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Chapter 2

Immunotherapy in Autoimmune Diabetes

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66909

Abstract

Autoimmune diabetes is a chronic autoimmune disease caused by the loss or selective destruction of the insulin-producing cells, called pancreatic beta cells. Damage to beta cells results in an absence or insufficient production of insulin produced by the body. Most cases of autoimmune diabetes have an autoimmune basis, and the immune system mistakenly attacks and destroys beta cells. The immune system plays a critical role in controlling the development of autoimmune diabetes. Over the past years there have been significant progress and an accumulation of scientific evidence for the concept of immunotherapy. Immunotherapy for the prevention and treatment of autoimmune diabetes has become the main focus of the research community. Three regimens of immunotherapy have been investigated: (1) Antigen-specific vaccines: Insulin-related molecules have attracted great interest in vaccine development, including the whole recombinant human GAD65 (rhGAD65) and the DiaPep277 peptide of HSP60. (2) Systemic immunomodulators: A large number of non–antigen-specific immunomodulators have been studied, including monoclonal anti-CD3 antibody, anti–CTLA-4 Ig, TNF-a, IFN-a, IL-1R antagonist, regulatory T cells, and dendritic cells. (3) Combination treatments: Combination therapies have the ability to enhance efficacy and will become the standard of care for autoimmune diabetes. Development of safe and efficient prevention of autoimmune diabetes is a general public health object in modern countries now. Although large numbers of preventive modalities including immunotherapy have been accomplished in animal models of autoimmune diabetes, prevention of human autoimmune diabetes remains indefinable. Genetic and environmental factors that control the relapsing-remitting course of β-cell destruction, terminating in complete insulin addiction are being determined. In the long run, initial prevention of islet autoimmunity will likely be the optimal approach to the prevention of autoimmune diabetes. However, environmental causes of islet autoimmunity need to be well stated. Modest predictive assessment of the existing genetic screening tools also means that the number of children requiring intervention will stay great, concerning the number of autoimmune diabetes cases prohibited. Nevertheless, combination treatments are more likely to be used for autoimmune

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diabetes. Primary systemic immunosuppression followed by antigen-specific induction of tolerance or islet regeneration is a sound approach.

Keywords: autoimmune diabetes, immunotherapy, immune cells, tolerance

1. Introduction

Autoimmune diseases arise due to loss of self-tolerance caused by tissue injury by T cells or antibody reactivity to self. There are several causes of autoimmunity that are not fully understood. One of the major causes of autoimmune disease is the activation of self-reactive T and B lymphocytes. During T and B cell development, Self-reactive T and B cells should be eliminated by antigen ligation of T cell receptor or B cell receptor. This is known as the mechanism of self-tolerance. To maintain the self-tolerance and eliminate the autoreactive cell, T cells and B cells undergo a selection process in primary lymphoid organs, the thymus and the bone marrow, respectively [1–3]. After somatic mutation of immunoglobulin genes, B cells need to go through a second process of selection failing which somatic mutation generates auto reactivity. This is called central tolerance. If somehow, central tolerance is not maintained, autoimmunity develops. Several autoimmune diseases have been reported until now. Among them, type-1 diabetes (T1D) is one of the major autoimmune diseases that develop due to the selective autoimmune destruction of pancreatic beta cells that leads to the insulin insufficiency. There is no definite treatment for T1D except life-long insulin therapy. Hence, the generation of insulin secreting beta cells and transplanting it to the diabetic patients is an unmet need.

Pluripotent stem cells (PSCs) have the ability to grow indefinitely while maintaining pluripotency. Under the right circumstances, mouse and human stem cells have the potential ability to differentiate into disease-relevant cells [4]. The generation of exogenous beta cells and its transplantation to replace dead or dysfunctional endogenous beta cell is a potential strategy for controlling blood glucose level in diabetic patients. Stem cell-derived beta cells have already been generated previously, and it was successfully able to control the blood glucose in clinical settings [5]. As autoimmune disease is a continuous process, it is possible to develop diabetes again by destructing the pancreatic islets by pathogenic T cells. As a result, this will not be a permanent solution for the control of blood glucose level.

It is already well established that regulatory T cells (Tregs), one of the subtype of T cells, are able to suppress the hyper activity of other T cells including beta cell-destructing pathogenic T cells. But the number of Tregs is relatively limited in mice and human being. The generation of Tregs *in vitro* and adoptively transfer them into the diabetic mice will be a great strategy for the treatment of diabetes that will reduce the hazards of complicated surgery events throughout the life. We already showed that retroviral transduction of genes with T cell receptor (TCR) and the transcriptional factor (FoxP3) into PSCs following coculture with stromal OP9-DL1/DL4 cells differentiate them into antigen (Ag)-specific Tregs. Our *in vitro* generated Tregs were able to suppress the autoimmune arthritis in a well-established mouse model of Ag-induced arthritis (AIA) [6, 7]. In this chapter, we will discuss how Ag-specific

Tregs can be generated from PSCs and how they are able to reduce blood glucose level in a mouse model of diabetes.

1.1. Epidemiology: incidence and prevalence

T1D is one of the most common chronic diseases in children. Children under 18 years of age are mostly affected. In 2012, 29.1 million Americans, or 9.3% of the population, had diabetes. More than 150,000 children in the United States have T1D; 1.4 million Americans are diagnosed with diabetes each year [8]. In children, T1D develops between 5 and 7 years of age and at puberty. The incidence for the development of T1D also varies with seasonal changes and geography. It seems that autumn and winter are the seasons for higher incidence of diabetes as compared to summer. The incidence and prevalence dramatically vary around the world, where some countries have 400-fold higher incidence rate compared to the others. The incidence rates of diabetes in China, India, and Venezuela are 0.1 per 100,000 and are far more common in Finland. In Finland, the incidence is approaching 50 cases per 100,000 individuals per year. Wide variations have been observed between neighboring areas in Europe and North America. Estonia is very close to Finland but the incidence of diabetes is less than one-third as that of Finland. Puerto Rico has an incidence similar to that of the mainland United States, whereas neighboring Cuba has an incidence of less than 3 cases per 100,000 [9].

The incidence for the development of T1D is increasing throughout the world. These changes are markedly observed in young children from countries with historically high incidence rates. Sweden and Norway have reported 3.3% annual increase in T1D rates, and Finland has observed a 2.4% annual rise in incidence. Hence, the increase in T1D incidence is not correlated with socioeconomic condition. Most of the autoimmune diseases disproportionally affect women but T1D seems to affect men and women equally. Therefore, T1D is different in disease prevalence and incidence that suggests that it is a combination of multiple genetic and environmental factors.

1.2. Etiology

T1D is the result of an autoimmune reaction to the proteins of pancreatic islets. There is a strong association between T1D and other autoimmune diseases such as Addison's disease. It is also notable that the incidence of autoimmune diseases is increased in family members of T1D patients. T1D develops due to the destruction of pancreatic beta cells by autoreactive T cells. Other types of diabetes may also develop due to a combination of reduced insulin sensitivity and impaired beta cells function [10]. Diabetes can be inherited or caused by mutations in an autosomal dominant gene resulting in the disruption of insulin production. There are three types of autoantibodies that are involved in the development of T1D:

1. Islet cell cytoplasmic antibodies: the presence of islet cell cytoplasmic antibodies indicates the future development of diabetes. Note that 90% of T1D antibodies are against islet cell cytoplasmic protein.
2. Islet cell surface antibodies: there are some other antibodies that are directed toward the islet cell surface Ags. Autoantibody against islet cell surface Ag is also detected in almost 80% of the cases. These antibodies are also positive in type 2 diabetes.

3. Specific antigenic target of islet cell: 80% newly diagnosed patients represent with autoantibody to glutamic acid decarboxylase (GAD). Presence of this antibody is also a strong predictor for future development of T1D. Anti-GAD antibody declines over time in T1D. In some cases, anti-insulin antibodies are also detected in T1D patients and in relatives [11].

In some cases, some viruses like B4 strain of the coxsackie B virus, German measles, Mumps, and Rota viruses are also responsible for the development of diabetes. When a virus invades the body, T cells start to produce antibodies against that virus. If some viruses have the same Ag as the beta cells, T cells can actually turn against the beta cell and start destroying it.

There is a strong genetic correlation for the development of diabetes though it is not an inheritance. It is considered as a complex and multifactorial disease. In the United States, individuals who have first-degree relative with T1D have 5% risk for the development of diabetes. But in general population this percentage is very low. Monozygotic twins have a high risk whereas dizygotic twins have a lower risk. There are a significant percentage of people developing diabetes without any family history. Differences in risk are also developed in the parents of children. Children who have their mother suffering from T1D have 2% risk of developing T1D, but children whose fathers have diabetes have a greater risk [12]. No single gene is predicted to develop diabetes, but more than two dozen susceptibility loci have been associated with susceptibility to T1D.

2. Pathophysiologic mechanism of T1D

The main factor for the development of autoimmune diabetes is loss of immunologic tolerance to β cells. β cells are selectively destructed by autoimmune reaction. Due to loss of immunologic tolerance, autoreactive $CD4^+$ and $CD8^+$ T cells as well as macrophages are infiltrated into the pancreatic islet and develop insulitis. During the disease process, several autoantigen that are targeted by autoantibodies are detected into the islet. The main autoantigen that may be found in the islets are insulin, glutamic acid decarboxylase (GAD65), islet Ag-2 (IA-2), and zinc transporter (ZnT8). These autoantibodies are predominantly associated with the development of insulitis [13]. Destruction of β cells is not dependent only on autoantigen, it is also related to the presence of high-risk human leukocyte antigen (HLA) haplotypes like DR3-DQ2, DR4-DQ8, or both [14]. HLA-class II molecules are mainly expressed by Ag-presenting cells (APC) like dendritic cells (DCs) and macrophage. In some cases, they are expressed by activated $CD4^+$ and B cells, even on activated endothelial cells. The presence of high-risk HLA molecules on APC may activate $CD8^+$ T cells through $CD4^+$ T lymphocytes. Then $CD8^+$ T cells become hyperactivated and initiate the destruction of β cells. This phenomenon is implicated in T1D siblings who share the high-risk HLA DR3-DQ2/DR4-DQ8 genotype [15].

Some other pathophysiological mechanisms have been documented. But the two most common mechanisms for developing T1D are:

1. Gradual β-cell destruction associated with one or multiple islet cell autoantibodies.

2. Development of glucose intolerance and hyperglycemia due to loss of β-cell secretory function.

The autoimmune process starts with the infiltration of mononuclear cells including autoreactive $CD8^+$ T cells into the pancreas that leads to the destruction of β cell. In the disease process, both the cellular and humoral pathways of immunity are involved. However, the role of B lymphocytes is not evident in human, only in the laboratory animal such as nonobese diabetic (NOD) mice [5]. In NOD mice, B cells infiltrate in the islets of young mice and play a role in the initiation of β-cell destruction by the autoimmune response. In some other autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis B-cell-targeted therapy has been used successfully.

There are several features that characterize the T1D as an autoimmune disease. These are because of the presence of mononuclear and immunocompetent cells in the infiltrated pancreatic islets; association with the class II major histocompatibility complex; islets cell-specific auto-antibodies; increase in the number of $CD8^+$ T cells in the pancreas and reduction in the number of $CD4^+$ T cell; and response to immunotherapy and other organ-specific autoimmune diseases.

The hallmark of T1D is the selective destruction of pancreatic islets. But due to marked heterogeneity, it is difficult to follow the destruction of beta cells within the islets. At the onset of hyperglycemia islet contain numerous components like infiltrating lymphocytes and monocytes, a mixture of pseudo-atrophic islets, pancreatic polypeptide, and somatostatin [16]. Lymphocyte infiltration is prominent when diabetes becomes chronic. Another important prerequisite for the development of diabetes is the activation of islet Ag-specific $CD4^+$ T cell [17]. Transferring of $CD4^+$ T cells into the nondiabetic mice from diabetic mice also developed insulitis and diabetes. It is already reported that $CD4^+$ T cells are able to induce diabetes initially and $CD8^+$ T cells participate in damaging the pancreas [18]. Some cytokines are also responsible for the development of autoimmune diabetes. High level of IL-2 and IFN-α correlates or enhances the induction of autoimmune diabetes by activating the macrophage in experimental models [19]. In the process of infiltration, macrophages are the first cell type invading the islets where they produce TNF-alpha and IL-1. TNF-alpha and IL-1 play an important role for inducing the structural changes of beta cells and suppression of their insulin releasing capacity.

3. T1D and Tregs

The role of Tregs has been focused in various autoimmune diseases. The vast majority of $CD4^+$ and $CD8^+$ T cells are eliminated in the thymus through central tolerance induction mechanism. But in some cases, few autoreactive T cells are not eliminated and are released to the peripheral circulation. These autoreactive T cells migrate into the pancreas that causes the destruction of islets cells and develop diabetes if they are not actively suppressed by Tregs. T1D is mainly a T cell-mediated autoimmune disease where pancreatic beta cells are destroyed due to the breakdown of tolerance to islets Ag. Initially, autoreactive $CD4^+$ T cell subset recognizes self-Ag and produce T helper (Th) 1 cytokine spectrum that initiate the autoimmune process. For further processing, $CD8^+$ T cells are necessary [20]. Tregs have

the ability to prevent self-reactivity through active suppression [21]. Several studies have demonstrated that $CD4^+$ $CD25^+$ Tregs expressing FoxP3 play an indispensable role in the maintenance of immune homeostasis by regulating inflammatory response against invading pathogens and preventing destruction of autoimmunity [22, 23].

There are a number of autoantigens that are involved in the pathogenesis of T1D. But the true primary autoantigen in T1D has not yet been definitively identified. It is very important to identify the islet-specific autoantigen for the development of autoantigen-specific tolerance induction immunotherapy and for establishing diagnostic and predictive markers of T1D. Until now, the most accepted autoimmune features of T1D are the presence of autoreactive T cells and autoantibodies in the pancreas. It is also proposed that autoantibody against islet autoantigen may appear many years before clinical diagnosis; 90% patients with T1D exhibit autoantibodies against islets autoantigen. But it is not clear whether these autoantibodies play a pathogenic role for the development of diabetes. Studies of different animal and human models lacking with certain types of cells demonstrated that the lack of Tregs or impairment of their function lead to the development of autoimmune disease, including diabetes [24, 25].

A number of mouse models suggests that Tregs play an important role to prevent the onset of diabetes. But how the Tregs can function *in vivo* to block the development of diabetes are still under investigation. There are some mechanisms that were proposed on the basis of mouse models, and its clinical significance is yet to be proved. As autoreactive T cells are the main culprits for the development of diabetes, it is essential to control the migration or differentiation of autoreactive T cells into the pancreatic lymph node. Tregs present in the pancreatic lymph node have the ability to regulate the priming of autoreactive T cells by limiting their expansion and differentiation. Tregs also have the ability to interrupt the development of autoreactive T cells through limiting the access of autoreactive T cells to DCs [26]. By limiting the priming of T cells in the lymph nodes, Treg cell also prevent the T cells becoming an effector T cells. Infiltration of autoreactive T cells into the islet is a crucial step for the development of inflammation and leading to the destruction of islet. Some chemokine receptors like chemokine receptor 3 (CXCR3) secreted by effector T cells are essential for infiltrating them into the islets. By exerting their suppressing mechanism, Tregs inhibit the expression of CXCR3 and ultimately prevents the infiltration of these cells into the pancreatic islets. The most common suppressive cytokine IL-10 and TGF-beta are secreted by inducible Tregs [27, 28]. These two cytokines play an important immunoregulatory role in T1D. TGF-beta secreted by Tregs in the islet during the priming phase stimulates the expansion or generation of intra-islet FoxP3 expressing Tregs [29]. It is already established that increased numbers of Tregs are essential for the suppression of autoreactive T cells that are destructive to the islets. Migration of autoreactive T cells into the islet worsens the disease condition. Intracellular adhesion molecule 1 (ICAM1) is one of the potential factors that helps to migrate the autoreactive T cells into the islets. ICAM1 is exclusively expressed by autoreactive $CD4^+$ and $CD8^+$ T cells [30, 31]. IL-10 secreted by Tregs downregulates the expression of ICAM1 on effector T cells, which prevents their migration to the target organ [32]. IL-10 also reduces the hyperactivity of T cells by modulating the function of APC and reduces the inflammation [33].

http://dx.doi.org/10.5772/66909

4. Role of Tregs in autoimmunity

Tregs are a subset of T cells that exhibit inhibitory or regulatory effects on effector T cells. Previously it was known as suppressor T cells. There is a phenotypical variation of Tregs, such as $CD4^+CD25^+FoxP3^+$ Tregs, $CD8^+$ Tregs, and $CD3^+$ $CD4^-CD8^-$ Tregs [34, 35]. A dysfunction, defect, or absence of Tregs has been implicated in the pathogenesis of many autoimmune diseases [36] as they are indispensable to maintain the immune homeostasis. However, how they control the development of autoimmunity is still under debate. Previous data suggests that several numbers of genetic and mechanistic defects may arise leading to defective regulation by Tregs [37]. Though all different types of Tregs work together to maintain the homeostasis, $CD4^+$ $FoxP3^+$ Tregs play the major role because they are the long lasting and produce most of the suppressive cytokines. There are several mechanisms by which Tregs exert their regulatory effects on effector T cells. These are cell-to-cell contact, secretion of IL-10 and TGF-beta-like immunosuppressive cytokines, modification or killing of APC, and competition for growth factor [38, 39]. $CD4^+$ $FoxP3^+$ Tregs suppresses the immune response, inflammation, and tissue destruction by inhibiting the function of classical $CD4^+$ Th cells, antibody production of B cells, and $CD8^+$ cytotoxic T lymphocyte granule release. Inducible $CD4^+$ $foxP3^-$ type 1 Tregs or $CD4^+FoxP3^+$ Tregs can exhibit their suppressive function through IL-10 secretion. Though some other functions of Tregs have been documented but the major function is to maintain the immune homeostasis. Deficiency in Treg frequency or function results in imbalance in the immune system. But in some cases there are no apparent defects in Treg frequencies like multiple sclerosis [40]. The result in other autoimmune settings have been mixed, but overall in most autoimmune patients, ample number of Tregs appear in the circulation. Any discrepancies in the results reflects the nonspecific phenotypic markers available or due to contamination with non-Tregs. Until now, almost all studies have been limited to analysis of peripheral blood so it is difficult to understand what is happening at the site of inflammation. A number of studies showed reduced frequencies of Tregs in peripheral blood, but an increased number or potency of cells isolated from inflammatory sites [41]. This may be a compensatory mechanism in response to ongoing inflammation during the disease process. Treg stability is another important issue when assessing the frequency of Tregs. Many autoimmune diseases are thought to undergo periods of relapse and remission [42]. These variations are susceptible to the influence of immunosuppressive regimens used in the treatment of autoimmune disease. Moreover, during the progression of disease Tregs in local sites can change phenotypically.

5. Management of T1D

T1D is a chronic metabolic disorder characterized by deficiency of insulin production by pancreatic beta cells. Insulin is essential for maintaining the normal blood glucose level. As it is an autoimmune destruction caused by endogenous autoreactive T cells, exogenous insulin supply is required to maintain normoglycemia in many diabetic patients. This is a lifelong treatment that is not convenient. Another option for treatment is replacement of beta cell therapy where sufficient amount of beta cells need to be included to control the blood

glucose level without repeated insulin injection. However, the beta cell transplantation did not achieve a satisfactory result. In 2000, Shapiro and colleagues achieved independence of insulin injections in seven T1D patients by transplanting a large number of islet cells combined with the use of glucocorticoid-free immunosuppressive regimen [43]. But the insulin independency was not sustained for long. Some patients even had complete graft loss 1 year after the final transplantation. The main reason for poor long-term outcome is continuous immune destruction of the transplanted islet as autoimmune destruction is a continuous process. Beta cell transplantation also has a major obstacle, shortage of donors when compared with large population. As cadaver tissue provides a low yield of islet cells, it requires a large number of donor cells to generate sufficient insulin-producing beta cells that are capable of producing and releasing adequate amount of insulin in response to normal physiological signals. Furthermore, chronic immunosuppression is also necessary after allograft transplantation. Patient-specific islet-like cells from adult tissues may compensate both the shortage of organ donors and allograft rejection. Several groups were successful to generate functional islet-like clusters from adult progenitor cells, but their success were limited [44, 45]. Therefore, it is highly demanding to explore some other option for searching more defined sources of beta cells.

Generation of induced PSCs (iPSCs) opens a new era in the treatment of autoimmune diseases. IPSCs have the ability to become all kinds of cells if they are maintained properly [46]. As Tregs have the ability to Suppress the hyper activity of autoreactive T cells and they can be expanded *in vivo* after one-time transplant, it is ideal to generate Tregs from iPSCs for the treatment of autoimmune diabetes.

6. iPSCs

Due to restricted use of human embryonic stem cells (ESCs) in both research and clinical settings, induced pluripotent stem cells (iPSCs) serve as an attractive potential alternative to ESCs. Human somatic cells can be reverted back to pluripotent stem cells by expression of defined transcription factors. Mouse and human somatic cells have already been converted into iPSCs by introducing transcription factors OCT4, and SOX2 in combination with KLF4, c-MYC, NANOG, and lin-28 homolog A [47]. iPSCs are similar to ESCs in morphology, gene expression, epigenetic status, and *in vitro* differentiation. C-MYC and KLF4 are known oncogenes and their use to generate iPSCs raises concerns about potential tumor formation. However, this can be overcome by the use of a histone deacytylase inhibitor, valproic acid, which facilitates the reprogramming of primary human fibroblasts with only two factors, OCT4 and SOX2. Thus, the reprogramming of cells to pluripotency has become potentially safer and practical for therapeutic use [48]. Another challenge was the use of retrovirus or lentivirus to deliver transcription factor genes into the somatic cells. This also raised the concern about viral integration into the host genome that increases risk of tumorigenicity. To avoid this risk, Yamanaka used a novel repeated transfection protocol for the expression of plasmids that resulted in iPSCs without evidence of plasmid integration [49]. Other groups also generated iPSCs from umbilical cord blood by lentiviral overexpression of the reprogramming

factor OCT4, SOX2, NANOG, and LIN28 [50]. The reprogramming efficiency was almost the same as keratinocytes and fibroblast. However, use of umbilical cord blood also leads to a possibility that it may be mutated over the lifetime of an organism. Thus, it is still under debate whether iPSCs are truly equivalent to human ESCs or not with respect to pluripotency.

iPSCs have already been used for the generation of Insulin secreting cells. iPSCs were generated from skin biopsies of a patient with T1D by using three transcription factors OCT4, SOX2, and KLF4 [51]. These cells were differentiated into insulin-producing cells. These cells were found to be released human C-peptide and exhibited a five-fold increase in the secretion of C-peptide in response to 20 mM glucose, which reveals that functional beta cells can eventually be derived from iPSCs.

Generation of functional beta cells for the immunotherapy of T1D is not the only challenge; there is a need to overcome the immune response both in terms of autoimmunity and rejection of allogenic tissue. It is also unknown whether these *in vitro* generated cells will migrate to the target tissue or not. Since beta cells will continuously be destroyed upon development of autoimmunity, it is ideal to generate Ag or tissue-specific Tregs from iPSCs for the treatments of autoimmune diabetes.

7. Generation of Ag/tissue-specific Tregs from iPSCs

Tregs have been used for the treatment of autoimmune diseases because it modulates the autoimmune response by immune suppression. A number of mouse models demonstrated that Tregs are potent inhibitors of polyclonal T cell activation [52]. This Treg-mediated suppression is achieved by cytokine-independent and cell-contact-dependent mechanisms that require activation by TCR. When its cognate Ag activates Tregs, they can suppress the conventional T cells within the immediate vicinity regardless of the specificity. In this phenomenon, Treg does not need to recognize any specific Ag; they exert their suppressive efforts by recognizing the Ag on APC. Thus, any autoimmune affected organ or tissue can be targeted without the knowledge of the causative Ag by using Tregs. By utilizing this procedure, the maximal therapeutic effect will not be achieved, as it is not Ag-specific. Polyclonal Tregs also inhibit a wide range of other immune cells such as B cells, DCs, and monocytes [53–55]. It has also been observed that polyclonal Tregs failed to reverse ongoing autoimmunity because Tregs require Ag specificity to home/be retained at the appropriate site and exert active suppression. Within a polyclonal population of Tregs, Ag specificity against autoantigen exists in a small proportion of cells, which is not sufficient to exert sufficient amount of suppression. Therefore, it is crucial to generate a large number of Ag-specific Tregs for adoptive immunotherapy to reverse the ongoing autoimmunity.

Since it has been established that Tregs are the most potent to suppress the overactivity of hyperactive T cells, our approach was to generate a large number of Ag-specific Tregs from iPSCs. It is already published that hematopoietic stem cells (HSCs) and ESCs are able to differentiate into T cells in an *in vitro* culture system and we have utilized a similar approach to test whether iPSCs could follow the same trend [6, 56]. In that study, mouse iPSCs were cocultured

with Notch ligand expressing bone marrow stromal cell line (OP9-DL1) as Notch ligand signaling is essential for T lineage differentiation [57]. At different days of cell culture, iPSCs were collected and evaluated for morphology, cell surface Ag, and their functional ability. It is found that iPSC-derived cells differentiated from stem-like cells to T cell-like cells, expressing T cell surface markers. Morphologically, dome-like stem cell colony was transformed to grape-like colony, which is a characteristic of lymphoid cells. iPSCs usually express CD117 and Nanog surface markers. After differentiation, the cells stopped expressing stem cell-like markers and expressed T cell markers such as CD4 and CD8. *In vitro* differentiated cells were also tested for their functional ability and it is found that they secrete IL-2 and IFN-α upon stimulation with anti-CD3 and anti-CD28 antibodies.

Since we could differentiate iPSCs into functional T cells, we proceeded to generate Ag-specific Tregs. First, we generated a construct called MiDR-TCR-FoxP3 where ovalbumin $(OVA)_{323–339}$ specific TCR OTII and FoxP3 were cloned into MiDR vector (**Figure 1**).

MiDR-TCR-FoxP3 vector was retrovirally transduced into mouse iPSCs and cocultured onto with OP9-DL1-DL4-I-A^b in the presence of recombinant cytokines of rIL-7 and rFLt3L. TCR and FoxP3 gene-transduced iPSCs were checked for differentiation by observing their morphological change. We found that iPSCs differentiated into mesoderm-like cells, and were associated with nonadherent grape-like clusters. On day 22 of culture, lymphocyte-like cell Spread fully across the plate (**Figure 2**).

In vitro cocultured cells were analyzed for cell surface markers. We found that the iPSC-derived cells substantially expressed CD3- and Ag-specific TCR, two T cell markers. The

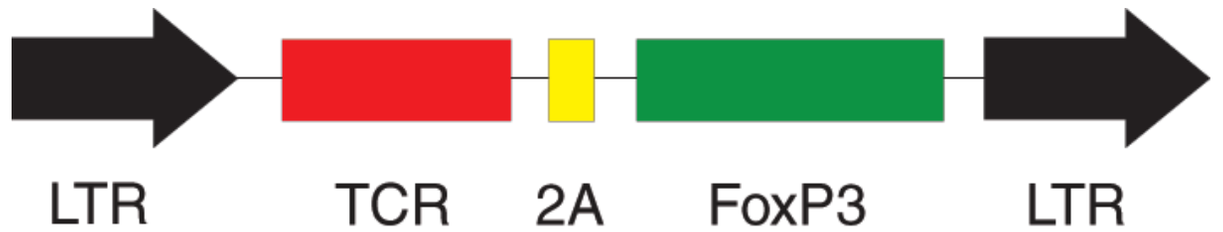


Figure 1. Generation of MiDR-TCR-FoxP3 retroviral construct. Schematic representation of the retrovirus construct MiDR-TCR-FoxP3 expressing OVA-specific TCR and FoxP3. Ψ, packaging signal; 2A, picornavirus self-cleaving 2A sequence; LTR, long terminal repeats.

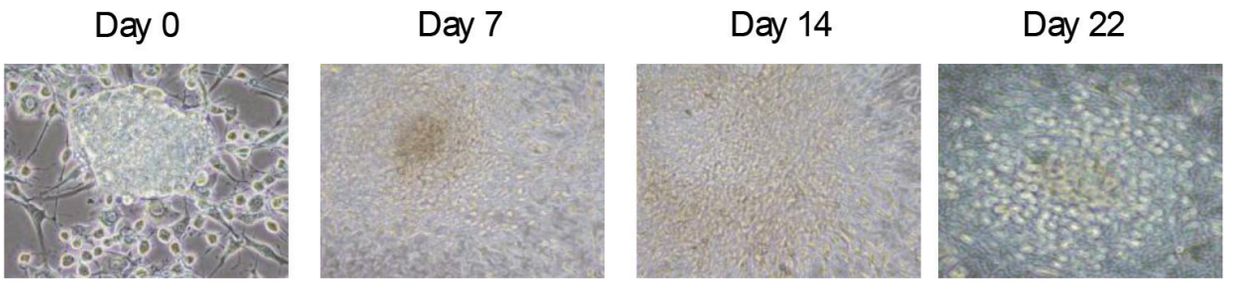


Figure 2. Morphology of Treg differentiation on days 0, 7, 14, and 22. The TCR/FoxP3 gene-transduced iPSCs were cocultured with OP9 stromal cell expressing Notch ligands DL1, DL4, and I-A^b in the presence of rIL-7 and r-Flt3L. Morphology was visualized under a microscope.

$CD3^+TCRV\beta5^+$ population expressed CD4. Most of the $CD3^+TCRV\beta5^+CD4^+$ cells expressed CD25, CD127, and CTLA-4, which are typically expressed at elevated levels in naturally occurring Tregs (iTregs) [58]. Subsequently, we also investigated the functional capability of iPSC-derived Ag-specific Tregs. After adoptive transfer, $CD4^+FoxP3^+$ Tregs were isolated from pancreatic lymph nodes and checked for expression of two suppressive cytokines, IL-10 and TGFβ. The result showed that significant amount of IL-10 and TGFβ were secreted by Tregs that supported that iPSC-derived Tregs are functional.

8. Utilization of iPSC-derived Tregs for the treatments of autoimmune diabetes

We developed a mouse model for autoimmune diabetes by crossing B6 mOVA transgenic (Tg) mice with OT I TCR Tg mice. In B6 mOVA Tg mice, membrane bound form of OVA expressed in the pancreatic islet β cells and the renal proximal tubular cells [59]. Once they are interbred, the resulting mice will be B6 mOVA-OT I where T cells from OT1 Tg mice will be directed to the pancreas as the pancreas expressed OVA autoantigen. The OT I OVA-specific T cells will begin to target and destroy pancreatic islet cells and mice will subsequently develop diabetes. Once pups reached 8 weeks, blood sugar level was measured and it was observed that only 30% mice developed diabetes. Subsequently, OT I Tg T cells were further triggered by injecting vaccinia virus expressing OVA (VV-OVA) into the mice. After vaccinia immunization, 100% mice developed diabetes with more urine discharge. After confirmation of disease developed in mice, we injected iPSC-derived Tregs into the mice. One-week post cell transfer, we checked the blood glucose level and found that more than 80% of the mice had reduced glucose level in their blood. Mice were sacrificed for histological evaluation. Pancreas were isolated from treated and untreated mice and it was observed that inflammation was markedly decreased in iPSC-derived Treg-transfer mice compare to untreated mice (**Figure 3**).

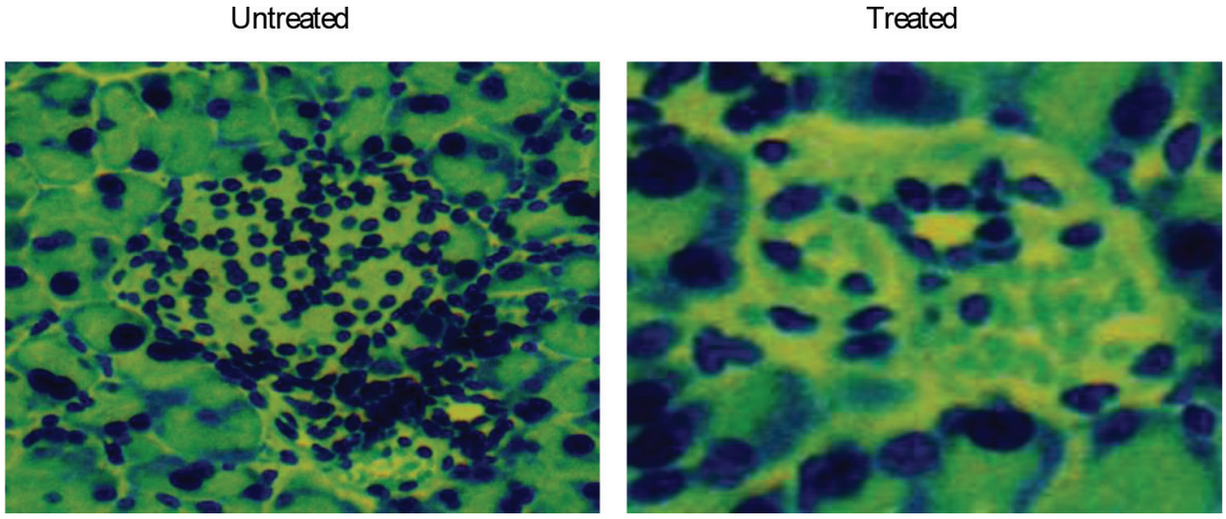


Figure 3. Inflammatory cells are accumulated in diabetic mice: diabetic and Treg-transferred mice were sacrificed and pancreases were prepared for HE staining. Untreated mice show large accumulation of inflammatory cell infiltration in the pancreas.

Further analysis was done to check the islet destruction in iPSC-derived Treg-transfer and nontransfer mice. Islet sizes were markedly reduced in nontransfer mice, whereas islet sizes were normal in iPSC-derived Treg-transfer mice (**Figure 4**).

We investigated the mechanisms of how iPSCs-derived Tregs controlled blood sugar levels and prevented the destruction of islet in autoimmune diabetes mice. Adhesion molecule

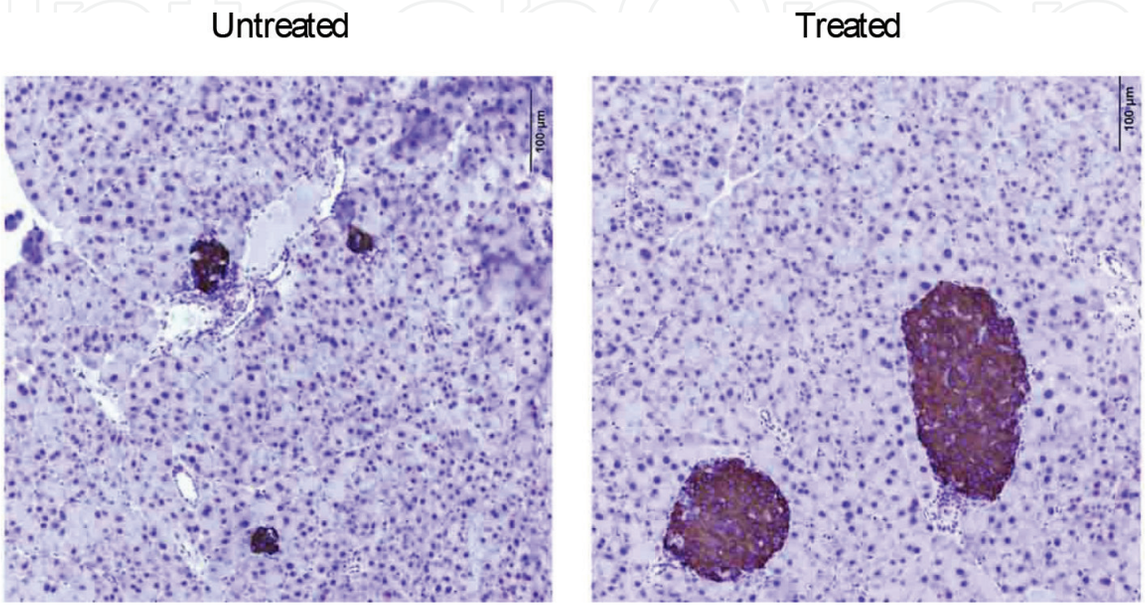


Figure 4. Islet size and numbers were reduced in diabetic mice. Diabetic and Treg-transferred mice were sacrificed and their Pancreas were stained with insulin to detect the beta cell. In diabetic mice, islets size and number were reduced markedly, whereas islet size and numbers were normal in Treg-transferred mice.

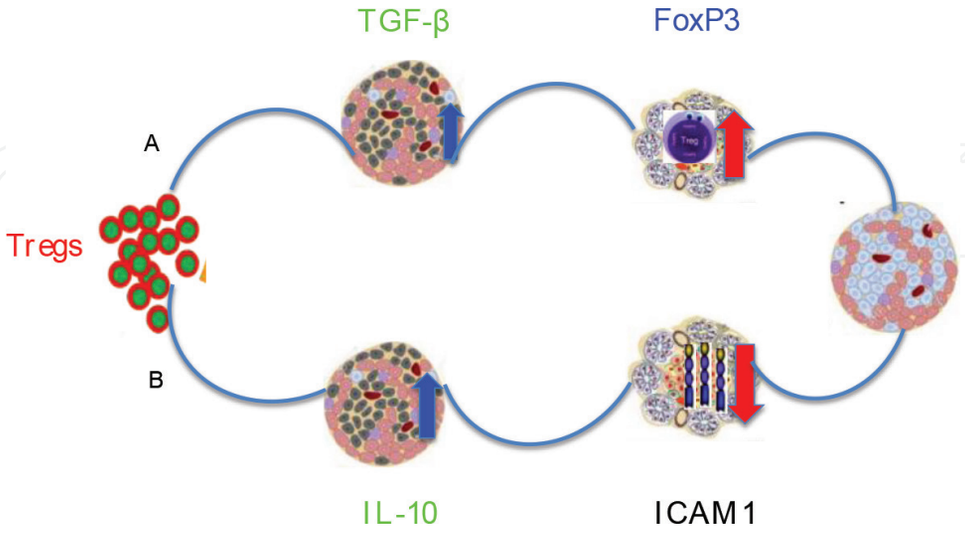


Figure 5. Stem cell-derived OVA-specific Tregs were adoptively transferred into diabetic mice. (A) Tregs induced the expression of TGF-β into the destructed islet leading to increase the expression of intra-islet FoxP3 that protected the islet from further destruction. (B) Tregs induced the expression of IL-10 into the destructed islet leading to reduce the expression of ICAM1 that prevented the migration of $CD8^+$ T cells into the destructed islet and protected the islet from further destruction.

ICAM1 is important for targeting autoreactive $CD8^+$ T cells into the pancreatic islet [32]. We determined the expression of ICAM1 into the pancreatic lymph nodes, and found that ICAM1 expression was dramatically increased in diabetic mice. Conversely, its expression was markedly reduced in iPSC-derived Treg-transfer mice. Previously, we have showed that iPSC-derived Tregs were able to secret IL-10 and TGF-β. Therefore, expression of TGF-β by iPSC-derived Tregs into the islet increased the intra-islet FoxP3 expression that protected the islet from further destruction. Moreover, IL-10 secreted by the Tregs reduced the expression of ICAM1, which prevented the migration of autoreactive $CD8^+$ T cell into the damaged islet and prevented further destruction of the islet (**Figure 5**).

9. Conclusion

PSCs have the ability to differentiate into Ag-specific Tregs and they are also found to be similar morphologically and functionally to iTregs. However, in autoimmune diabetes, it is important to mitigate the disease by increasing the activity of islet cells or preventing their destruction from autoreactive T cells. In our study, iPSC-derived Tregs were successful in reducing the blood sugar level and restoration of the islet size. By utilizing the knowledge from iPSC differentiation, We will be able to generate Ag-specific T cells that are more closely associated with the development of autoimmune diabetes. It is already known that heat shock proteins (HSPs) are an islet tissue-associated auto-Ag and involved in the islet cell destruction of T1D [60]. HSPs can modulate chronic inflammatory diseases and can be a target of immunotherapy of T1D. In our preliminary study, we checked the expression of HSPs in our diabetes model and found that diabetic mice substantially expressed HSPs. Therefore, it will be ideal to generate HSP-specific Tregs from PSCs for the treatment of autoimmune diabetes. For this study, HSP-specific TCR needs to be genetically processed and cloned into a viral vector to be retrovirally transduced into PSCs and follow the general protocol to allow for *in vitro* differentiation for the development of HSP-specific Tregs. In recent years, not only our efforts in utilizing PSC derived T cells for therapeutic purposes, but also other groups have made considerable efforts in understanding the PSC function in hematopoietic development. PSCs also could be differentiated into DCs, NK cells, and B cells. Consequently, by using patient-derived iPSCs, autoantigen-specific Tregs could be generated to specifically treat diabetic patients.

Acknowledgements

This project is funded, in part, under grants with the National Institute of Health Grant R01AI121180, R21AI109239, and K18CA151798, American Diabetes Association 1-16-IBS-281, and the Pennsylvania Department of Health using Tobacco Settlement Funds.

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