant te versterken en transplantvernietiging te bestrijden kan de resultaten van eilandjestransplantatie in geselecteerde patiëntenpopulaties verbeteren.

In de eerste dagen na transplantatie ontbreekt een adequaat vasculair network in de eilandjes, wat leidt tot ernstige hypoxie en celdood. Men gelooft dat dit één van de belangrijkste oorzaken is voor de slechte prestaties van eilandjestransplanten onmiddellijk na implantatie. Daarom hebben we in **hoofdstuk 5** een nieuwe klasse van progenitorcellen MAPC® gebruikt en deze ge-cotransplanteerd met muiseilandjes om de resultaten van de transplantatie te bevorderen. Ten eerste hebben we aangetoond dat menselijke MAPC grote hoeveelheden angiogene groeifactoren (bv. VEGF) produceren *in vivo* en *in vitro,* wat o.a. blijkt uit de inductie van neo-angiogenese in de CAM assay. Vervolgens toonden we voor de eerste keer aan dat de menselijke MAPC co-getransplanteerd als samengestelde pellets met de muiseilandjes de eilandjestransplantfunctie kon verbeteren in een syngeen “marginale massa” eilandjestransplantatiemodel zoals gemeten kon worden aan de snelheid van diabetescontrole

en functionalitiet van de eilandjes vergeleken met een transplantatie van eilandjes alleen. Bovendien stelden we vast dat transplanten samengesteld uit muiseilandjes opgemengd met menselijke MAPC een verbeterd revascularisatieproces hadden. In de transplanten van muizen waarin eilandjes tesamen met menselijke MAPC werden getransplanteerd was er een duidelijke verbetering in de ontwikkeling van een nieuw capillair network. Een grotere hoeveelheid capillair-achtige structuren met een voering van endotheelcellen kon aangetoond worden in de periferie en in de eilandjes van de eilandjes-humane MAPC composieten 5 weken na implantatie. Dit suggereert dat gastheer-afkomstige bloedvaten rechtstreeks de getransplanteerde eilandjes voeden en dat de nabijheid, en zelfs het direct contact tussen de getransplanteerde eilandjes en de menselijke MAPC, van cruciaal belang is voor de verbetering van diabetescontrole en de verhoogde revascularisatie. Op basis van deze gegevens stellen wij voor dat een co-transplantatie van pancreaseilandjes tesamen met MAPC, welke hoge concentraties aan angiogene groeifactoren kunnen afscheiden, de eilandjestransplant-revascularisatie kunnen verbeteren en dus ook de eilandjestransplant-functie bevorderen.

Als algemene conclusie stellen we voor dat de bevindingen in dit proefschrift kunnen leiden tot nieuwe veelbelovende therapieën die de resultaten van eilandjestransplantatie kunnen verbeteren. Ons werk geeft ook aan dat deze therapieeën moeten inwerken op verschillende niveaus om de vele hindernissen van beta-celvervanging door middel van eilandjestransplantatie te overwinnen. We toonden aan dat genetisch gemodificeerde *L. lactis*-gebaseerde antigeenspecifieke vaccins, gecombineerd met een korte behandeling bestaande uit lage dossisen anti-CD3, het heroptreden van autoimmmune diabetes na eilandjestransplantatie kunnen voorkomen afhankelijk van het tijdstip van eilandjesimplantatie met betrekking tot T-cel-targetingstrategie en tot diabetesduur. We toonden ook aan dat het gebruik van menselijke MAPC in eilandjestransplantatie de transplantmorfologie alsook de functie ervan kan verbeteren door deze tesamen als composieten te transplanteren. We hebben voldoende bewijzen om aan te nemen dat dit gebeurt via het bevorderen van transplant-revascularisatie gemediëerd door de menselijke MAPC. We hopen dat het in de nabije toekomst mogelijk zal zijn om de langetermijn resultaten van eilandjestransplantatie te verbeteren door het implementeren en het combineren van onze vernieuwende therapieën. Onze aanpak zou zowel de immunologische problemen alsook de moeizame revascularizatie na eilandjestransplantatie kunnen oplossen.

CHAPTER 9

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CURRICULUM VITAE

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Additional Courses and Events Participation

- *Laboratory Animal Science Training Course, 2015.*
- *25th meeting of the Belgian Endocrine Society, 2015.*
- *European Society for Organ Transplantation Congress, 2015.*
- *Islet Study Group & Beta Cell Workshop, 2015.*
- *World Transplantation Congress, 2014.*
- *Lecture Scientific Integrity, 2014.*
- *Academic Writing Course, 2013.*
- *V Brazilian Congress of Stem Cells And Cell Therapy, 2010.*
- *IX Brazilian Congress of Veterinary Surgery and Anesthesiology, 2010.*
- *VIII Brazilian Congress of Veterinary Surgery and Anesthesiology, 2008.*
- *Latin America Congress of Emergency and Critical Care of Small Animals, 2008.*
- *Video-laparoscopy course, 2008.*
- *III International Symposium of Cellular Therapy, 2008.*
- *28th Congress of Brazilian Association of Veterinary Clinicians of Small Animal, 2007.*

Pre-reviewed journal articles

- Vangoitsenhoven R., van der Ende M., **Cunha J.P.M.CM.**, Corbeels K., Lannoo M., Bedossa P., van der Merwe S., Mertens A., Gesquiere I., Meulemans A., Matthys C., Mathieu C., Overbergh L., Van der Schueren B. (2016) Dietary composition determines the degree of metabolic improvement at similar weight loss. Submitted

- **Cunha J.P.M.C.M**., Leuckx G., Sterkendries P., Korf H., Bomfim-Ferreira G., Overbergh L., Vaes B., Heimberg H., Gysemans C., Mathieu C. (2017) Human Multipotent Adult Progenitor Cells Enhance Islet Function And Revascularization When Co-Transplanted As A Composite Pellet In A Mouse Model Of Diabetes. **Diabetologia** 60:134-142

- **Cunha J.P.M.C.M.**, Gysemans C., Gillard P., Mathieu C. (2016) Stem-Cell-Based Therapies for Improving Islet cell transplantation Outcomes in Type 1 Diabetes. **Current Diabetes Review** (in press)

- M.G.M.C.M. Cunha, Raiser A.G., Martins D.B., Lopes S.T.A., Freitas G.C., Beckmann D.V., **Cunha J.P.M.C.M**., Teixeira L.V., Pippi N.L. (2016). Fluid therapy rate in postrenal azotemia stabilization in cats. **Symbiosis Journal of Veterinary Science** (in press)

- Ferreira G.B., Gysemans C.A., Demengeot J., **Cunha J.P.M.C.M**., Vanherwegen A.S., Overbergh L, Van Belle T.L., Pauwels F., Verstuyf A., Korf H., Mathieu C. (2014). 1,25-Dihydroxyvitamin D_3 promotes tolerogenic dendritic cells with functional migratory properties in NOD mice. **The Journal of Immunology** 192:4210-4220.

- Freitas, G.C., Cunha, M.G.M.C.M., Carregaro, A.B., Gomes, K., **Cunha, J.P.M.C.M.**, Tognini, M., Pippi, N.L. (2012). Acid-base and biochemical stabilization and quality of recovery in male cats with urethral obstruction and anesthetized with propofol or a combination of ketamine and diazepam. **Canadian Journal of Veterinary Research** 76:201-208.

- Maris, M., Overbergh, L., Gysemans, C., Waget, A., Cardozo, A.K., Verdrengh E., **Cunha, M.C.M.J.P**., Gotoh, T., Cnop, M., Eizirik, D.L., Burcelin, R., Mathieu, C. (2012). Deletion of C/EBP homologous protein (Chop) in C57Bl/6 mice dissociates obesity from insulin resistance. **Diabetologia** 55:1167-1178.

- Treichel, T. E., Cunha, M. G. M. C. M., **Cunha, J. P. M. C. M.**, Pippi, N. L., Lopes, S. T. A., Santos Junior, E. B., Fighera, R. A., Rosa, M. B. (2011). Total fraction of mononuclear cell or stromal vascular fraction transplant associated with cellulosic membrane in experimental cutaneous wounds in rabbits. **Brazilian Journal of Veterinary Research and Animal Science (Impresso)** 48:62-72

- Müller, D. C.M., Basso, P.C., Serafine, G.M. C.., Rosa, M.B., Sprada, A.G., **Cunha, J.P.M.C.M.**; Pippi, N.L. (2011). Replacement of the cranial and caudal cruciate ligaments in dogs by double t polypropylene implant. **Ciência Rural (UFSM)** 41:487-491

- Cunha, M.G.M.C.M., Freitas, G.C., Carregaro, A.B., Gomes, K., **Cunha, J.P.M.C.M.**, Beckmann, D.V., Pippi, N.L. (2010) Renal and cardiorespiratory effects of treatment with lactated Ringer's solution or physiologic saline (0.9% NaCl) solution in cats with experimentally induced urethral obstruction. **American Journal of Veterinary Research**. 71:840-846.

- Cunha, M.G.M.C..M.**,** Pippi. N.L., Gomes. K., **Cunha, J.P.M.C.M.** (2010). Cerclage with Nylon Cable Tie Band or Stainless Steel Wire for Repair of Experimental Mandibular Symphyseal Fractures in Cat. **Acta Scientiae Veterinariae.** 38:363-369.

- Cunha, M.G.M.C.M.**,** Gomes, K., **Cunha, J.P.M.C.M.**, Pippi, N.L., Rappeti, J.C. (2009). Catheter needle for osteosynthesis of the rib in a dog. **Acta Scientiae Veterinariae.** 37:205-209.

Oral Presentation and Poster presented during PhD

- Cunha JPMCM, Leuckx G, Sterkendries P, Korf H, Ferreira GB, Vaes B, Heimberg H, Gysemans C Mathieu C. **Human multipotent adult progenitor cells enhance islet function and revascularization when co-transplanted as a composite pellet in a mouse model of diabetes** 25th meeting of the Belgian Endocrine Society. Antwerp, Belgium, October 23-24, 2015. ***Award best poster.***

- Cunha JPMCM, Leuckx G, Sterkendries P, Korf H, Ferreira GB, Vaes B, Heimberg H, Gysemans C Mathieu C. **Improved islet function and revascularization by co-transplantation of human multipotent adult progenitor cells in diabetes mouse model** European Society for Organ Transplantation Congress, Brussels, Belgium, September 13-16, 2015.

- Cunha JPMCM, Leuckx G, Sterkendries P, Korf H, Ferreira GB, Vaes B, Heimberg H, Gysemans C Mathieu C. **Human multipotent adult progenitor cells enhance islet function and revascularization when co-transplanted in a mouse model of diabetes**.

Poster presentation at the Islet Study Group & Beta Cell Workshop, Jerusalem, Israel, May 3-7, 2015. ***Award with a scholarship which covered registration fees and cost of accommodation.***

- Cunha JPMCM, Leuckx G, Sterkendries P, Korf H, Ferreira GB, Vaes B, Heimberg H, Gysemans C Mathieu C. **Human multipotent adult progenitor cells enhance islet function and revascularization when co-transplanted in a mouse model of diabetes**. Poster and oral presentation at the Belgian Diabetes Excellence Summit, Brussels, Belgium, April 25, 2015. ***Award and oral presentation*.**

- Cunha JPMCM, Takiishi T, Belle T, Korf H, Rottiers P, Steidler L, Gysemans C, Mathieu C. **Antigen-specific therapy with human proinsulin and IL10 in combination with short-course monoclonal CD3 antibody in preclinical models of islet transplant** Poster presentation at the World Transplantation Congress, San Francisco, USA, July 26-31, 2014. ***Poster distinction after review in the top 20% of the topic.***

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