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Chapter 24

Cell Replacement Therapy in Type 1 Diabetes

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Additional information is available at the end of the chapter

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1. Introduction

Type I diabetes (insulin-dependent diabetes mellitus, IDDM) is a chronic autoimmune disease caused by the selective destruction of insulin-producing β-cells in the pancreatic islets of Langerhans, which results in severe insulin deficiency. Insufficient circulating levels of insulin lead to potentially fatal metabolic dysfunction. Although the exact mechanism of islet cell destruction is unclear, a T-cell-mediated autoimmune process seems to be the most likely explanation. Other factors, genetic and environmental, are likely contributing causes, but have not been fully identified as of yet.

Although whole pancreas transplantation has been considered as a therapeutic option for selected patients with IDDM, most individuals with the disease are not likely candidates for this therapy. Since the discovery of insulin in the 1920's, the main therapeutic approach to treating IDDM patients has been insulin replacement [1]. The standard of care for most patients with Type 1 diabetes is based on exogenous insulin therapy delivered through several daily injections. Despite great improvements in insulin delivery systems seen in the last two decades, it's still difficult to provide the precise amount of insulin that is required by the patient at any given time. This results in hypo- and hyperglycemic episodes, potentially leading to cell damage in many tissues, ultimately resulting in the development of severe long-term complications. Therefore, insulin delivery systems which can quickly and continuously respond to constantly changing physiological needs of the organism by adjusting the amount of insulin released into the circulation would be of great benefit.

Due to the fact that IDDM is a disorder in which β-cells in the pancreatic islets of Langerhans are selectively destroyed by an autoimmune attack, cell replacement strategies offer a very attractive treatment option. Recent successes in the field of islet cell transplantation have led to renewed optimism in this area. Clinical trials clearly demonstrated that islet transplantation not only offers a viable option for patients with severe forms of IDDM, but can successfully

treat the disease [2,3,4]. It is, however, apparent that islet transplantation is not currently a viable option for the treatment of all potential recipients, due to the limited source of islet cells, i.e. limited number of available donors. Another feature of islet transplantation, as currently performed, is the requirement for life-long immunosuppression that limits the patients' eligibility to individuals with the most severe cases of IDDM. These issues have driven the investigation of alternative cell sources, which include xenografts from other species, embryonic and adult stem cells, and gene therapy products. Such therapies will also likely require immunological protection provided by means such as conventional immunosuppression, administration of immunomodulatory cell subsets or a combination and manipulation of the islets by shielding and/or encapsulation, which can protect transplanted cells from recognition by the immune system and, in particular, from recurrence of autoimmunity.

An adult pancreas contains approximately one million (1×10^6) islet cells, which represent a minor part of the organ, i.e. 2-3 % of the pancreatic tissue. Islets designated for transplantation must be isolated from the whole pancreas using the method that combines enzymatic digestion with mechanical disruption. Despite considerable improvements made in the islet isolation process (the process itself, the reagents used during the procedure), that led to improved quantity and quality of islet preparations, it still remains a largely inefficient process. Clinical symptoms of Type 1 diabetes do not develop until 60-80% of the β-cell mass is lost to the autoimmune attack [5]. This means that adequate glycemic control can be maintained with as little as 20-40% of the normal β-cell mass. Intrahepatic islet transplantation is the accepted gold standard at the present time. Ample scientific evidence suggests [4] that a significant number of islets are lost during the immediate post-transplant period, mostly due to the inflammation and thrombosis following initial islet-blood contact and activation of hepatic microenvironment. Thus, if the goal of islet transplantation is to replace 1×10^6 islets to achieve long-term normoglycemia, several donors may be requires for each recipient. In fact, it has been previously demonstrated [2,3] that insulin independence is achieved with ≥13,000 islet equivalents (IEQ)/kg of recipient body weight, using more than one islet preparation per recipient, at the same time or in succession. This means that a single islet transplant may require 3-4 donor pancreata. At the present time, the only source of islet cells are pancreata obtained from a deceased, heart-beating, brain-dead donor. This type of donor, especially of suitable age, is rare, making current protocols for human islet transplantation an unlikely candidate for widespread treatment for patients with IDDM. In the US alone, there are approximately 2 million people diagnosed with Type 1 diabetes. This demand is driving the current research trends into alternative functionally competent, i.e. insulin secreting and sensing, β -cell sources as potential replacement therapies for IDDM.

A number of different cell types have been proposed as a starting material to generate sufficient cell mass for transplantation; these include insulin-secreting cell lines, non-β-cell sources engineered through gene therapy, β-cells from non-human species, and β-cells generated from adult (bone marrow, pancreas, liver and neural tissue) and embryonic stem cells [5]. Regardless of the cell source, i.e. β- or non-β cells, many agree that the optimal treatment for Type 1 diabetes should ideally consist of an autologous cell source, which can synthesize, store and release insulin in a highly regulated fashion to maintain glucose homeostasis. Too much or too

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little is potentially lethal, so the cells must be able to rapidly respond to changes in plasma glucose in either direction.

This chapter will offer a detailed discussion of the latest developments in islet transplantation and its future direction. In addition, attention will be paid to alternative approaches for achieving insulin homeostasis and glycemic control through various novel cell replacement therapies, as well as potential advantages and risks associated with each therapeutic option.

1.1. Allogeneic islet transplantation

Diabetes Mellitus (DM) poses a significant challenge in the United States and around the world. It's increasing in prevalence and, at the present time, affects almost 20 million people in the United States alone [1]. DM is considered to be the sixth leading cause of death in the USA and is a major morbidity hazard [6,7] because of its associated complications that may negatively impact a patient's quality of life. Presently, the disease lowers average life expectancy by about 15 years, increases cardiovascular disease (CVD) risk by about two- to four-fold, and is the main cause of kidney failure, lower limb amputations, and adult-onset blindness. DM is a costly disease: its estimated attributable costs in 2010 were approximately 135 billion dollars [6].

IDDM has an early childhood or young adulthood onset, although it can be diagnosed at any age. It is characterized by profound deficiency in insulin secretion caused by the autoimmune destruction of insulin-producing cells in the pancreas, the pancreatic β-cells. IDDM accounts for approximately 5-10% of all disease cases. Factors that have been associated with the development of Type 1 DM are both genetic and environmental [8-10]. In animal models such as the NOD mouse and BB rat, and in human Type 1 diabetes, there is strong evidence of a role of the class II gene, I-A in NOD mouse (equivalent to human DQ beta gene), most probably in combination with lack of I-E expression (equivalent to human DR) [11]. Although it is entirely possible that the genetic response can be triggered by environmental factors such as infections or drastic change in diet, the clear definition of such factors has been elusive to date [11]. Ultimately, though, it is the autoimmune component of Type 1 diabetes that is responsible for the progressive and selective autoimmune destruction of insulin-producing β-cells in the pancreas. Due to the fact that the disease is the result of the loss of a single cell type, i.e. β-cell, it is considered to be amenable to treatment by cell replacement therapy.

The discovery of insulin in 1922 by the Canadian physician Frederic Banting brought about the realization that it was the pancreas that produced the "sugar-reducing substance" [1], i.e. insulin. Since then scientists have been interested in how this hormone is synthesized and secreted, and the main therapeutic approach to IDDM has been focused on insulin replacement. Until recently, the only available treatment for Type 1 diabetes was the administration of exogenous insulin. The Diabetes Control and Complications Trial (DCCT) [12] demonstrated that, in patients with Type 1 diabetes, intensive insulin replacement therapy can control blood glucose levels to a certain extent [12]. Unfortunately, even intensive care it is not able to mimic normal hormone release that regulates glucose homeostasis [8] and results in the fine-tuned physiological balance [13]. Even in patients with good glycemic control achieved through intensive insulin therapy blood glucose lev-

els can vary greatly outside the normal range [13]. In addition, tight control of blood glucose levels often results in frequent episodes of hypoglycemia. The DCCT trial [12] clearly demonstrated that although intensive insulin therapy is able to delay the onset of diabetes-associated complications, it doesn't result in complete prevention of their development [12,13]. It is also not clear as to how early in the progression of the disease glucose homeostasis must be restored to affect a near-positive outcome.

Thus, the need for alternative or additional therapies has been apparent for some time. Endocrine replacement has been but one approach in the quest for tight glycemic control. Achieved either through transplant of a whole pancreas [14] or allogeneic islet cells [13-15], it has been investigated for quite some time now. There is little doubt that pancreas transplants, especially when performed simultaneously with a kidney, favorably impact metabolic control [14]. Eighty percent of the patients receiving simultaneous kidney-pancreas transplants demonstrate good graft function and insulin independence at one year following surgery, with 50% of the recipients maintaining euglycemia at 5 years [16]. Pancreas transplantation results in independence from exogenous insulin, normalization of glucose levels (both fasting and post-prandial), normal Hemoglobin A1c (HbA1c) levels, and freedom from hypoglycemia [16]. However, pancreas transplantation is still associated with significant morbidity and mortality rates [16-18]. Thus, most patients with Type 1 diabetes are not candidates for pancreas transplantation.

In contrast, islet transplantation requires only a safe interventional radiology technique to implant the graft, and doesn't require general anesthesia, does not call for post-transplant management of pancreatic secretions, and is not associated with post-transplant morbidity and mortality. In addition, in patients with Type 1 diabetes, pancreatic exocrine tissue, which represents the vast majority of the organ, is not affected. These are all factors that contribute favorably toward a wider application of islet cell transplantation.

Of the 159 islet cell allografts reported to the International Pancreas Transplant Registry [18] in 1983, none resulted in insulin independence that could be clearly linked to the implanted graft. These unsatisfactory results could be attributed to the suboptimal islet isolation methods and variable immunosuppressive regiments utilized at the time. It is now apparent that islet isolation methods used at that time - originally developed for the isolation of rat islets by Moskalewski[19] and further improved upon by Lacy [20] - were not entirely adequate for the isolation of human islet cells. The use of unpurified islet preparations was not particularly safe, resulting in reported cases of portal hypertension and even death [21].

Introduction of collagenase through the pancreatic duct during the distension of the organ, and purification of the islet cells from the exocrine tissue using discontinuous Ficoll gradients [22, 23] resulted in the optimization of the islet isolation method, i.e. improved isolation yield and islet purity of up to 90% [9]. These continued improvements in the islet isolation methodology provided a new impetus to continued attempts at islet transplantation during the 1980's. Although none of the islet allografts resulted in insulin independence, clinical trials conducted during this period proved islet cell transplantation to be safe, and for the first time demonstrated a sustained C peptide production [24].

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In the 1990's, the International Islet Registry [25] reported that 10% of the patients receiving allogeneic islet grafts could maintain insulin independence at ≥1 year following transplant. Although the majority of transplant recipients continued to require some exogenous insulin, their daily insulin intake was reduced, HbA1c decreased, and they reported fewer episodes of hypoglycemia unawareness. At this point, transplantation of allogeneic islet cells became a reality. However, questions related to partial graft function and eventual graft failure due to recurrence of auto-immunity or rejection - both difficult to predict - remained. Animal studies of glucose metabolism in rat [26], dog [27-29] and cynomolgus monkey [30,31] models demonstrated that long-term normoglycemia could be achieved provided that a sufficient islet mass was transplanted. These studies also showed that, in dog and simian models, the site of implantation did not play a significant role in graft failure. These findings demonstrated that islet transplantation could be successful, and represented a sustainable cell-based treatment for patients with Type 1 diabetes.

Of significant, positive impact was the introduction of the Ricordi automated method for islet isolation [32] which allowed for continuous release of large numbers of islet cells during the digestion phase, protecting them from any further enzymatic action, thereby preventing over-digestion of the islet tissue, and significantly reducing islet cell loss as a result of the isolation process. The digestion process was allowed to proceed until only a fibrous network of ducts and vessels of the pancreas remained. In contrast with previous methods utilized to isolate human islets, the Ricordi method allowed for the digestion of the whole pancreas and a significant improvement in the quantity and quality of the isolated cells [32]. Introduction of more efficient enzyme blends [33,34], development of more effective organ preservation methods [35-37], effective use of semi-automated large-scale purification techniques [38-41], and the introduction of additional reagents during various phases of islet cell processing, all contributed to the improved islet recovery and the utilization of islet preparations for transplantation. Islet preparations can be transplanted fresh, i.e. immediately following isolation, or following culture [2,3], which is of substantial benefit. This window offers sufficient time for both the detailed characterization and quality assessment of the islet preparation, and shipment of the cells to satellite transplant centers, when necessary.

Islet preparations of various degree of purity are normally implanted into the recipient's liver portal vein by transhepatic cannulation using minimally invasive interventional radiological techniques [42-47]. This approach has been demonstrated to be safe, is associated with low morbidity and is well tolerated by the transplant recipients. In fact, when additional islet mass is required to improve recipient's metabolic control, additional preparations of islet cells are delivered using the same route of administration.

New immunosuppressive protocols designed for the recipients of solitary islet allografts, i.e. islet transplant alone (ITA), and the publication of the results of the Edmonton Protocol in the year 2000 [2,3] lead to further improvement in the clinical outcomes reported by a number of centers [42,48-50]. These new protocols moved away from the use of glucocorticoids and calcineurin inhibitors (CNI, cyclosporin A (CyA)) that have diabetogenic effects, and potential islet toxicity [2,3,42,48-50]; and utilized alternative strategies as immunosuppressive therapy. On-going clinical studies clearly demonstrated that allogeneic islet transplantation has the

potential to become a viable therapy for patients with severe forms of Type 1 diabetes. However significant challenges need to be overcome before islet transplantation can be considered as the treatment of choice.

Some of the critical questions that remain to be addressed include: (i) definition of an adequate supply of donor organs which can meet the existing need; (ii) isolation of a sufficient number of high quality islet cells from the exocrine tissue, which comprises 98-99% of the pancreas; (iii) improvements in the immunosuppressive strategies that are currently used, by either the development of less toxic drugs or the induction of tolerance; (iv) preventing the recurrence of autoimmunity, demonstrated to have successful outcomes in murine models [5]; (v) identifying the early occurrence of immune rejection, which is quite challenging to monitor given the very small volume of the transplanted tissue and our limited ability to characterize the process.

1.2. Islet cells from xenogeneic sources

At the present time xenogeneic islet cells isolated from pig pancreata offer the most promising alternative to human islets as a treatment for Type 1 diabetes. This is based on a number of observations: (i) there is a large number of facilities in the US with capabilities for high-throughput breeding, rearing and slaughter of pigs; (ii) pig insulin differs from human insulin by just one amino acid and has been successfully utilized as a source of exogenous insulin for many years before the advent of recombinant insulin; (iii) large numbers of islet cells can be isolated from a single pig pancreas using techniques similar to those developed for human islet isolation; (iv) pig donors can be genetically manipulated to increase insulin production, and to protect the islet cells from immune and cytokine assault [5,51].

Several limitations have restricted the use of pig islets in human recipients. The first one is the hyperimmune response, possibly mediated by the galactoseα -1,3-galactose (Gal) epitope. Elimination of this epitope was shown to prevent hyperacute rejection of pig-to-nonhuman primate solid organ xenografts. Immune protection of xenografts utilizing encapsulation techniques resulted in progressive loss of graft viability and insulin secretion over prolonged period of time, during which transplanted islets were expected to function [52]. The second one is the possibility of transmission of porcine endogenous retroviruses (PERV), several copies of which are present in the genome of all pigs and able to infect human cell *in vitro*, with unknown consequences [53]. The possibility of novel viral infections in recipients of porcine islet grafts raised serious safety and ethical concerns, as C-type retroviruses related to PERV have been demonstrated to associate with hematopoietic cell malignancies in the natural hosts [53].

The interest in porcine islets peaked when it was demonstrated that T-cell immunomodulatory therapies which target indirect co-stimulatory pathway, i.e. CD28-CD154, supported prolonged engraftment of unmodified pig islet cells in non-human primates [54, 55] Furthermore, published data drew attention to the fact that, in contrast to human islets that produce copious amounts of islet amyloid polypeptide (IAPP) capable of inducing β -cell apoptosis, pig neonatal and adult islets do not form amyloid deposits. This could be due to the fact that pig IAPP is considerably less amyloidogenic [56]. Recently published data, however, have suggested that

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the PERV scare may have been overestimated: long-term immunosuppressive regimens and exposure to porcine islet grafts did not result in any detectable PERV transmission. These data clearly showed (a) no expression of PERV in porcine islets in either *in vivo* or *in vitro* studies, and (b) no integration of PERV sequences into recipient cell or organs [55, 57-58]. Additionally, Koulmanda et al successfully demonstrated that, following anti-CD4 treatment, pig islet grafts became resistant to autoimmune destruction in non-obese diabetes (NOD) recipients, suggesting that CD4-mediated autoimmunity, rather than hyper-acute immunological response, might be the cause of the destruction of xenogeneic islet grafts [59].

It is also difficult to overlook the fact that large numbers of porcine islets can be isolated with considerable ease, using protocols similar to those developed for the bulk isolation of human islet cells [32,60]. Since the introduction of highly efficient semi-automated methods for bulk islet isolation of pig pancreatic islets by Ricordi et al [32], the quantity and quality of islet preparations from a pig donor has been consistently higher. Pig donors are healthy and avoid cell senescence due to various co-morbidities, brain death, and cold and warm ischemia injury, as these factors can be controlled and, under normal circumstances, kept to a minimum [60,61]. Using standard purification methods [61] a purity of 70-90% of islet can be achieved. Islet cells isolated from adult pigs are functionally competent, and graft function can be recorded shortly following transplantation [61]. In addition, adult pig islets have appropriate glucose-sensing and insulin release mechanisms, as demonstrated by prolonged diabetes reversal when porcine islets were transplanted into nonhuman primates [534,55]. However, the fragile nature of adult porcine islets leads to significant loss as a result of ischemia and inflammation, during cell culture and early engraftment process. It also makes it challenging to maintain them in culture, and may result in the loss of a significant proportion of cells following isolation. Although a reduction in islet mass and cell viability has been reported when adult porcine islets were maintained in culture, short-term culture is desirable to reduce cell immunogenicity or combine preparations from several donors, prior to transplant [61].

In comparison to adult pig islets, fetal islets have been isolated with even greater ease. Once isolated these require several weeks of culture to facilitate re-aggregation of the endocrine tissue and elimination of exocrine tissue, and to mature to glucose-sensing and insulin production [60]. Additionally, immature cells are much more resistant to the ischemia and inflammation-related injury. Fetal islet isolation is very simple and highly reproducible, and can be accomplished using an exogenous solution of digestive enzymes with minimal loss of immature islet cells [61]. This is due to the fact that fetal islet tissue is not prone to ischemic damage, most probably because of its inherent relative lack of exocrine tissue, capable of inducing damage as a result of the release of proteolytic enzymes from damaged exocrine cells [62,63]. At the same time, there is a relative abundance of endocrine tissue which makes the isolation of fetal islets an easier and more efficient process. Additionally, the copiousness of immature precursor cells in the ductal tissue and their possible presence in the islet-like cell clusters (ICCs) that form during culture, contributes to high capacity of ICC tissue for post-transplant proliferation, a key feature lost in adult pig islets [63]. Thus, small numbers of ICCs can eventually produce large-size grafts, provided that rejection, recurrence of autoimmunity and hyperglycemia can be overcome and controlled [63]. Considering the small size of the fetal

pancreas, the capability of a small number of ICCs to mature into a functionally competent graft speaks to the use of this tissue. As mentioned above, a major drawback with using functionally immature cells is their delayed function. ICCs require several weeks, and even months, of development before normal glucose levels in the recipient can be achieved, during which time a poor response to physiological glucose has been observed [64]. This on-going hyperglycemic state during the period of functional maturation can lead to possible damage of the transplanted fetal tissue. Thus, while transplanting immature ICCs in diabetic recipients who are early in the course of their disease might not represent a problem, it is potentially a serious drawback for patients with brittle diabetes and declining kidney function [51,62]. A second disadvantage to using ICCs is the high expression of α-1,3-Gal epitope on the surface of fetal islets, making these cells more susceptible to rejection than adult pig islets, which in contrast express little Gal [62].

Neonatal pancreatic cell clusters (NPCCs) obtained from 1-5 day-old piglets can be also easily procured and successfully isolated in a relatively quick and efficient manner [61,65], using culture media supplemented with collagenase. Due to their availability and inherent capacity to differentiate *in vitro* and *in vivo,* NPCCs represent an attractive source of xenogeneic tissue for clinical transplantation. Although freshly isolated cell clusters contain only 7% endocrine cells, 11% epithelial cells, and ~74% exocrine tissue, this content undergoes dramatic transformation following a 9-day culture [51,61]. Published data indicates that during *in vitro* culture the acinar tissue undergoes apoptosis resulting in the enrichment of the endocrine component to 35% [61] of the total cellular content, with 25% of the cells capable of insulin production. The rest of the tissue is characterized as non-granulated epithelial cells [61]. *In vitro* culture results in the formation of NPCCs [61,65], as well as the proliferation of β-cell as assessed by studies using bromodeoxyuridine (BrdU) [65]. NPCCs have been demonstrated to be more responsive to glucose challenge compared to the fetal ICCs, but not as fully functional as adult islets [61]. Although NPCCs were showed not to correct diabetes immediately following transplantation, the insulin content of the grafts was reported to increase by ~20 fold [61], confirming either NPCCs' capacity for β-cell proliferation, or differentiation of epithelial precursor cells into β-cells, or both. During the period of hyperglycemia, none of the transplanted immunodeficient mice were lost, suggesting that even in the immediate post-transplant period NPCCs are capable of producing small, but sufficient, amount of insulin to keep the recipients alive, stopping short of achieving normoglycemia [61]. This speaks to the fact that compared to the adult porcine islets, NPCCs have an extensive *in vivo* and *in vitro* proliferative capacity [61,65], as well as the ability to acquire endocrine function in a time-dependent manner. In addition, data showing that NPCC can be successfully and reproducibly transfected with a non-immunogenic, non-pathogenic recombinant AAV demonstrated a possible strategy for gene delivery to improve transplantation outcome [65].

On the other hand, NPCCs require long periods of *in vivo* maturation before developing functional competence [65], which represents a potential draw-back with respect to the clinical utilization of this xenogeneic islet cell source.

Small and large animal models to study the potential clinical use of porcine islet transplantation to treat Type 1 diabetes have been developed and success has been reported [54,55,59,61,

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62]. Reversal of diabetes with prolonged restoration of insulin independence has been achieved in several porcine-to-nonhuman primate xenogeneic transplant models [54,55] in recipients that developed diabetes as a result of chemical treatment, surgical intervention, i.e. pancreotectomy, or spontaneously. Long-term insulin independence has also been achieved when neonatal and fetal pig pancreatic precursors were implanted intraportaly, subcutaneously, and into the peritoneal cavity [66,67]. The choice of the anatomical implantation site for not only porcine, but human islets is crucial. At the present time, the accepted clinical practice is to deliver islets to the liver, through the portal vein. However, it has been demonstrated that using this route of administration, low oxygen tension, and an active innate immune response that results in complement activation and immediate blood-mediated inflammatory response (IBMIR) contribute to significant islet mass loss in the immediate post-transplant period [68,69]. Different challenges arise when the graft is placed under the kidney capsule, i.e. islets in this case may be damaged by stress as a result of ischemic injury. However, implantation of encapsulated porcine islets under the kidney capsule of non-diabetic Cynomolgus Macaques resulted in low levels of porcine C-peptide, with islet grafts surviving for up to 6 months [70]. Reports of other implantation sites, such as subcutaneous and peritoneal space, have been published, but both have been reported as relatively immunoreactive [51], unless the islets were protected by an immune barrier in the form of a capsule.

Although most of the data regarding the possible use of porcine islets as an alternative treatment modality for Type 1 diabetes became available as a consequence to the extensive effort undertaken in a number of small and large animal models, a number of reports of controversial clinical trials have been published in the last several years. An Australian biotechnology company, Living Cell Technologies Ltd., reported a clinical trial in Moscow where 10,000 encapsulated porcine islet equivalents (IEQ)/kg recipient body weight isolated from adult virus-free pigs (DiabeCell®) were implanted into several adult recipients with brittle form of Type 1 diabetes, leading to reduced insulin requirements and detectable porcine C-peptide 11 months following transplant [71]. Follow-up dose-finding clinical trial conducted in New Zealand, however, produced less optimistic results. Although a statistically significant reduction in hypoglycemic unawareness was demonstrated, insulin requirements and C-peptide were reported to be largely unchanged. Additional dose-finding trials using DiabeCell® are currently in progress. Earlier clinical trials conducted in Mexico utilized neonatal islets co-cultured with Sertoli cells in a collagen-coated device which was implanted subcutaneously into 12 adolescent Type 1 diabetic patients [72]. Although pioneering in nature, this work drew a certain amount of criticism with regard to the ethical implications of conducting clinical trials in countries without strict regulatory oversight, the dearth of relevant pre-clinical data to warrant Phase I clinical trials to assess the safety of the investigational therapy, i.e. porcine islet transplants, as well as the efficacy of the treatment [73].

It's hard to dispute potential clinical and commercial implications of porcine xenotransplantation as a potential therapy for patients with severe forms of Type 1 diabetes. Although not a new idea, recent developments in this field are likely to drive larger, more tightly controlled pre-clinical and clinical studies to explore its enormous potential as a substitute for human islets. However, if xenotransplantation is going to be the way to solve inherent supply

problems with allogeneic organs, a much better understanding of the immunological processes involved in the destruction of xenogeneic tissue is necessary.

1.3. Stem cell as β-cell replacement therapy

The most promising cell source for β-cell progenitors is embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts during the early stages of embryogenesis. ECSs offer several notable advantages. First, ESCs differ from adult stem cells in that under the right growth and differentiation conditions they have the potential to differentiate into any cell type *in vitro* and *in vivo*, a potential termed pluripotency. Given the capacity for pluripotency, there is an interest to explore guided *in vitro* differentiation into a desired cell type for the purpose of cell replacement therapy, in this case for the treatment of Type 1 diabetes. Second, ESCs' potential to self-renew while maintaining their stem cell properties is of immeasurable advantage, as it allows for unlimited cell expansion, while the cell differentiation capacity is preserved. Given the need for large number of cells for therapeutic applications, this favors ESCs over the cells at more advanced stages of maturation which, in general, are reported to have a much more limited proliferative capacity [74]. Here, of course, certain precautions are necessary. Directed cell differentiation and proliferation also results in the differentiation of associated cell types, which are not necessarily desired and need to be inhibited. This represents a challenge. It's been previously postulated that to successfully differentiate a cell type such as insulin-producing β-cells, an ideal protocol would involve culture steps that mimic a differentiation process taking place during normal embryonic development. That involves certain signaling pathways and transcription factors necessary to guide the development of undifferentiated progenitor cells into fully mature, metabolically functional insulin-producing β-cells [74-76].

First attempts to generate insulin producing islet-like cells (IPCs) were centered on the selection of cells positive for nestin, an intermediate filament protein which serves as a neural stem cell/progenitor marker [77-79]. The reason behind the focus on nestin-positive cells is that in some species neural cells, namely brain neurons in Drosophila, are the source of circulating insulin. In addition, insulin gene transcription is found in the vertebrate brain, although it's not clear if vertebrate neurons produce or secrete the actual protein [74,78]. Recent reports, however, have demonstrated that selection of nestin-positive cells from ESCs leads to generation of neural cell types [80-82], although differentiation into insulin-producing cells was also achieved. This is consistent with the fact that nestin is a marker of neural and pancreatic exocrine progenitors, but does not indicate endocrine progenitor cells. Attempts to differentiate brain-derived neural ESCs into insulin-producing cells resulted in the formation of glucose-sensing insulin producing cell clusters following the exposure to multiple signals that regulate *in vivo* islet pancreatic development [83]. Following transplantation into immunocompromised mice islet-like clusters were demonstrated to release insulin and C-peptide. However, the C-peptide content of these islet progenitor clusters was estimated to be 0.3% of the normal level found in isolated human pancreatic β-cells [83], suggesting that the resulting islet-like cell clusters were not bona-fide β -cells. In addition, temporal sequence of expression of gene products active during the development of pancreatic islet cells, such as glucokinase,

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Glut-2 and Pdx1, did not exactly resemble that observed in the embryonic pancreas; nor was the transcription of other genes normally expressed in β-cells, such as Nkx2-2 and Nkx6-1, detected in the later stage islet-like clusters. Some of these insulin-producing cell clusters [77], while staining positive for insulin, were - in all likelihood - the result of insulin uptake from the culture medium, rather than activation of robust insulin transcription, as demonstrated by other studies [84]. These data pointed to the fact that evidence demonstrating the equivalence of these islet-like cells clusters to mature β-cells was lacking; and that a better understanding of the signaling pathways and transcriptional factors regulating the development of pancreatic β-cell identity during embryogenesis was necessary.

In 2005, D'Amour clearly demonstrated that cells closely resembling fully mature native β-cells could be generated by replicating the culture conditions that closely mimicked embryonic development [85,86]. Utilizing a step-wise approach, ESCs were first directed into definitive endoderm stage, a pre-requisite for all pancreatic cell types, followed by a pancreatic endoderm, and subsequently into β-cells with an insulin content similar to that observed in native islets [86]. However, similar to fetal β-cells, the resulting cells were able to release C-peptide in response to multiple secretory stimuli, but only minimally to glucose. These studies were followed by others [87-89] in which these cells were implanted into immunocompromised mice half way through the differentiation process. When the cells were allowed to mature *in vivo*, the efficiency of the differentiation process was improved, glucose-responsive insulin secretion observed, and chemically induced diabetes reversed [89]. Progress in this area has been rapid and recently, California-based ViaCyte (previously Novocell) has reported positive pre-clinical results with Pro-Islet™, a material based on the technology discussed above, in conjunction with a retrievable encapsulation device. On-going efforts to translate this strategy into pre-clinical and clinical applications were supported by a recent $20 million award from the California Institute of Regenerative Medicine. This works favorably towards ESC-based clinical approaches becoming available in the very near future.

Upon demonstration that the mature state of somatic stem cells can be redirected toward the a progenitor state similar to that of ESCs [90-92], the field of stem cell-based strategies has been further expanded and now includes an attractive alternative to ESCs, i.e. induced pluripotent stem cells (iPSCs). Potentially, iPCSs offer a practical solution to the ethical dilemma posed by the destruction of human embryos necessary for the production of ESCs-based cell therapies. These cells, they are virtually undistinguishable from ESCs in terms of their molecular and biological characteristics. They offer a possibility of generating autologous patient-specific cell therapies directed to treat a variety of medical conditions, including diabetes. This means that depending on the specific illness, desired cells can be differentiated from the patient's own cells. This is certainly attractive as the cells designated for re-transplantation would have the same genetic makeup as those of the patient, and would alleviate the challenges posed by the activation of the recipient's immune system that would occur when allogeneic cells are transplanted.

In 2006, Takahashi et al [90] demonstrated that pluripotent stem (iPS) cells could be generated from mouse fibroblasts by the retrovirus-mediated transfection of four transcription factors, namely Oct3/4, Sox2, c-Myc, and Klf4. Since then, following the main steps of the original β-

cell differentiation protocol and retroviral expression of the same four transcription factors, it was reported that differentiation into insulin-producing islet-like clusters was possible. Islet-like clusters were obtained from iPSCs using a serum-free, feeder-free protocol [93]. Following initial reports, a number of modifications to the original protocol have been introduced. These included substituting the originally-described transcription factors with oncogenic potential with stable recombinant proteins [94], episomal constructs [95], DNA minicircles [96], modified mRNAs [97], and small molecule compounds with re-programming properties [98]. Despite these efforts, generation of patient-specific cell lines form iPSCs remains inefficient and expensive, hindering progress in this area. Additionally, there seem to be an inconsistency in the methods utilized for the successful differentiation of insulin-producing islet-like cells, leaving the field open for a much wanted universal protocol utilized to generate a wide variety of patient-specific cell lines, much like that developed by ViaCyte for the Pro-Islet™ technology. Then, of course, the risks inherent to the use of iPSC-and ESC-based approaches must be carefully considered, as these seem to be almost identical.

First, there are the reports of teratoma formation when undifferentiated (cultured *in vitro* for ~12 days) ESCs are utilized in pre-clinical models [89]. Interestingly, when cells cultured under similar conditions for extended period of time were utilized [87], no teratoma formation was observed in recipient animals suggesting that more extensively differentiated ESCs lose their ability for neoplastic transformation. Hence, teratoma formation can probably be controlled through elimination of less differentiated cells via advanced purification techniques, as well as more efficient machinery for cell differentiation.

Another critical aspect that deserves serious consideration is related to the full cell complement present at the final ESC differentiation stages. Transplantation of pancreatic progenitor cells results in the development of not only the endocrine cell types, the full complement of which are probably required for fully functional islet structures, but also the exocrine pancreas, i.e. acinar and ductal cells, albeit at much lower frequency [89]. The presence of these cells that have the ability to produce and release various enzymatically active proteins is worrisome. In addition, under conditions of stress caused by inflammation and injury, acinar cells can develop into cells with progenitor-like activity, able to result in neoplastic lesions as a result of oncogenic mutations. While the possibility of such events is small, detailed investigation into these issues needs to continue to assure that the function of ESC-derived endocrine cells are not compromised by the cancer-related risks associated with exocrine cell populations.

Another issue that needs to be explored is the immune response of the host following transplantation of an allogeneic ESC-derived cellular graft. In the last decade or so sophisticated immunosuppressive regiments have been developed to protect allogeneic islet grafts obtained from deceased donors [46-50] long term, following transplantation. In case of ESC-related therapies, not only the graft must be protected from the immune insult by the recipient's immune system, but the cells with tumorigenic capacity need to be isolated and sequestered. This can be, most probably, achieved with the use of sophisticated immunoisolation / encapsulation devices that have become available in the last few years [4].

Finally, what needs to be ascertained is the fact that stem-cell derived β-cells have the same ability to synthesize, store and release insulin in a highly regulated fashion similar to that of

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native pancreatic islets. Extensive efforts should be undertaken to understand whether the same regulatory mechanisms are in place in stem cell-derived insulin producing cells to control prolonged and uncontrolled insulin release which would result in sever hypoglycemia. Only when it is clearly and unequivocally ascertained that stem cell-derived insulin producing cells are true equivalents of endogenous pancreatic β-cells, can clinical application of such therapies become a reality.

1.4. Immunotherapy for the prevention and treatment of Type 1 diabetes

As Type 1 diabetes is an autoimmune disease characterized by the selective and progressive destruction of insulin-producing β-cells via the cumulative attack by autoantigen-specific CD4+ and CD8+ T-cells, autoantibodies, and functionally defective bone marrow derived antigen-specific cells, development of various immunotherapeutic options has been the major focus for prevention and treatment of IDDM. Multiple studies in the NOD mouse model demonstrate that islets are attacked in step-wise manner, with benign insulitis being the starting point of this assault. With time and not fully defined qualitative changes, overt diabetes characterized by the efficient destruction of β-cells ensues. It is generally acknowledged that diabetes in animal models and men is strongly associated with the changes in more than 20 genetic loci - with genes encoding MHC class II molecules playing the major role, most probably influenced by a number of environmental factors, although it's been quite challenging to identify either in detail [11].

Animal studies indicate that a number of pathogenic events contribute to the progressive loss of T-cell tolerance to β-cell proteins, and, therefore, expansion of β-cell specific pathogenic CD4+ and CD8+ T-cells. These events seem to take place during the early stages of the pre-clinical IDDM. These include defective negative selection in the thymus, inefficient peripheral tolerance characterized by low frequencies of IL-4, IL-10 and TGF-β secreting CD4+ T helper 2 (Th2) cells, as well as diminished numbers of "natural" immunoregulatory FoxP3 expressing CD4+CD25+ Regulatory T (Treg) cells and invariant natural killer T (iNKT) cells. These events, coupled with reduced frequency / function of immunoregulatory effector cells within the islets, reduced sensitivity of T cells to immunoregulation, and increased levels of pro-inflammatory cytokines produced by macrophages and dendritic cells (DCs), result in the severe loss in the balance between pathogenic effector and immunoregulatory T cells, especially during the later stages of the disease [99-101]. Effective prevention / treatment strategies for Type 1 diabetes must focus on the restoration of this balance..

The progression course of diabetes offers obvious time points for interventional immunotherapeutic strategies. The first opportunity is presented during the pre-clinical stages of diabetes, when the goal is to suppress the on-going β-cell autoimmune process and prevent the development of overt diabetes. Undiagnosed at risk individuals - family members of patients with the previously diagnosed diabetes - can be monitored by screening for autoantibodies specific for several autoantigens found in the serum. These include insulin, glutamic acid decarboxylase 65 (GAD65), and insulinoma–associated tyrosine phosphate (IA-2) [102]. The second time point for intervention is at clinical onset, in an attempt to preserve 10-15% of the β-cell mass that is usually still present at the time of diagnosis.

There is a definite therapeutic potential through the rescue of the residual β-cells, and there are reports that halting autoimmunity at this stage can potentially lead to β-cell regeneration and/or replication and, in ideal circumstances, remission of diabetes [103,104].

Further down the line, when all β-cells are lost, the likelihood of remission through immunoregulation becomes slim, but recent observation in clinical trials performed in patients with undetectable C-peptide suggest that restoration of β-cell function is not impossible in these circumstances. A recent clinical trial conducted in China demonstrated that following treatment with autologous lymphocytes and allogeneic cord blood-derived stem cells, patients with and without residual β-cell function demonstrated improved C-peptide levels, reduced median Glycated hemoglobin A_1C (HbA_1C) values, and decreased daily insulin requirements [105].

The development of specific immunotherapeutic strategies that effectively target pathogenic effector cell populations, promote β-cell tolerance, while maintaining a "normal" immune function, i.e. balance between pathogenic effector and immunoregulatory T-cells, is the ultimate goal. This means that different immunotherapeutic strategies, alone or in combination, must be considered to effectively suppress β-cell autoimmunity at different stages of the disease progression.

There is sufficient information that deals with various immunotherapeutic strategies to prevent / treat Type 1 diabetes, for which both clinical and experimental findings are available. Two major approaches have received most attention, although others have been discussed, namely, antigen- and antibody-based immunotherapies.

1.4.1. Antigen-based immunotherapy

Antigen-based immunotherapy has to do with selectively targeting disease-specific T cells to maintain the normal function of the immune system. β-cell antigen-specific vaccination has proved to be an effective strategy for the induction of the immunoregulatory T cells and suppression of autoimmune pre-clinical diabetes in rodent models and NOD mice. Vaccination of 12-week old NOD mice with GAD65 protein resulted in the inhibition of the progression of insulitis and long-term protection mediated by the GAD65-specific CD4+ T cells [106]. Successful application of antigen-based immunotherapies in the clinical setting has yet to be reported, although some evidence does exist of the successful application of this methodology. The Diabetes Prevention Trial-1 (DPT-1), during which participating pre-diabetic subjects received insulin either orally or parentally demonstrated no significant effect on the development of diabetes or β-cell autoimmunity [107]. Although the reason for why the treatment failed to prevent diabetes in the majority of subjects was never clearly identified, it was thought that insufficient dose of insulin administered to trial participants was the main culprit. One interesting observation had to do with the fact that some effect was observed in subjects receiving oral insulin that presented with high titers of insulin autoantibodies. It is, therefore, entirely possible that success or failure, as well as efficacy, of a given antigen-based immunotherapy is related to the severity of the existing autoimmunity.

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It's been demonstrated that treatment with antigen-based therapy can have a dual effect on autoreactive T-cells: induction of T-cell deletion and the induction of immunoregulatory T- cell population [108,109]. The number of immunoregulatory β-cell specific T-cells induced as result of treatment is critical. As diabetes progresses and the pro-inflammatory milieu is established, a relatively high number of immunoregulatory T-cells would be required to effectively suppress β-cell autoimmunity and to restore the balance between pathogenic effector and immunoregulatory T-cell subsets. The number of inducible immunoregulatory effector cells is, at least in part, dependent on the size of the pool of naive precursors for a given β-cell autoantigen [101,106]. As the pool of β-cell specific T-cell precursors actively involved in the autoimmune process is limited, minimizing the pool of immunoregulatory T-cells that can be induced, it is of critical importance to choose the autoantigen utilized for treatment at late stages of the disease wisely. While in experimental models it's been demonstrated that administration of a combination of β-cell autoantigens suppresses β-cell autoimmunity during the late stages of the disease, the same has not been clearly defined in patients [101]. Although some progress has been made towards the development of methods that can be used to detect β-cell specific T-cells, more development is necessary before this approach can become a standard immunotherapeutic approach. In addition, as demonstrated by the DPT-1 [107], the efficacy of a given treatment, i.e. β-cell autoantigen, may vary significantly between individuals, probably based on the extent of autoimmunity, i.e. β-cell specific T-cell precursors. This means that, similarly to the NOD model, immunization with the cocktail of various β-cell specific peptides would be necessary to achieve a measurable degree of success in abating the progress of the autoimmune process taking place during the advanced stages of Type 1 Diabetes.

The number of immunoregulatory β-cell specific T-cells induced as result of treatment also depends on the efficiency of the process involved in the induction of immunoregulatory T-cell population. What complicates matters is the fact that this induction must take place *in vivo*, under the same conditions that favor the expansion of autoreactive β-cell specific T-cell subsets. Hence, strategies that preferentially promote the expansion of immunoregulatory T-cell populations are necessary. Properties of mucosal tissues [110,111], co-administration of various types of adjuvants and cytokines, as well as manipulation of the way the autoantigen is presented have been investigated in both experimental and clinical settings [107], with some degree of reported success. In addition, variety of inducible immunoregulatory T-cell populations has been reported to be of importance as induction of different types of immunoregulatory cells, each with a distinct mode of action, would be expected to increase the overall efficacy of a given immunotherapy [107].

1.4.2. Antibody-based immunotherapy

Various monoclonal antibodies have been utilized to target a wide range of immune components actively involved in the progressive autoimmune process. Most of these focus on directly or indirectly targeting the T-cell compartment [101], but also include soluble mediators such as cytokines and chemokines, and antigen presenting cells (APC). Several recent reports suggest that B-cells may also represent a useful target to alter the progression of β-cell autoimmune process.

There is an abundance of literature that discusses the efficacy of monoclonal antibodies targeting T-cells, in a number of experimental models. Following the administration of a short course of depleting CD4 antibody or anti-lymphocyte serum in NOD mice, suppression of β-cell autoimmunity and, in some cases, remission of the recent onset of diabetes is achieved [112,113]. There is, however, a drawback to this approach. Depleting antibody immunotherapy resulted in the indiscriminate depletion of not only the pathogenic, but also non-autoimmune T-cell populations, and induced long-term state of immunosuppression. In addition, after the depleting antibody was cleared from the system the number of T-cells that reappeared was significantly reduced, compared to normal levels. At this same time, the use of non-depleting anti-CD4 and CD8 antibodies resulted in tolerance induction in the antigen-specific manner, with the T-cell numbers intact [114], induction of apoptosis in activated T-cells, and activation of the CD4+CD25+FoxP3+ cell population demonstrated to have a suppressive effect on the differentiation of pathogenic effector T-cells [114].

Studies investigating the efficacy of anti-CD3 monoclonal therapy for the treatment of Type 1 diabetes have been generating a lot of interest ever since they've been first reported [104], Chatenoud demonstrated that a short course treatment of NOD mice with low dose anti-CD3 antibody resulted in long-term remission of recent onset diabetes and β-cell specific tolerance [104]. The mode of action of this therapy proved to be multi-faceted. A critical observation was of the anti-CD3 antibody preferentially affecting activated rather than naïve T-cells by down-regulating the T-cell receptor and reducing TCR signaling, enhancing apoptosis, and altering T-cell trafficking [115]. This treatment was also demonstrated to promote the expansion of the immunoregulatory T-cells with CD4+CD25+ phenotype. Utilization of a non-mitogenic anti-CD3 antibody in a clinical setting, during the first 6 weeks following diagnosis, resulted in the preservation of C-peptide response over a 2-year period in certain patients relative to untreated controls. The fact that residual β-cell function was reported in some patients at the time of treatment speaks to the importance of therapeutic administration at "earlier" stages in the disease progression [116]. Although efficacy with this treatment was observed, the protection offered by the anti-CD3 antibody treatment was nevertheless transient. This suggests that this type of therapy needs to be refined either in terms of schedule or route of the administration, or the therapeutic dose, before it can be applied to a larger patient population.

Studies with monoclonal-based therapies targeting co-stimulatory pathway of immune activation such as CD40-CD40L, and APCs such as DC's and B-cells have also been reported. Blocking the CD40-CD40L pathway proved highly effective in abrogating T-cell responses in autoimmune and transplantation models [117]. However, before this approach could be investigated further the anti-CD40L antibody was withdrawn from use in various clinical trials due to serious adverse events that came into view as a result of treatment.

Targeting B-cells, whose primary role in Type 1 diabetes is that of APCs to T-cells, was never high on the list of targets for potential immunotherapy. The reason behind this is simple: islet-specific autoantibodies have never been considered the primary culprits of β-cell destruction. However, this pathway may prove to be the indirect approach to targeting β -cell autoreactivity [118]. Despite an initial skepticism, some work has been done in this area. Recent studies performed in an experimental setting reported that depleting B-cells with a short course of

monoclonal anti-CD20 antibody proved beneficial in abrogating diabetes in young NOD mice, and significantly delaying the onset of the disease in older animals [119]. A recent clinical trial conducted by the TrialNet group confirmed these findings by demonstrating that selective and transient depletion of B-lymphocytes with rituximab, an anti-CD20 monoclonal antibody, partially preserved β-cell function in patients with recent onset of Type 1 diabetes, for a period of 1 year [120].

1.4.3. Regulatory R (Treg) cells

Although this cell subset with unique immunomodulatory properties has been briefly discussed above, these cells deserve special attention and are discussed in more detail in this section. Ever since the realization that Treg cells have an innate capacity to maintain tolerance to self-antigens in peripheral organs under immune assault, this population has attracted great attention with respect to their potential role in the prevention of autoimmune disorders which include Type 1 diabetes. The interest in these immune traffic regulators peaked when it was demonstrated that they represented an inducible population able to halt the progression of IDDM, while curbing autoimmune responses not only to antigens responsible for the induction of autoimmunity but others involved in this process as well. This represents an attractive therapeutic alternative for IDDM as to date no specific antigen(s) has been identified as a causative agent for the diabetogenic response.

As discussed elsewhere in this chapter, autoimmune response aimed at the progressive destruction of pancreatic β-cells can be manipulated through antigen-based manipulation and non-antigen-based treatments, possible though the involvement of Treg cell population. Although immunoregulatory capacity has been demonstrated in several T-cell subsets, the main players in the field are "natural" CD4+CD25+, and "adaptive or induced" regulatory T-cells of various phenotypes. "Natural" CD4+CD25+ regulatory T-cells require a variety of co-stimulatory interactions for their development, and are mainly identified by the FoxP3 transcription factor necessary for the development and function of this cell subset. *In vitro*, natural CD4+CD25+ cells have been demonstrated to have an uncanny ability to inhibit T-cell proliferation and cytokine production, most probably, via cell-cell contact [121]. Despite previously published dissenting reports, there is an agreement that during the development of diabetes, the autoreactive T-cell subsets become unresponsive to CD4+CD25+ mediated suppression mechanism. This could be due to the fact that CD4+CD25+ cell are present in reduced numbers during the development of the IDDM in humans. At the same time, opposing results have been obtained in an NOD model: at the time of diabetes onset, CD4+CD25+ cells exist in equal numbers compared to non-diabetic animals [121]. It's been also demonstrated that while CD4+CD25+ cells are relatively abundant in normal individuals, data obtained from various animal models suggest that antigen-induced Treg cells are present in relatively low numbers. Despite this fact, of most benefit is the data that demonstrated that once induced, Treg cells become activated in the immediate tissue where the given autoantigen is expressed. Of added benefit is the realization that in addition to suppressing the responses of an autoantigen in question, Treg cells are able to modulate other autoreactive T-cell responses as well, most probably via production of anti-inflammatory soluble cytokines such as IL-4, IL-10 and

TGF-β. Pre-clinical studies in non-obese diabetic mice have demonstrated that adoptive transfer of Tregs can slow diabetes progression and, in some cases, reverse new onset diabetes. Clinical trials investigating the effect of natural expanded and patient-specific Treg cells on autoreactive T-cell responses, preservation of β-cell function and other outcomes related to diabetes management are in progress at the present time [122].

The effect of antigen-based immunotherapy has been discussed earlier in this chapter. However, to recapitulate, the available data demonstrates that antigen-based immunotherapeutics probably favor the induction of immunoregulatory T-cell subsets by reacting with endogenous reactive autoantigens, and halting the progression of diabetes. In animal models of IDDM, amplification of Treg cell responses has been achieved using several self-antigens administered using tolerogenic means such intravenous, intranasal or subcutaneous injection, or oral feeding. It has also been shown that Treg cells are able to exert their modulatory effector function through the action of several cytokines, namely IL-4 and IL-10. The situation with TGF-β is much more complex. When administered as a vaccine, it was shown to confer protection from diabetes in NOD mice, but not in other animal models [123].

"Adaptive or induced" Treg cells comprise a group of heterogeneous T-cell subsets that arise as a function of a specific context in which they are generated [120]. These normally go along with antibody-specific approaches to treating IDDM. For example, treatment with CD3 antibody, a potent treatment option for autoimmune disease, has been associated with a marked increase in Treg cell populations, although the mode of action was never elucidated [101,121]. The results of the administration of non-mitogenic anti-CD3 therapy proved to be encouraging [116]. Recent-onset IDDM patients treated with FcR-nonbinding humanized anti-CD3 monoclonal antibody were found to maintain their insulin production for ~2 years following treatment. Although the mechanism of action is well understood, it was thought that the treatment had a direct effect on pathogenic T-cells and resulted in the induction of Treg cell population, or both [116,121]. Data from several other clinical trials seems to indicate that anti-CD3 monotherapy could neither elicit long-term protection, nor protect from adverse effects. Hence, it is possible that combination of immunotherapeutic options might offer a better sustained protection against the disease over time.

1.5. Bone Marrow Chimerism

It was Owen, back in 1945, who made an observation that bone marrow cells have the ability to induce transplantation tolerance to donor histocompatibility antigens. Billingham, Brent and Medawar confirmed and expanded on this idea by transplanting Major Histocompatibility Complex (MHC)-disparate bone marrow cells (BMC) into neonatal recipient mice which resulted in the induction of specific, systemic, stable tolerance to the donor, while preserving immunocompetence required to reject genetically disparate third party grafts [124]. Fetuses and neonates, of course, offer an immunoprivileged state, during which pre-conditioning is not required for the successful BMC engraftment that leads to chimerism. The situation changes after that. Over the last several decades, numerous investigators working in the area of bone marrow (BM) conditioning to reduce the immunogenicity of solid and cellular grafts,

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demonstrated that adult recipient pre-conditioning is necessary to "make space" for the successful engraftment of donor BMC and induction of donor-specific chimerism.

It was initially thought that lethal recipient conditioning which leads to complete BM ablation was necessary for engraftment of allogeneic BMC. Over time, however, it has become clear that stable engraftment can be achieved using partial pre-conditioning strategies [125,126]. Conditioning approaches to allow for stable engraftment of donor cells have included total body irradiation, total lymphoid irradiation, cytoreductive approaches, low dose irradiation with polyclonal or monoclonal antibodies, single or multiple infusions of large doses of donor BMC with T-cell co-stimulatory blockade, anti-CD4 and anti-CD8 antibodies with local thymic irradiation, and targeted BM ablation using bone seeking ^{153}Samarium-Lexidronam (^{153}Sm) compound with transient T-cell co-stimulatory blockade [125-127]. The fact that hematopoietic chimerism induces donor-specific tolerance, while preserving third-party reactivity, has been established in experimental animal models, i.e. rodents [127,128], large animals [129], primates [130] and in humans [131]. Using conditioning approaches listed above full or mixed chimerism leading to stable, long-term donor-specific tolerance has been achieved. Although both full and mixed chimerism can be achieved in animal models, fully chimeric animals demonstrate immune-incompetence for antiviral activity and antibody production [125,126]. Mixed allogeneic chimerism is much more preferable in tolerance induction protocols, as both donor and recipient antigen presenting cells can be found in the recipient [125].

The realization that BM transplantation represents a credible treatment for diabetes came as a result of animal studies that demonstrated the interdependence between BMC transplantation and autoimmune disease: the disease could be transferred from NOD mice to mouse strains resistant to autoimmunity, while BM from disease-resistant mouse strains could prevent the development of autoimmunity in NOD mice [125,126]. BMC-associated tolerance to islet cell grafts has been achieved in a number of animal models and human subjects [125,126,132]. Donor-specific tolerance has been demonstrated in both animals that were first preconditioned, treated with donor-specific BMC, with the islet graft placed at a later date, and those that received islet grafts 24-48 hours after BMC infusion [125,126]. Over the last several decades a profound contribution has been made to the understanding of underlying processes in the induction of BM-derived tolerance to pancreatic islet grafts in the later stages of diabetes, prevention of recurrence of autoimmunity in the graft, and reversal of overt diabetes once the pre-diabetic state is identified [125,126].

Animal Type 1 diabetes models fall into two groups, which deal with etiology of the disease. Diabetes can be induced chemically or surgically, or developed spontaneously as in BB rat or NOD mouse model. In the first case autoimmunity is not an underlying factor of the disease. In the second case, however, the disease progresses spontaneously, similarly to the clinical course of Type 1 diabetes, which is autoimmune in nature. BMC transfer experiments between the NOD mouse and disease-resistant mouse strains discussed earlier have suggested that it is a BMC-derived stem cell that is associated with the development of the autoimmunity observed in Type 1 diabetes. Both unmodified and T-cell depleted NOD-derived BMC can transfer autoimmunity followed by diabetes development [125,126]. Conversely, BMC from diabetes-resistant mouse strains, when transferred to a lethally, or sub-lethally conditioned

NOD mice and rendering these recipients mixed chimeras, reverses insulitis and the autoimmune process, halting the development of overt diabetes. Ildstad proposed two possible explanations for how allogeneic BMC-derived chimerism can prevent diabetes. First, donor BMC activates a regulatory cell instrumental in suppressing the activation of autoreactive lymphocytes identified as a culprit in the progression of autoimmunity, development of the overt diabetes and fully developed disease. Second, BMC can cause clonal deletion of autoreactive T lymphocytes via donor-specific disease resistant APCs [126].

Taking into an account that it is a BM stem cell that's involved in the development of autoimmune disease, the timing of BMC administration for the treatment of autoimmune diabetes is critical. Due to the fact that autoimmunity results in the progressive destruction of pancreatic β-cells, the ultimate timing for BMC infusion is during the early stages of the disease, when overt diabetes ensues, exogenous insulin is administered, and return to normoglycemia and even production of endogenous insulin are observed. However, the main drawback for the widespread use of BMC therapy to treat Type 1 diabetes is harsh, often lethal, recipient preconditioning regiments. Although non-lethal conditioning protocols, discussed earlier, have been developed, donor-specific chimerism reported under such circumstances is often transient [126]. However, encouraging results in terms of the induction of stable chimerism in kidney transplant recipients have been recently reported by Leventhal et al [132]. He used mobilized cells enriched for hematopoietic stem cells (HSC) in combination with a graft-facilitating cell (FC) population ($CD8^{dim}$, $CD3^+/CD45R^+/Thy1^+/Class\ II^{dim/intermediate}$, $\alpha\beta\text{-}TCR^-$ and $\delta\lambda\text{-}TCR^-$) and nonmyeloablative conditioning in recipients of MHC mismatched, unrelated kidney grafts. Five out of eight transplant recipients exhibited stable donor-specific chimerism, and were weaned of immunosuppression at 1 year following transplant. None of the transplant recipients were reported to show signs of GVHD. As previously reported by Ildstad [125], the FC is not a stem cell, but this population seems to be necessary to enable successful BMC engraftment in MHC disparate environment. Although the mechanism by which FC aids engraftment is not clear, it was characterized previously and found to be necessary to prevent GVHD and promote engraftment in standard BMC transplant protocols [125,126]. These results are exciting and offer much optimism towards treatment strategies applicable to patients with Type 1 diabetes.

Type 1 diabetes is a multifaceted disease, for which no single arm immunotherapeutic approach is possible. It has been long established that immunotherapies that target early vs. later pre-clinical stages in the disease progression offer a treatment approach with higher likelihood of success. However, even that might not be enough to effectively solve this formidable problem. It is possible that no single immunotherapeutic approach will offer long-term protection from diabetes onset and progressive autoimmune destruction of β-cells, in either prevention or treatment setting. A number of immunological approaches, in combinatorial manner, that exploit the strengths and circumvent the adverse events of potential therapies at the same time, might prove to be the answer. At this point such approaches are still in the development stage, albeit many hurdles have been overcome to move this approach forward. Latest developments in this area do offer much optimism.

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1.6. Concluding remarks

Cell replacement strategies offer an enormous potential for the treatment of patients with Type 1 diabetes, in both clinical and economic terms. The availability of unlimited amounts of functionally competent graft material to treat millions of patients suffering from IDDM and its dreadful, debilitating complications can move this field forward from the experimental stage it has found itself in for the last several decades to the forefront of transplantation medicine. The fact that allogeneic islet transplantation offers the most extensively studied and sensible solution to potential cure for IDDM is clear. However, this therapeutic option is far from a perfect solution, and comes hand-in-hand with several problems in the form of serious shortages of the available organs and resulting tissue to satisfy the ever-growing demand, recurrence of autoimmunity and rejection and life-long immunosuppression. Porcine islets offer a viable substitution or addition to the allogeneic islet therapy, offering both a functionally competent adult cell source with already developed insulin-sensing machinery, and sufficient quantities of tissue immediately available for transplant. However, before persistent problems with immune rejection and destruction of the graft can be overcome, porcine islets do not have a hope of replacing or supplementing allogeneic islet cells as a viable treatment option. Embryonic stem cells have the required proliferative potential, with recent studies clearly demonstrating that ESCs provide a definitive platform for differentiation into insulin producing structures. However, it remains to be seen whether (a) current experimental protocols can be scaled-up to generate sufficient number of cells for transplant; (b) current purification methods offer sufficiently stringent protocols to be able to transplant glucose-sensing β-like cells only, all the while unequivocally excluding potentially oncogenic "other types" of cell populations; (c) functional equivalency of the resulting glucose-sensing β-like cells to native β-cells can be clearly confirmed; and (d) the cell graft can be adequately protected to avoid efficient immune surveillance systems of the host. This is where the concept of generating sufficient insulin-producing tissue from an autologous, i.e. patient-specific, source becomes attractive. However, the early promise of this iPSCs has not translated from it early success in the experimental setting to the clinical model, mostly due to the same problems that are associated with ESCs. These, however, are multiplied by the limited proliferative capacity of these cells, as well as issues with inadequate function, i.e. poor insulin expression coupled with very low insulin secretion. The problems that stem from immunogenicity of the graft tissue are intrinsic to cells from various sources, including a tailored patient-specific iPSCs-derived approach. With the number of factors impacting the way β-cell autoimmunity can be manipulated, several key issues might be considered when it comes to the development of immunotherapeutic solutions to diabetes. These include the requirement for the suppression of the diabetogenic response early in the course of the development of the disease, as well as clear understanding of the autoreactive antigen(s) that might be defined by particular genotype and/or environmental exposure. The complexity of IDDM means that immunomodulatory therapies, antigen- and antibody-specific, might offer a solution when utilized in combination. The goal here is to preserve the functional capacity of the cellular graft or innate islet cells, while at the same time attempting to restore the unique balance between the pathogenic effector and the immunomodulatory T-cell population eroded by the autoimmune assault brought forth by the onset of the disease. Combination immunotherapy will likely

prove the most effective by exploring the strength of each approach, while limiting the adverse effects associated with each. Despite significant success attained in this area, most progress so far has been made in experimental models, while clinical applications are still relatively early in their development. Although the challenge of bench-to-bedside technology transfer is significant, success of the last few years give much hope and even optimism for future clinical developments.

Various types of cellular therapies discussed in this chapter might offer multi-faceted and practical approaches to the treatment of diabetes. It is entirely possible that a choice of several different therapeutic options is of great benefit, and might provide a platform to avoid frustrating developmental pains towards a "universal cure". While the prospect of developing patient-specific, i.e. personalized, cellular therapy is appealing, it is complicated, quite expensive and, it's tempting to say, unrealistic to develop. Each of the allogeneic cell replacement approaches towards a potential therapeutic option discussed here needs to be carefully studied, dissected, and defined regardless of the costs associated with it. Further development in the area of immunotherapeutic approaches and various immunoisolation methodologies, which are beyond the scope of this chapter, will be able to help move cell replacement therapy to the forefront of transplant science. Given the fact that for almost a century administration of exogenous insulin was the only real available therapeutic alternative to the treatment of IDDM, the developments of the past several decades are exiting. It is quite possible that the following decade will see clinical application of a whole gamut of therapeutic options to treat this devastating disease.

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