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Stem cell-derived tissue-associated regulatory T cells suppress the activity of pathogenic cells in autoimmune diabetes

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Conflicts of interest statement

The authors have declared that no conflict of interest exists.
Abstract

The auto antigen (Ag)-specific regulatory T cells (Tregs) from pluripotent stem cells (PSCs), i.e., PSC-Tregs, have the ability to suppress autoimmunity. PSC-Tregs can be programmed to be tissue-associated and to infiltrate into local inflamed tissues to suppress autoimmune responses after adoptive transfer. Nevertheless, the mechanisms by which the auto Ag-specific PSC-Tregs suppress the autoimmune response remain to be fully elucidated. In this study, we generated the functional auto Ag-specific Tregs from the induced PSC (iPSCs), i.e., iPSC-Tregs, and investigated the underlying mechanisms of autoimmunity suppression by these Tregs in a type 1 diabetes (T1D) murine model. A double transgenic (Tg) mouse model of T1D was established in F1 mice in which the first generation of RIP-mOVA Tg mice that were crossed with OT-I T cell receptor (TCR) Tg mice was challenged with vaccinia viruses expressing OVA (VACV-OVA). We show that adoptive transfer of OVA-specific iPSC-Tregs greatly suppressed autoimmunity in the animal model and prevented the insulin-secreting pancreatic β cells from destruction. Further, we demonstrate that the adoptive transfer significantly reduced the expression of ICAM-1 in the diabetic pancreas and inhibited the migration of pathogenic CD8+ T cells and the production of the pro-inflammatory IFN-γ in the pancreas. These results indicate that the stem cell-derived tissue-associated Tregs can robustly accumulate in the diabetic pancreas, and through down-regulating the expression of ICAM-1 in the local inflamed tissues and inhibiting the production of pro-inflammatory cytokine IFN-γ, suppress the migration and activity of the pathogenic immune cells that cause T1D.
Introduction

Type 1 diabetes (T1D) develops due to autoimmune self-destruction of pancreatic beta (β) cells that produce insulin, and life-time administration of insulin is required for treatment of this disease (1). Although accumulating knowledge has contributed greatly to our understanding of the autoimmune pathogenesis of T1D, the precise causes remain unclear. It has been generally appreciated that autoimmune diseases arise from the breakdown of immune tolerance (2, 3). A number of studies found the massive infiltration of CD8+ T cells into the pancreas of newly diagnosed T1D patients, whereas the number of CD4+ T cells was greatly reduced (4, 5). Infiltrated CD8+ T cells are pathogenic to the islets, can cause the destruction of pancreatic β cells and ultimately reduce the secretion of insulin.

Currently, there is no definitive treatment for controlling blood glucose level in T1D except durable insulin therapy. As a result, generations and transplantation of exogenous β cells to replace dead or dysfunctional endogenous β cells are considered as a promising strategy for controlling blood glucose level in patients with T1D. However, since the autoimmune disease is a continuous process, after β cell transplantation, diabetes may develop and progress again through destructing the pancreatic islets by pathogenic T cells. Therefore, β cell transplantation might not be a long-lasting solution for the control of blood glucose level in T1D.

Over the past several years, there has been an increasing interest in the regulatory T cells (Tregs), which play a fundamental role in controlling various autoimmune responses. Numerous preclinical studies suggest that adoptive transfer of Tregs can prevent or cure the T cell-mediated autoimmune diseases such as T1D and arthritis (6, 7). There are several advantages of the Treg adoptive transfer over conventional treatments. These advantages include: (i) potential of antigen (Ag) specificity without general immunosuppression; (ii) option of inducing ‘physiological’ long-
lasting regulation in vivo; and (iii) possibility of Treg-based immunotherapy as a customized or personally designed agent for each patient, with reduced side effects (8, 9). It is now generally believed that adoptive transfer of in vitro generated Tregs can reduce the hazards of complicated surgery events throughout the life. However, the use of Tregs has been complicated due to difficulties in expanding and characterizing this minor subset of T-cells.

Here, we report the development of a robust technique for producing a large amount of auto Ag-specific Tregs from induced pluripotent stem cells (iPSCs), i.e., iPSC-Tregs that retain all the quintessential characteristics of this T cell subset, including expressions of CD25, CTLA-4 and FoxP3, and production of IL-10. We show that adoptive transfer of these auto Ag-specific iPSC-Tregs significantly reduces the high ratio of CD8⁺ to CD4⁺ T cells in the pancreas of diabetic mice, and markedly decreases the expression of intracellular adhesion molecule-1 (ICAM-1) in the diabetic pancreas of the pre-diabetic and diabetic mice. These results demonstrate a great potential of stem cell-derived auto Ag-specific Tregs in T1D immunotherapy. Moreover, we show that the stem cell-derived tissue-associated Tregs control autoimmune diabetes via preventing the ICAM-1-mediated migration of pathogenic CD8⁺ T cells in the pancreas and suppressing the production of pro-inflammatory cytokine IFN-γ.
Results

Characterization of auto Ag-specific naturally-occurring Treg (nTreg)-like iPSC-Tregs

Although FoxP3 is a master regulator and a specific molecular marker for nTregs, there are evidence that FoxP3 expression is not a distinct and reliable marker or a sole regulator of functionally stable Tregs. In addition, recent evidence showed that various transcription factors are critical for the development of FoxP3+ Tregs including Nr4a1, Ikzf4, Tnfrsf18, and Tbx21 (10). These genes, which are substantially expressed in nTregs, are essential for regulating Treg transcriptional programs and maintaining the lineage stability in Tregs.

We previously showed the generation of functional Ag-specific iPSC-Tregs, which had the ability to suppress the development of autoimmune arthritis after adoptive transfer in murine models (11, 12). Because the iPSC-Tregs are retrovirally transduced with FoxP3 and TCR genes and have the similar phenotype with nTreg-like expression of IL-10 and TGF-β, we examined the Treg signature genes in the auto Ag-specific iPSC-Tregs. We adoptively transferred Rag 1− mice with pre-iPSC-Tregs that had been co-cultured with OP9-DL1/DL4/I-A b cells for 7 days. Six weeks later, mice were sacrificed and their spleens and LNs were removed for the isolation of CD4+CD25+ cells. Conversely, CD4+CD25+ cells were sorted from the spleens and LNs of normal C57BL/6 mice. The RT-PCR analysis of the Treg signature genes showed that there were no significant differences between both of the mice in the gene expression of the transcription factors (Nr4a1, Il2ra, Ikzf4, Tnfrsf18, and Bcl6) involved in the regulation of Treg functions (all p>0.05. Fig. 1). Collectively, these results and suggest that the auto Ag-specific iPSC-Tregs are nTreg-like suppressive cells.

Accumulation of activated CD8+ T cells in auto Ag-expressing pancreases of diabetic mice
To show the expression of auto Ag in the pancreases of double Tg mice (B6-mOVA with OT-I), we performed immunofluorescence examination. The results showed the expression of OVA auto Ag in the double Tg mice, but not in the B6 or OT-I control mice (Fig. 2A). Because the OVA-specific CD8+ T cells have the ability to migrate into the pancreases of the F1 generation by crossing B6 mOVA with OT-I TCR Tg mice and to cause destruction of the islets, we examined the fate of OVA-specific CD8+ T cells in vivo (Fig. 2B). The migrations of CD8+ T cells in diabetic mice were further confirmed by flow cytometry in which more numbers of both CD8+ and TCRVβ5+ cells in diabetic mice (Fig. 2C). In the F1 mice post VACV infection (Supplementary Fig. 3), the draining LNs and spleens of the F1 mice were analyzed for the presence of OT-I CD8+ T cells. The proportions of OVA-specific CD8+ cells among the total CD8+ cells in the pancreatic LNs were significantly higher than in the pyloric, mesenteric, inguinal, cervical, or splenic LNs. No apparent accumulation or homing was observed in the non-Tg control B6 mice (Fig. 2D). Additionally, all F1 mice post VACV infection developed autoimmune diabetes at age of 9 weeks, which was confirmed by the measurement of blood sugar (Fig. 2E). These results verify that auto Ag-specific CD8+ T cells are the main pathogenic immune cells that induce autoimmune diabetes in the murine model.

In vivo specificity of nTreg-like auto Ag-specific iPSC-Tregs

To exert their suppressive effects, Tregs need to migrate to specific tissues or organs, and this requires Ag specificity (8). We and others have previously reported that Tregs are detected within the inflamed tissues and transplanted grafts, suggesting that these Tregs could control effector T cells in the peripheral tissues at sites of ongoing immune responses (12-14). Tregs may block the pathogenic cells from filling up their appropriate niche by taking up the space. Treg population
was reduced in autoimmune prone animals and patients, and Tregs were defective in NOD mice (15). In this study, we observed that mice receiving auto Ag (OVA)-specific iPSC-Tregs maintained a high population of both CD4+ and FoxP3+ cells in the pancreas, as compared with the diabetic mice receiving cell control (iPSC-derived cells with empty vector) or non-tissue-associated SM1-specific iPSC-Tregs by immunofluorescence examination (Fig. 3A) and by flow cytometric analysis (90.1% vs. 12.5% or 16.8% at week 13. Fig. 3B). These results suggest that the auto Ag-specific iPSC-Tregs are organ/tissue-associated nTreg-like suppressive cells.

**Auto Ag-specific iPSCs-Tregs can prevent destruction of pancreatic β cells and reduce the autoimmune diabetes**

Next, we injected the diabetic mice with the auto Ag-specific iPSC-Tregs. Two weeks after the cell transfer, blood glucose level was measured. This cell transfer with auto Ag (OVA)-specific iPSC-Tregs but not cell control or non-tissue-associated SM1-specific iPSC-Tregs significantly reduced blood glucose level with urine discharge in all of the diabetic mice (p<0.0001. Fig. 4A and Supplementary Fig. 3). T1D develops due to destructive auto reactive immune response in which CD8+ T cells play a critical role. CD8+ T cells infiltrated into the pancreatic islets of the T1D patients at both initial and final destructive phases of autoimmune β cells attack (16). The blood glucose level was increased because of the destruction of β cells in the islets by pathogenic CD8+ T cells in the diabetic pancreas, which was evidenced in the above mouse diabetic model. More CD8+ T cells were accumulated in the pancreases of mice receiving cell or non-tissue-associated Ag-specific iPSC-Treg control than auto Ag-specific iPSC-Tregs by immunofluorescence examination (Fig. 4B) and by flow cytometric analysis (49.8% or 46.1% vs. 6.5%. Fig. 4C). Only mice receiving auto Ag-specific iPSC-Tregs significantly reduced percent
A large number of inflammatory cells were infiltrated into the pancreases of mice receiving cell controls, whereas the infiltration was significantly reduced in mice receiving auto Ag-specific iPSC-Tregs by HE staining (Fig. 4E). The total numbers of the islets were also reduced in mice receiving cell controls as compared with auto Ag-specific iPSC-Tregs ($p<0.01$ or $p<0.001$. Fig. 4F). To detect the destruction of $\beta$ cells in the islets of diabetic mice, we stained the pancreases with insulin. The islets were reduced in size and number in mice receiving cell controls, whereas mice receiving auto Ag-specific iPSC-Tregs were protected from the destruction by immunofluorescence examination (Fig. 4G). The islets were partially destructed by pathogenic CD8$^+$ T cells, which caused the increase of blood sugar and developed autoimmune diabetes, but some positive insulin staining in diabetic mice still presented. Collectively, these results indicate that the auto Ag-specific iPSC-Tregs are specific and effective in protecting the hosts from the islet destruction and in promoting insulin secretion to prevent the mice from diabetes mellitus.

**Auto Ag-specific iPSC-Tregs can decrease the expression of ICAM-1**

A number of mononuclear cells including macrophages and CD4$^+$ T cells were found to be initially predominant in the autoimmune process taking place in T1D (17, 18). In autoimmune diabetes, these cells played an important role in the early infiltrating process and were shown to express ICAM-1, which served to bind to lymphocytes, and possibly to monocytes and polymorphonuclear leukocytes (19). The infiltrated ICAM-1 can promote the release of several cytokines that modulate the expression of the adhesion molecules, thus increasing the adhesion of leukocytes and other autoreactive T cells (20). These auto reactive T cells, after migrating into the pancreas, start to destroy $\beta$ cells. We thus examined the expression of ICAM-1 in the pancreases. The high
expression of ICAM-1 in the pancreases of the diabetic mice, which was undetectable in the normal pancreases, was significantly reduced in the diabetic mice receiving auto Ag-specific iPSC-Tregs, but not other cell controls (Fig 5 A - B). Also, the high expressions of ICAM-1 on the effector CD4+ T cells in the pancreases of the diabetic mice receiving cell controls (84.4% or 78.9%), which was undetectable in the normal pancreases (0.35%), was significantly reduced in those of mice receiving auto Ag-specific iPSC-Tregs (18.4%) (Fig 5C). In addition, the expressions of ICAM-1 on CD11b+ cells in the pancreases of the diabetic mice receiving cell controls (15.64% or 12.83%), which was at a low level in the normal pancreases (4.89%), was considerably reduced in those of mice receiving auto Ag-specific iPSC-Tregs (5.68%) (Fig 5D). Because CD11b is expressed on the surface of various leukocytes including monocytes/macrophages, granulocytes, neutrophils, NK cells, and dendritic cells, the reduction of CD11b+ ICAM-1+ cells indicated the inhibition of leukocyte adhesion and migration to mediate the inflammatory response. Taken together, these results suggest that the auto Ag-specific iPSC-Tregs can reduce the expression of ICAM-1 in the diabetic mice, inhibit the migration of autoreactive CD8+ T cells into the pancreas and ultimately protect the pancreatic β cells from destruction.

Auto Ag-specific iPSC-Tregs can migrate to the pancreas and produce a large amount of suppressive cytokines

In the BDC2.5/NOD mice, FoxP3 was highly expressed in insulitic lesions where the function was impaired due to alterations in their gene expression profile (21, 22). We previously showed that the iPSC-Tregs exhibited a similar expression of Treg signature genes and produced IL-10 and TGF-β (12). In the current study, we observed very few numbers of FoxP3-expressing T cells in the pancreases of diabetic mice, and these cells did not secret IL-10 and TGF-β, which are the two
key cytokines that are the hallmark for functional Tregs and secreted by activated and functional Tregs.

We further showed that the pancreatic CD4+ T cells from mice receiving auto Ag-specific iPSC-Tregs produced significantly more IL-10 (66.1% vs. 12.9% or 15.1%) and TGF-β (5.61% vs. 0.62% or 1.68%), as compared with those from mice receiving control cells (Fig. 6). These results support the assumption that auto Ag-specific iPSC-Tregs accumulating in the pancreas can secrete large amounts of suppressive cytokines (IL-10 and TGF-β), reduce the expression of ICAM-1 and prevent the migration of pathogenic CD8+ T cells into the pancreas, thus protecting the pancreas from destruction.

**Auto Ag-specific iPSC-Tregs can decrease the production of IFN-γ by pathogenic immune cells in the diabetic pancreatic islets**

Treatment of NOD mice with an anti-IFN-γ monoclonal antibody could block the diabetes (23); conversely, over-production of IFN-γ in the pancreatic islets provoked the disease (24). We next determined the secretion of IFN-γ in the diabetic islet. We found more IFN-γ-producing pancreatic CD8 and CD4 T cells in mice receiving control cells (31.4% and 30.4% or 29.7% and 28.6%); by contrast, both of the IFN-γ-producing pancreatic CD8 and CD4 T cells were significantly reduced in mice receiving auto Ag-specific iPSC-Tregs (14.6% and 10.9%) (Fig. 7). These results indicate that the auto Ag-specific iPSC-Tregs can protect the islets from destruction by suppressing the secretion of IFN-γ produced by the pathogenic immune cells.
Discussion

We and others have previously reported that adoptive transfer of stem cells or stem cell-derived Tregs have the ability to suppress the development of autoimmunity in various animal models (11, 12, 25, 26). However, the suppressive mechanisms behind remain to be fully defined. By investigating the adoptive transfer of auto Ag-specific iPSC-Tregs in a murine model of T1D, we demonstrate that the adoptive transfer significantly reduced the high ratio of CD8^+ to CD4^+ T cells in the pancreases of diabetic mice. We also show a critical role of this transfer in reducing the expression of ICAM-1 in the inflamed pancreatic tissues and preventing the accumulation of pathogenic CD8^+ T cells in the pancreases and diabetic pathogenesis. We further demonstrate that auto Ag-specific iPSC-Tregs were inflamed tissue-associated and protected the islets from destruction through suppressing the production of pro-inflammatory cytokine IFN-γ. These findings may help better understand the pathogenesis of autoimmunity in T1D and provide a foundation for therapeutic use of the stem cell-derived tissue-associated Tregs in the treatment of T1D (27).

Among animal models of autoimmune diabetes, NOD mouse is a widely-used animal of autoimmune T cell-mediated T1D (spontaneously non-obese diabetic). The NOD mouse model clearly shows the leukocytic infiltration into the pancreatic islets (insulitis) and autoimmune destruction of the pancreatic β cell in female mice (2–4 weeks) and later in male mice (5–7 weeks). Insulitis in NOD mice shows a combination of T cells (both CD4^+ and CD8^+), B cells (28), and inconstant numbers of macrophages/dendritic cells. In addition, a dominant negative mutation in the mouse insulin 2 gene (Ins2^{Akita}) produces a severe insulin deficiency syndrome exclusive of autoimmune participation and various transgenes overexpressed in β cells. Moreover, pharmacologically-induced T1D (without autoimmunity) by alloxan (AL) or streptozotocin (STZ)
produces hyperglycemia in most strain of mouse. Several low doses of STZ combing direct β cell toxicity with local inflammation also elicit T1D in a male sex-specific fashion (29). To determine the suppressive mechanisms of stem cell-derived tissue-associated Tregs, we employed a mouse model of T1D by crossing B6-mOVA Tg mice with OT-I TCR Tg mice. Around 8 weeks of age, blood glucose level was measured in F1 mice. Approximately 20 - 40% of the mice were found to be diabetic, one potential explanation for this occurrence is that the OT-I CD8+ T cells may be tolerated during T cell development in the thymuses of F1 mice. As we propose in our model that mice will develop autoimmune diabetes due to a large amount and the auto reactivity of CD8+ T cells, we activated tissue-associated CD8+ T cells by inoculating VACV-OVA into the mice in which the viruses-induced CD8+ T cells became highly poly-functional (30). All mice developed diabetes with high blood glucose level (Supplementary Fig. 3) and more urine discharge for which mice needed additional animal care. In this mouse model of T1D, OVA protein serves as inflamed tissue-associated auto Ag and is highly expressed in the pancreas. OVA-specific CD8+ T cells act as central pathogenic immune cells that accumulate in the pancreas and cause pancreatic β cell destruction and T1D (Fig. 2). In addition, in this mouse model of T1D, we observed a non-OVA-specific CD4+ population existing in the pancreas (Fig. 3), and these CD4+ T cells produced IFN-γ (Fig. 7), which might also involve to the disease pathogenesis. These FoxP3+ CD4+ T cells in the pancreas were TCRα2/Vβ5+, indicating that the T cell activation was not through OVA-specific TCR. Of note, the number of these effector CD4+ T cells was also dramatically reduced in mice receiving OVA-specific but not SM1-specific iPSC-Tregs (Supplementary Fig. 4). These results also indicate tissue-associated (OVA-specific) iPSC-Tregs are more effective than non-tissue-associated (SM1-specific) iPSC-Tregs. Because of the challenge of VACV-OVA, these non-OVA-specific CD4+ T cells mainly originating from the RIP-mOVA descent may undergo
bystander activation (31, 32). However, addition studies are needed on the pathways leading to bystander T-cell activation under the condition.

Tregs are an important component of self-tolerance, and numerous studies have demonstrated the effects of Tregs on natural and induced autoimmune diseases in various mouse models (33, 34). Targeting Tregs as a treatment for autoimmune disorders is an attractive approach, as there is an emerging consensus that many patients suffering from autoimmune disease have dysfunctional Tregs (35, 36). Given the manageability of murine models, the evidence for Tregs in control of autoimmune diseases has recently become clearer, particularly as a therapeutic intervention. In well-established models of rheumatoid arthritis (37), multiple sclerosis (38) and systemic lupus erythematosus (39), adoptive transfer of polyclonal Tregs could prevent or slow disease progression when administered prior to disease occurrence. In multiple sclerosis, Ag-specific Tregs targeting the disease-associated Ags were highly efficacious in reverting the ongoing disease (8, 40). Specifically, the non-obese T1D NOD.Cd28−/− mouse model deficient in Tregs developed diabetes at an accelerated rate as compared with NOD mice. Injection of NOD Tregs into NOD.Cd28−/− mice could delay and, in some cases, prevent the development of diabetes (41). In the current study, we have confirmed that adoptive transfer of stem cell-derived auto Ag-specific Tregs but not cell control, non-specific iPSC-Tregs or non-tissue-associated Ag-specific iPSC-Tregs greatly accumulated in the pancreas and reversed the disease process of autoimmune diabetes in the mouse model of T1D that was caused by pathogenic CD8+ T cells (Fig. 4 and Supplementary Fig. 3). A small number of Ag-specific iPSC-Tregs were observed in LNs and spleen (Supplementary Fig. 5). A possible working mechanism of stem cell-derived auto Ag-specific Tregs is mediated through the secretion of large amounts of suppressive cytokines (e.g., IL-10 and TGF-β) that was identified (Fig. 6). Of note, Ag-specific iPSC-Tregs have a functional
stability for which we had previously demonstrated in an autoimmune condition (12), and FoxP3 expression in these cells persisted for six weeks after adoptive transfer in the diabetic mice (Fig. 3).

We further show that ICAM-1 (also known as CD54), a member of the immunoglobulin (Ig) like superfamily of adhesion proteins, plays a critical role in the accumulation of the pathogenic CD8\(^+\) T cells in the pancreases of the mice with autoimmune diabetes. ICAM-1 is a cell surface glycoprotein expressed in endothelial cells and some immune cells. ICAM-1 expressed in the diabetic pancreatic tissues can bind to integrins of type CD11a/CD18, or CD11b/CD18 on pathogenic CD8\(^+\) T cells, and this binding facilitates the accumulation of pathogenic CD8\(^+\) T cells in the panaceas. The expression of ICAM-1 in the pancreases of diabetic mice is much higher than that in normal mice. In contrast, in the pancreases of diabetic mice, the adoptive transfer of auto Ag-specific iPSC-Tregs can dramatically reduce the expression of ICAM-1. This cell transfer can also reduce the accumulation of autoreactive CD8\(^+\) T cells in the pancreases and eventually protect the pancreatic β cells from further devastation (Fig. 5). This conclusion is in line with the previous studies showing that the expression of ICAM-1 is critical for the development of experimental autoimmune encephalomyelitis (42) (43).

The role of IFN-γ, a prototypical Th1 cytokine, has been appreciated in the development of T1D, because the treatment of NOD mice with an anti-IFN-γ monoclonal antibody prevented the development of the autoimmune diabetes (23, 24). We detected an elevated amount of IFN-γ in both of effector CD8 and CD4 T cells of the diabetic islets and showed that adoptive transfer of stem cell-derived auto Ag-specific Tregs markedly decreased the production of the pro-inflammatory cytokine in the pathogenic T cells. We demonstrated that the auto Ag-specific iPSC-Tregs protected the islets from mass destruction via suppressing the production of pathogenic IFN-
\( \gamma \) (Fig. 7). As other pro-inflammatory cytokines such as interleukin-21 (IL-21) (44), IL-17 (45), tumor necrosis factor-alpha (TNF\(\alpha\)) (46), and IL-1\(\beta\) (47) also play roles in the pathogenesis of T1D, studies on these pro-inflammatory cytokines would be needed to better exploit the stem cell-derived tissue-associated Tregs as a therapeutic approach against autoimmune disorders.

The transgenic model used in this study highly expressed OVA as surrogate auto Ag and received approximately two million of OVA-specific iPSC-Tregs for the treatment, which may be much higher than in clinical setting with diverse low abundance target peptides. However, the understanding of islet Ags has previously resulted in translation into new strategies by targeting tissue-specific immune interventions to prevent disease progression as well as to reverse T1D. A big advantage of our approach is to develop large numbers of auto Ag-specific nTreg-like suppressive cells (Fig. 1 and Supplementary Figs. 1-2) which can accumulate in the inflamed tissues and suppress local pathogenic immune cells. In addition, the stem cell-derived Tregs maintain the stability up to 3 months (11, 12), because the over-expression of FoxP3 has the ability to suppress the potential switch of T\(_{\text{regs}}\) to Th17 cells. Of note, the selection of optimal auto Ags for the development of tissue-associated Tregs is critical for the success of the Treg-based T1D immunotherapy.

Taken together, the current study provides a new insight into the mechanisms by which stem cell-derived tissue-associated Tregs suppress autoimmunity in T1D and suggests that stem cell-derived auto Ag-specific Tregs have a great potential to be adapted as an immunotherapy for T1D.
Methods

Cell lines and mice

Mouse iPS-MEF-Ng-20D-17 cell line was obtained from the RIKEN Cell Bank (48). The OP9-DL1/DL4/I-A^b cell line was generated by a retroviral transduction of the OP9 cells (12). SNL76/7 cell line (ATCC® SCRC-1049™) was purchased from ATCC (Manassas, VA). C57BL/6 (B6), Rag1^−/−, B6 mOVA RIP (RIP-mOVA) Tg and OT-I TCR Tg mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Cell culture

iPSCs were maintained on feeder layers of irradiated SNL76/7 cells in 6-well culture plates (Nunc), and were passaged every 3 days (48).

Retroviral transduction and generation of OVA-specific iPSC-Tregs

cDNA for FoxP3 with ovalbumin (OVA) 322-339 (ISQAVHAAHAEINEAGR)-specific I-A^b-restricted TCR genes (Vα2 and Vβ5; obtained from Dr. Dario A. Vignali, University of Pittsburgh, Pittsburgh, PA) or LCMV (SMARTA1; SM1) gp61 (GLKGPDITYKGYQFDSVEFD)-specific I-A^b-restricted TCR genes (Vα2 and Vβ8; obtained from Dr. Matthew A. Williams, University of Utah, Salt Lake City, UT) was used for retroviral transduction of mouse iPSCs and the generation of OVA or SM1-specific iPSC-Tregs (12).

Antibodies

PE-, PE/Cy7, Alexa 647, APC or APC/Cy7-conjugated anti-mouse TCRVβ5 (MR9-4), CD4 (GK1.5), CD11b (M1/70), TGF-β1(TW7-16B4) and FoxP3 (MF-14) were obtained from
Biolegend (San Diego, CA). FITC- or PE-conjugated anti-mouse CD8 (6A242) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). APC-conjugate IL-10 (JES5-16E3) was purchased from BD Biosciences (San Diego, CA). Rabbit insulin antibody (C27C9; #3014) was purchased from Cell Signaling (Beverly, MA) and anti-ICAM1 (YN1/1.7.4; ab25375) antibody was obtained from Abcam (Cambridge, MA).

**Flow Cytometric Analysis**

T cells from the pancreatic lymph nodes (LNs) were collected and intracellular IL-10 and TGF-β and were analyzed by flow cytometry after gating on live CD4⁺ or CD8⁺ cells.

**RT-PCR**

MiDR OTII 2A FoxP3-transduced mouse iPSCs were co-cultured with OP9-DL1-DL4-I-Aᵇ in the presence of mFlt-3L for 7 days. Differentiated iPSCs were separated and adoptively transferred into Rag1⁻/⁻ mice. Mice were housed for 6 weeks for *in vivo* maturation of the iPSC-Tregs. Six weeks later, spleen and LNs were collected from normal B6 mice and cell transferred Rag1⁻/⁻ mice. CD4⁺CD25⁺ cells were sorted and total RNA was extracted from sorted cells using QIAgen RNeasy mini kits. Samples were subjected to reverse transcription using a high capacity cDNA synthesis kit (Applied Biosystems). PCR analysis was performed by TaqMan real-time PCR (Thermo Fisher Scientific).

**Ex vivo stimulation assay**

Mice were sacrificed 4 weeks after adoptive cell transfer and single cell suspension was then prepared from the pancreatic LNs. Cells were stimulated with plate-coated CD3 plus soluble CD28
antibodies. Productions of IL-10 and TGF-β were determined by intracellular cytokine staining (12).

**Murine autoimmune diabetes model**

Autoimmune diabetes was induced in F1 mice that were crossed between RIP-mOVA Tg with OT-I TCR Tg mice by *i.p.* injection with vaccinia viruses expressing OVA (VACV-OVA, 2×10⁶ PFU/mouse) (49). All of the mice developed diabetes after viral injection. Blood glucose was measured one week after the VACV-OVA injection.

**Blood glucose measurement**

Blood glucose levels were determined using the Glucometer Ascensia Elite XL (Bayer). Six hundred milligrams per deciliter is the maximum measurable glucose reading. Mice were typically considered diabetic with readings of >250 mg/dl.

**Adoptive cell transfer**

B6-mOVA Tg x OT-I TCR double Tg mice (F1) were immunized with VACV-OVA for a week. At week 10, OVA-specific pre-iPSC-Tregs or iPSC-derived control cells (3×10⁶) were *i.v.* transferred into diabetic mice. Four–six weeks later, mice were euthanized and the pancreas tissues were removed for histopathological examination.

**Histology and immunohistochemistry**

For H&E staining, pancreas tissues were fixed with 10% neutral formalin solution (VWR, West Chester, PA), and the fixed samples were prepared and stained as described (48).
immunofluorescent microscopy, the pancreas tissues were frozen in cryovials on dry ice immediately after resection. Cryo-sectioning and immunofluorescent staining were performed as described (48).

Statistical analysis

Multiple Student’s 1-tailed t test, one-way ANOVA or two-way ANOVA analysis was performed to analyze the differences between the groups, using GraphPad Prism (GraphPad Software, San Diego, CA), and a P value less than 0.05 was considered significant.

Study approval

The present studies in mice were reviewed and approved by The Texas A&M University Animal Care Committee (IACUC; College Station, TX) and in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.
Author contributions

J.S. and J.Y. designed the experiments, analyzed data, and contributed to the writing of the paper. M.H., F.L. and J.K.D. performed the experiments. D.F. and S.S. provided reagents in the animal model. X.X. and X.R. analyzed data.
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Guarantor’s statement: J.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References


Legends to Figures

Figure 1. Characterization of auto Ag-specific nTreg-like iPSC-Tregs. PCR analysis was performed by TaqMan real-time PCR. Primers for sequences were used as follows: (A) Nr4a1. fw 5'-TGTGAGGGCTGCAAGGGCTTC-3' rv 5'-AAGCGGCAGAACTGGGAGCGG-3'; (B) Il2ra. fw 5'-AECTGCCAGTGCAACCAGCAAC-3' rv 5'-GAGGTGGCTCCCTGCAAGTGAC-3'; (C) Ikzf4. fw 5'-CAATCTGCTTGCACATCAAG-3' rv 5'-GCCACAGTAGTTGCACCTTGAG-3'; (D) Tbx21. fw 5'-GGAGCCACCTGGATGCGCCAG-3' rv 5'-AGGCAGCCTCTGGCTCTCCATC-3', Tnfrsf18- fw 5'-CCTGCCAAACCAGGCCAGGG-3' rv 5'-GTCCAAAGTCTGACTGACAGCC-3'; and (E) Bcl6. fw 5'-CACACCCGTCATCATGAA-3' rv 5'-TGTCCTAAGGTCGGCTTTTT-3'. Data shown are representative of three identical experiments. The values represent mean ± S.E.M. (n = 3). ns, p>0.05, Student’s 1-tailed t test.

Figure 2. Accumulation of activated CD8+ T cells in auto Ag-expressing pancreas in diabetic mice. Pancreases were isolated from the B6-mOVA × OT-I TCR double Transgenic (Tg) mice and B6 mice at age of 9 weeks, including a week in which mice were challenged with vaccinia viruses expressing ovalbumin (VACV-OVA). (A) Detection of OVA expression by immunohistochemically staining. OVA expression (∩) is indicated (200x magnification). Data are representative of five mice per group of three independent experiments. (B) CD8+ T cell infiltration
in the pancreas. CD8+ T cells (↑) are indicated. Data are representative of five mice per group of three independent experiments. (C) OVA-specific CD8+ T cells in the pancreas. OVA-specific TCRVβ5 was analyzed by flow cytometry, after gating on CD8+ populations from the pancreas. Data shown are representative of three independent experiments (p<0.001, Student’s 1-tailed t test). (D) Summarized analyses of OVA-specific CD8+ T cells in various locations. Data shown are representative of five mice per group of three independent experiments. Data shown are representative of three individual experiments. The values represent mean ± S.D. **, p<0.01, ns, p>0.05, multiple Student’s 1-tailed t test. (E) Blood glucose measurement. Data shown are representative of three individual experiments (n=5). The values represent mean ± S.D. **, p<0.01, Student’s 1-tailed t test.
Figure 3. Accumulation of auto Ag-specific iPSC-Tregs in the pancreas of diabetic mice following adoptive transfer. B6-mOVA Tg x OT-I TCR double Tg mice were immunized with VACV-OVA. At week 10, cell control, OVA or SM1-specific pre-iPSC-Tregs were transferred into diabetic mice. Before or after the cell transfer, mice were sacrificed and their pancreas were isolated for analysis of CD4 and FoxP3. (A) Immunohistology at week 13 (200× magnification). (B) Flow cytometric analysis at week 10 (before the cell transfer), 13 and 16. Data are representative of five mice per group of three independent experiments.

Figure 4. Prevention of destruction of pancreatic β cells and suppression of autoimmune diabetes by auto Ag-specific iPSCs-Tregs. Cell control or pre-iPSC-Tregs were adoptively transferred into diabetic mice as described in Fig. 3. (A) Blood sugar measurement at various weeks. Data shown are representative of five mice per group in three independent experiments. **, p<0.001; ***, p<0.0001, two-way ANOVA analysis. (B) Immunofluorescence detection of pathogenic immune cells. Mice were sacrificed and the pancreases were isolated for immunohistochemistry staining with CD8+ T cells (200× magnification). Data are representative
of five mice per group in three independent experiments. (C) Flow cytometric analysis of pathogenic immune cells. Pancreatic lymph nodes (LNs) were isolated and single cell suspensions were prepared for OVA-specific CD8+ TCRVβ5+ staining and analyzed by flow cytometry. Data shown are representative of three identical experiments (p<0.001, Student’s 1-tailed t test). (D) Percent incidence of diabetes at week 22. Data shown are representative of five mice per group in three independent experiments. The values represent mean ± S.D. *** p<0.001, one-way ANOVA analysis. (E) Representative photomicrographs (HE staining) of the islet inflammation (200× magnification). Cellular infiltrations (↑) are indicated. Data are representative of five mice per group in three independent experiments. (F) Islet count from sections of 5 individual pancreases in each group. Data are represented as the mean ± S.D. of three independent experiments (*** p<0.001, one-way ANOVA analysis). (G) Representative photomicrographs (Immunofluorescence staining) of islet destruction (200× magnification). Insulin-producing cells (↑) are indicated. Data are representative of five mice per group in three independent experiments.
**Figure 5. Reduction of ICAM-1 expression in the diabetic pancreas by auto Ag-specific iPSC-Tregs.** Mice were sacrificed 4 weeks after adoptive cell transfer and the pancreases were isolated from non-diabetic, diabetic (cell transfer control) and diabetic (Ag-specific iPSC-Treg transfer) mice. Samples were prepared for immunohistochemistry staining. (A) ICAM-1. ICAM-1\(^+\) zones (↑) are indicated (200× magnification). Data are representative of five mice per group in three independent experiments. (B) Quantification of ICAM-1 expression from sections of 5 individual pancreases in each group. Data are represented as the mean ± S.D. from three independent experiments (** \( p<0.01 \), *** \( p<0.001 \), one-way ANOVA). (C, D) Flow cytometry analysis of ICAM-1 expression in pancreatic CD4\(^+\) T or CD11b\(^+\) cells. The pancreatic lymph nodes (LNs) were isolated and single cell suspensions were prepared for ICAM-1 staining on CD4\(^+\) or CD11b\(^+\) cells and analyzed by Flow cytometry. Data shown are representative of three identical experiments.
Figure 6. Induction of IL-10 and TGF-β by auto Ag-specific iPSC-Tregs. Mice were sacrificed 4 weeks after adoptive cell transfer, and single cell suspension was prepared from the pancreatic lymph nodes (LN). Cells were stimulated with plate-coated CD3 and soluble CD28 antibodies, and then stained with CD4, TGF-β and IL-10. CD4 population was gated and the production of TGF-β and IL-10 was analyzed. Data shown are representative of three identical experiments.

![Figure 6](image)

Figure 7. Down-regulation of IFN-γ by auto Ag-specific iPSC-Tregs in the diabetic pancreatic islets. Mice were sacrificed 4 weeks after adoptive cell transfer, and single cell suspension was prepared from the pancreatic lymph nodes (LN). Cells were stimulated with plate-coated CD3 and soluble CD28 antibodies, and then stained with CD4, CD8 and IFN-γ for flow cytometric analysis. (A) IFN-γ production in CD4+ or CD8+ population. Data shown are the
representative of three identical experiments. (B) Quantification of IFN-γ production. Data shown are the representative of three identical experiments (** p<0.01, *** p<0.001, one-way ANOVA).

Figure 7