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Stem Cell-Derived Regulatory T Cells for Therapeutic Use

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Abstract

CD4⁺ regulatory T cells (Tregs) are essential for normal immune surveillance, and their dysfunction can lead the development of autoimmune diseases. Pluripotent stem cells (PSCs) can be utilized to obtain a renewable source of healthy Tregs to treat autoimmune disorders as they have the ability to produce almost all cell types in the body, including Tregs. However, the right conditions for the development of antigen (Ag)-specific Tregs from PSCs (i.e., PSC-Tregs) have not been fully defined, especially the signaling mechanisms that the direct differentiation of such Tregs. Ag-specific PSC-Tregs can be tissue-associated and infiltrate to local inflamed tissue to suppress autoimmune responses after adoptive transfer, thereby avoiding potential overall immunosuppression from non-specific Tregs. Development of cell-based therapies using Ag-specific PSC-Tregs will provide an important step toward personalized therapies for autoimmune disorders.

Keywords: regulatory T cells, pluripotent state cells, cell differentiation, immunotherapy, autoimmunity

1. Introduction

Regulatory T cells (Tregs) are a component of the normal immune system and contribute to the maintenance of peripheral tolerance. Tregs are defined phenotypically by the expression of the interleukin (IL)-2 receptor α -chain (CD25) and the transcriptional factor, forkhead box P3 (FoxP3), which is required for the Treg development and controls a genetic program specifying cell fate. Tregs can down-regulate immune responses and are essential for immune homeostasis [1]. Tregs are key effectors in preventing and treating autoimmune disorders, for

example, rheumatoid arthritis (RA), type 1 diabetes (T1D), and inflammatory bowel diseases (IBD), and controlling both the transplant rejection graft-versus-host disease (GVHD) [2–5].

Tregs or suppressors of T cells, as they had been originally named, have been studied since early 1970s. In 1970, Gershon and Kondo reported that T cells not only augmented but also have the ability to dampen immune responses. The immune dampening was mediated by a special class of T cells that were different from helper T cells; this special type of T-cell population is known as suppressor T cells and is found in CD4⁺ population [6]. It is well understood that CD4⁺ Tregs are essential for normal immunological self-tolerance and immune homeostasis. Failure of immunologic self-tolerance often leads to the development of autoimmune disorders, which are estimated to afflict up to 5% of the population. Although there are a number of debates regarding the etiology of autoimmune diseases, it is well documented that T cells are the key mediators of many autoimmune disorders, such as autoimmune arthritis, autoimmune thyroiditis, and insulin-dependent diabetes mellitus (IDDM). There are various mechanisms for establishing and sustaining immunological self-tolerance and immune homeostasis. In addition to these, T cell-mediated suppression of immune responses toward self and non-self antigens (Ags) has recently attracted enormous interest [7]. T-cell-mediated suppression is mainly achieved by FoxP3⁺ Tregs, which play an important role in the prevention and suppression of autoimmunity. The deficiencies in Treg function have been identified in a wide variety of human autoimmune disorders, such as IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) [8–10]. As the Treg numbers are important for effective suppressive function, adoptive transfer of exogenous Tregs would be ideal for the treatment of autoimmunity or Treg-deficient diseases.

Tregs comprise of 5–10% of the mature CD4⁺ T helper cell subpopulation in mice and about 1–2% of CD4⁺ T cells in human. As a result, it is crucial to develop large numbers of Tregs *in vitro* and adoptively transfer them for cell-based therapies. There has been no approach described for isolating a large number of Tregs with % specificity. Therefore, the attention has now been focused on utilizing pluripotent stem cells (PSCs) as a source for obtaining Ag-specific Tregs. PSCs can be utilized to obtain a renewable source of healthy Tregs to treat autoimmune disorders as they have the ability to produce almost all cell types in the body, including Tregs. However, optimal conditions and signaling network for the development and differentiation of Ag-specific Tregs from PSCs (*i.e.*, PSC-Tregs) have not been fully defined [11]. Ag-specific PSC-Tregs serve as a better choice of Tregs for cell-based therapies as they can accumulate in local inflamed tissues to suppress autoimmune responses after adoptive transfer, thereby avoiding potential overall immunosuppression from non-specific Tregs.

2. CD4⁺ and CD8⁺ Tregs

Tregs are an integral component of the normal immune system and contribute to the maintenance of peripheral tolerance. Tregs can down-regulate immune responses and are essential for immune homeostasis. There are two major classes of Tregs: CD4⁺ and CD8⁺ Tregs. CD4⁺ Tregs consists of two types: Naturally occurring Tregs (nTregs) that is characterized by

constitutively expression of CD25 and FoxP3, and adaptive or inducible Tregs (iTregs) that are induced upon persistent Ag exposure in the periphery.

nTregs develop in the thymus. nTregs originate as CD4⁺ cells which highly express CD25, the alpha chain of the IL-2 receptor, and the transcription factor FoxP3. Of the total CD4⁺ T-cell population, approximately 5–10% are nTregs, which can be visualized at the single-positive (SP) stage of development in the thymus [12]. nTregs are positively selected in the thymus and represent the thymocytes with a relatively high avidity for self-Ags. The signals for the development of Tregs are the interaction between the T-cell receptor (TCR) and the complex of *MHC* (major histocompatibility complex) class II molecules with self-peptide expressed on the thymic stroma [13]. It is also known that nTregs are essentially cytokine independent during the development.

iTregs originate from the thymus as a single-positive CD4⁺ cells. They differentiate into CD25- and FoxP3-expressing Tregs following adequate antigenic stimulation in the presence of cognate Ag and specialized immune-regulatory cytokines, such as TGF- β , IL-10, and IL-4 [14].

3. Treg development

Till today, several models have been proposed for the development of Tregs in the thymus. The interaction of costimulatory molecule CD28 and its B7 family member ligands is important for the development of nTreg in the thymus. CD28 ligand B7.1 and B7.2 are primarily expressed on thymic *antigen-presenting cells* (APCs), including dendritic cells (DCs), and epithelial cells. Although the role of this costimulation in the development of nTregs is not clear, it has been reported that mice deficient in the costimulatory molecule CD28 or CD28 ligands B7.1/B7.2 has decreased numbers and percentages of thymic nTregs [15–17]. One possible function is that they provide a quantitative signal along with TCR stimulation that drives T cells to develop into nTregs. This costimulation has the ability to function in preventing negative selection and supporting nTreg development [18]. NF- κ B is a transcription factor that functions the downstream of the CD28/B7 costimulation. NF- κ B family member c-Rel has been shown to be a critical factor for the development of nTregs in the thymus [19]. TCR engagement leads to the expression of high-affinity IL-2 receptor (CD25), which causes IL-2-induced FoxP3 expression and nTreg commitment [20]. Published evidence showed that IL-2-deficient mice exhibited 50% reduction of nTregs in the thymus [21]. Therefore, IL-2 is an important cytokine required for the development of nTregs. FoxP3 is another important transcription factor for the development of nTregs. Mice deficient in FoxP3 or harboring a mutated FoxP3 gene developed lethal multi-organ inflammation. Adoptive transfer of FoxP3⁺ T cells into neonates protected FoxP3-deficient mice from their autoimmune pathology [22, 23]. Clearly, FoxP3 is required for the nTreg development and affects its functional activity.

Hematopoietic stem cell (HSC)-derived hematopoietic progenitors migrate into the thymus and develop into different types of T cells. The development of $\alpha\beta$ T cells in the thymus is a highly ordered process. The most immature thymocyte population (CD4⁻CD8⁻) is referred to as *double negative* (DN) cells. DN precursors are subdivided into sequential developmental

subsets as follows: DN1 (CD44⁺CD25⁻), DN2 (CD44⁺CD25⁺), DN3 (CD44⁻CD25⁺), and DN4 (CD44⁻CD25⁻). Recombination activating genes (*Rag*), including *Rag1* and *Rag2* catalyze the TCR β locus for rearrangement, which is initiated during cell transit from the DN2 to the DN3 stage. A functional TCR β chain generated only in DN3 cells has the ability to pair with the invariant pre-T α /CD3 and create a pre-TCR. The DN3 cells with pre-TCR are selected and can continue to differentiate to DN4. This episode is called β -selection, which is the initial checkpoint of T lymphocyte differentiation in the thymus. Pre-TCR formation in DN3 cells drives cell differentiation and ends the rearrangement of TCR β locus, resulting in the development of the CD4⁺CD8⁺ *double positive* (DP) cells from DN3 cells [24].

In summary, the Treg development within the thymus includes a series of processes—positive selection (*e.g.*, TCR rearrangement) and negative selection (*e.g.*, clonal deletion) [25]. The autoimmune regulator Aire (largely expressed in thymic medullary epithelial cells—TECs) [26] and FoxP3 have key functions in clonal deletion and Treg selection [27, 28]. There are links among Aire expression, FoxP3 up-regulation, and Treg selection [29–31]. Evidence suggests that Aire deficiency affects the negative selection of self-reactive T cells and FoxP3 controls the development and function of the nTregs [29].

4. Ag-specific induction of Tregs

The mechanisms of acquisition of self-tolerance by the immune system are still being investigated. However, a widely accepted mechanism is the deletion of immature thymocytes before acquiring functional maturity in the thymic cortex and medulla [32, 33]. However, some self-reactive cells can escape to the periphery by breaking central tolerance [34, 35]. It is possible that such cells contain TCRs that recognize weak self-epitopes and as such, these autoreactive cells require peripheral tolerance to counteract them. Maintenance of peripheral tolerance, such as the deletion or reversible anergy of T cells, is performed by Tregs [36, 37]. The identification and characterization of Tregs definitively confirmed the existence of dominant tolerance [23, 38, 39]. Generating the peripheral Tregs in diverse microenvironments, for example, the gut, as well as the specific locations with tumors or microbes maintains local homeostasis. Tregs engendered in the peripheral tissue, external of the thymus, stay by way of resting cells on inter-mitotic phase, self-regulating further supply of agonist ligand which drives the formation of Tregs. This critical feature of the immune system lets the Tregs an approaching production to conquer undesirable immunity. As soon as encountering agonist TCR ligand, Tregs have the ability to migrate to Ag-draining lymph nodes in which Tregs undertake substantial expansion [40, 41]. The specificity of Treg-mediated suppression results from the corecruitment of Tregs and other T cells in Ag-draining lymph nodes. As a result, Tregs with certain specificity have the ability to suppress various effector cells through distinctive specificity when restricted in the area of identical APCs [42, 43]. At locations of inflammation, suppressive function, including the suppression of Th1 and Th17 responses, needs Tregs' trafficking and migration in tissues and secondary or peripheral lymphoid organs, such as lymph nodes and the spleen [44]. In addition, compared with naive or activated T cells, FoxP3-expressing Tregs have a distinct transcriptional profiling, showing a different

number of differentially expressed genes, including certain genes generally up-regulated in activated T cells, for example, *IL2ra*, *Ctla4*, and *Tnfrsf18*, which individually contribute CD25, CTLA4, and glucocorticoid-induced TNF receptor (GITR). Therefore, these target genes represent the transcriptional induction of the Treg signature by FoxP3 [45, 46].

5. Treg-mediated suppression

Tregs play an important role in the maintenance of immune tolerance. Sakaguchi and colleagues first described the importance of Tregs in the prevention of autoimmune disease development. They demonstrated that adoptive transfer of CD4⁺ T cells, depleted of CD25⁺ T cells by a specific monoclonal antibody against CD25 into BALB/c athymic nude mice, caused spontaneous development of T-cell-mediated autoimmune disease [47]. When these mice were reconstituted with CD4⁺CD25⁺ T cells, the autoimmune disease development was successfully ameliorated within a brief period post transfer. The discovery of the X chromosome-encoded transcription factor FoxP3 as a specification and maintenance factor subsequently confirmed CD4⁺CD25⁺ T cells as a unique thymus-derived lineage. Examining their role in the murine immune system revealed that Tregs have a central role in immune homeostasis: Genetic defects resulting in the dysfunctional Tregs result in multi-organ autoimmune disease, and the depletion of nTregs induces autoimmunity. The next question to be addressed is to define the mechanisms of Treg-mediated suppression. It has already been established that Foxp3⁺CD25⁺CD4⁺ nTregs suppress the proliferation of naive T cells and their differentiation to effector T cells *in vivo*. They also have the ability to suppress effector activities of differentiated CD4⁺ and CD8⁺ T cells and the function of natural killer (NK) cells, natural killer T (NKT) cells, B cells, macrophages, osteoclasts, and DCs [48–50]. Tregs suppress the proliferation and cytokine production (in particular of IL-2) of responder T cells when the two populations are cocultured *in vitro* and stimulated by Ag in the presence of APCs [51]. Once activated by a particular Ag, Tregs can suppress responder T cells irrespective of whether they share Ag specificity with the Treg [52].

Several mechanisms of Treg-mediated suppression have been proposed, including the secretion of immunosuppressive cytokines, cell contact-dependent suppression, and functional modification or killing of APCs. For example, IL-10 and TGF- β contribute to the suppression of arthritis in Ag-induced arthritis mice [11]. Another study showed that IL-10 and TGF- β contribute to the suppression of IBD induced in mice by Treg depletion [53]. Another mechanism for the killing of responder T cells or APCs is cell-to-cell contact. Tregs can secrete granzyme or perforin to destroy target T cells or APCs by cell-to-cell interaction method, or deliver a negative signal by CTLA-4 or FasL to responder T cells. Potential critical signals involve up-regulation of intracellular cyclic adenosine monophosphate (cAMP) that results in the suppression of T-cell proliferation and cytokine production, such as IL-2, or the production of pericellular adenosine catalyzed by CD39, that is, ectonucleoside triphosphate diphosphohydrolase 1, and CD73, that is, ecto-5'-nucleotidase, which are presented through Tregs [50]. Activated Tregs can cause down-regulation of CD80/86 expression on APCs or stimulate DCs to form the enzyme indoleamine 2,3-dioxygenase, which catabolizes the essential amino acid tryptophan to kynurenines, causing toxicity to T cells. All these func-

tions are dependent on the expression of CTLA-4 on Tregs. Treg-mediated suppression can occur (1) *via* Ag-specific Tregs upon antigenic stimulation, which is highly mobile and swiftly recruited at the site of inflammation; (2) Ag-activated Tregs contacting DCs that restrict DC function, thereby hindering the activation of other T cells; and (3) through the secretion of granzyme/perforin, IL-10, or other immune suppressive cytokines, such as IL-35, depending on the strength and duration of antigenic stimulation and the local milieu of cytokines and chemokine.

CTLA-4 is particularly critical for Treg function in spite of a number of distinct molecules that are associated with Treg suppression. CTLA-4 is constitutively expressed on FoxP3⁺ Tregs, and FoxP3 regulates its expression. The blockage of CTLA-4 abolishes Treg-mediated inhibition. Moreover, fatal autoimmunity and inflammation appear in the germline removal as well as Treg-specific conditional deficit of CTLA-4. The fate of responder T cells that are suppressed by Tregs is also unclear, that is, whether they remain non-activated, die by apoptosis, or become anergic. Additional studies are required to elucidate the molecular basis of suppression mediated by Tregs.

Recent advances in the use of large-scale *in vitro* expansion of Tregs followed by *in vivo* reinfusion of these cells raises the possibility that this strategy may be successfully utilized for the treatments of autoimmune disorders. While cell-based therapies using Tregs are currently largely recognized in animal experimental tests, up to the present time, cell-based therapies using Tregs have not been clinically utilized in the suppression of autoimmune disorders.

There are numerous issues to be solved for using Tregs in humans, such as the requirement for vigorous methods to separate and grow these cells. *First*, only low numbers of Tregs can be harvested from the peripheral blood mononuclear cells (PBMCs). CD4 and CD25 have been used to isolate Tregs for *ex vivo* expansion. CD4⁺CD25⁺ T cells are not homogenous and contain both Tregs and conventional effector T cells (Teffs). Current expansion protocols activate both Tregs and Teffs, and because it takes a longer time for Tregs to enter the S phase of cell cycle, Teffs outgrow Tregs [54]. In addition, Tregs can lose suppressive activity after repetitive stimulation with α -CD3 plus α -CD28 Abs with or without rIL-2 *in vitro* [55–57]. *Second*, despite a growing number of published purification protocols for isolating subsets of Tregs, no approach to date has demonstrated the capacity to isolate the entire Treg population with 100% specificity from patients (the current clinical approach). Even FoxP3 or more recently Eos, a transcriptional factor, considered the gold standard for identification of Tregs, is expressed transiently in some activated non-regulatory human T cells [58], highlighting the difficulty in both identifying and isolating a pure Treg population. Adoptive transfer of non-regulatory Teffs with Tregs has a potential to worsen diseases. *Third*, gene transduction of CD4⁺ T cells from PBMCs with Ag-specific TCR [59–61] or chimeric Ag receptor (CAR) [59–61, 63] elicits generation of suppressive T-cell populations [64, 65] and overcomes the hurdle of the limited numbers of Ag-specific T cells. Moreover, gene transduction of human PBMC with Ag-specific TCR generated functional Ag-specific T cells, which targeted tissue-associated inflammation [63]. However, the engineered Tregs express endogenous and exogenous polyclonal TCRs, which may reduce their therapeutic potential (the current experimental approach) [66, 67]. Also, TCR mispairing is a concern with regard to the safety of TCR gene-transferred Tregs for

clinical use, because the formation of new heterodimers of TCR can induce immunopathology [68]. Therefore, there is a need to improve this strategy and generate monoclonal Tregs. *Fourth*, the differentiation state of Tregs is inversely related to their capacity to proliferate and persist [69, 70]. The “right” Tregs resist terminal differentiation, maintain high replicative potential (*e.g.*, expression of common- γ chain – γ_c , CD132), are less prone to apoptosis (*e.g.*, low expression of PD-1), and have a greater ability to respond to homeostatic cytokines [71], which facilitates their survival. In addition, the “right” Tregs express high levels of molecules that facilitate their homing to lymph nodes (LNs), such as CD62L and CC-chemokine receptors (*e.g.*, CCR4, CCR7), and maintain stability or plasticity under certain inflammatory conditions. Furthermore, after an effective immune response, the “right” Tregs persist in a variety of differentiation states, providing protective immunity. Thus, the “right” Tregs are the superior subsets for use in cell-based therapies. *Finally*, because there are too few cells, harvesting sufficient numbers of tissue-associated Tregs from patients’ PBMC for TCR gene transduction can be problematic.

Taken together, strong arguments support the development of Treg-based therapies in autoimmune disorders using engineered Tregs. While clinical trials show the safety, feasibility, and potential therapeutic activity of Treg-based therapies using this approach, concerns about autoimmunity due to cross-reactivity with healthy tissues remain a major safety issue [72, 73]. In addition, genetically modified Tregs using current approaches are usually intermediate or later effector Tregs [74], which only have short-term persistence *in vivo*.

6. Stem cell-derived Tregs

To date, stem cells are the only source available to generate a high number of the “right” Tregs [75, 76]. It has been already demonstrated that induced pluripotent stem cell (iPSCs) are like embryonic stem cells (ESCs) in different aspects, including the expression of definite stem cell genes and proteins, doubling time, embryoid body formation, viable chimera formation, potency and differentiability, chromatin methylation patterns, and teratoma formation [77]. However, the similarity between iPSCs and ESCs is still being assessed [78]. The generation of iPSCs from the mouse and human somatic cells has garnered considerable attention. Research has shown that iPSCs could be generated from the mouse and human somatic cells by introducing Oct3/4 and Sox2 with either Klf4 and c-Myc or Nanog and Lin28 using retroviruses or lentiviruses-mediated transduction [79–82]. Thus, iPSC technology continues to progress rapidly, and clinically applicable iPSCs can be generated from patients with noninvasive medical procedures. Many genetic methods as well as protein-based approaches have been developed to produce iPSCs with potentially reduced risks, including that of immunogenicity and tumorigenicity [83]. Because of the plasticity and the potential for an unlimited capacity for self-renewal, iPSCs have great potential to be used in cell-based therapies for autoimmune disorders comparable to ESCs and HSCs.

Previously, T cells have been demonstrated to be differentiated from ESCs and HSCs; recently T cells [84] and Ag-specific CD8⁺ cytotoxic T lymphocytes (CTLs) [85] have been confirmed to

be differentiated from iPSCs. In these investigations, the researchers genetically modified mouse iPSCs with Ag (ovalbumin, OVA)-specific MHC II (I-A^b)-restricted TCR and FoxP3, and then *in vivo* differentiated the iPSCs into functional Ag-specific CD4⁺ Tregs, which dramatically prevented the mice from Ag-induced arthritis. Thus, a new approach to generate a high number of functional Tregs from iPSCs may be used for the treatments of autoimmune disorders. In fact, Ag-specific Tregs can be *in vitro* generated from iPSCs through a Notch-mediated signaling. It has been shown that Ag-specific Tregs were generated from iPSCs or ESCs genetically modified with the FoxP3 and Ag-specific TCR followed by stimulation with an *in vitro* Notch signaling. Furthermore, adoptive transfer of these stem cell-derived Ag-specific Tregs had the ability to secrete a large amount of suppressive cytokines, including TGF- β and IL-10, and suppressed autoimmunity [11, 75]. Additionally, forced expression of Ag-specific TCR can suppress the expression of recombination-activating (*Rag*) genes, resulting in a uniform expression of Ag-specific TCR on iPSC or ESC-derived Tregs. As a result, this method has a potential to develop a great number of single-type Ag-specific Tregs.

Collectively, a large number of single-type Ag-specific Tregs can be generated by gene transduction of iPSCs with Ag-specific TCR with FoxP3 followed by T lineage differentiation through an *in vitro* Notch signaling or an *in vivo* approach. The Ag-specific Tregs may be applied for cell-based therapies of autoimmune disorders, such as T1D, and RA. Of note, these stem cell-derived Ag-specific Tregs are less differentiated and have the ability to persist *in vivo* after adoptive transfer. It can be predicted that the use of iPSCs as a mean to develop disease-specific immune cells for immunotherapy has a great potential in the prevention of many diseases. Therefore, iPSC-derived Ag-specific Tregs can be used in cell-based therapies for autoimmune disorders.

7. Stem cell-derived Ag-specific Tregs for therapeutic use

Treatments of autoimmune disorders with Tregs have been shown to work in a number of mouse models, such as T1D and RA. Tregs were activated with its cognate Ag and can suppress the conventional Tregs within the immediate vicinity regardless of specificity [86]. Additionally, this suppressive function extends to a wide range of other immune cells such as B cells, DCs, and monocytes, including naive, effector, and memory T cells. Ag specificity of Tregs is important to counteract the ongoing autoimmunity because high doses of polyclonal antibody may fail to keep in check autoimmune responses. Tregs require Ag specificity to home/be retained at the appropriate site or tissue, and exert active suppression where Ag specificity is chiefly determined by the individual TCR expressed. Under usual circumstances, a small population of Ag-specific Tregs exists within a polyclonal population. For therapeutic interventions, the generation of a large number of Ag-specific Tregs becomes essential. Furthermore, existing Tregs in patients are insufficient to prevent the initiation of autoimmunity, it is questionable whether simply putting a large number of these cells back into the patient, without modifying specificity or function, would have the desired effect. In addition, Tregs specific to tissue/organ (*e.g.*, joints, pancreas, intestine) facilitate stable FoxP3 expression and avoid the induction of a potentially harmful systemic immunosuppression [87, 88].

Therefore, in order for Tregs to be a viable treatment for autoimmune disorders, approaches for generating the populations of Ag-specific Tregs are essential.

Previous studies have demonstrated the broad application of genetic manipulation of PSCs for immunotherapy and have provided proof-of-principle data for using TCR gene-transduced PSCs for cell-based therapies [85, 89–91]. We also showed that functional iPSC-Treg differentiates *in vitro* mediated through the Notch signaling [11, 75]. Murine iPSCs were genetically modified with OVA-specific MHCII-restricted TCR (OTII) and FoxP3 by retrovirus-mediated transduction. Genetically modified iPSCs were stimulated with an *in vitro* Notch ligand to direct iPSC differentiation into functional OVA-specific Tregs, which were able to produce suppressive cytokines (TGF- β and IL-10), and inhibit other immune cell activities *in*

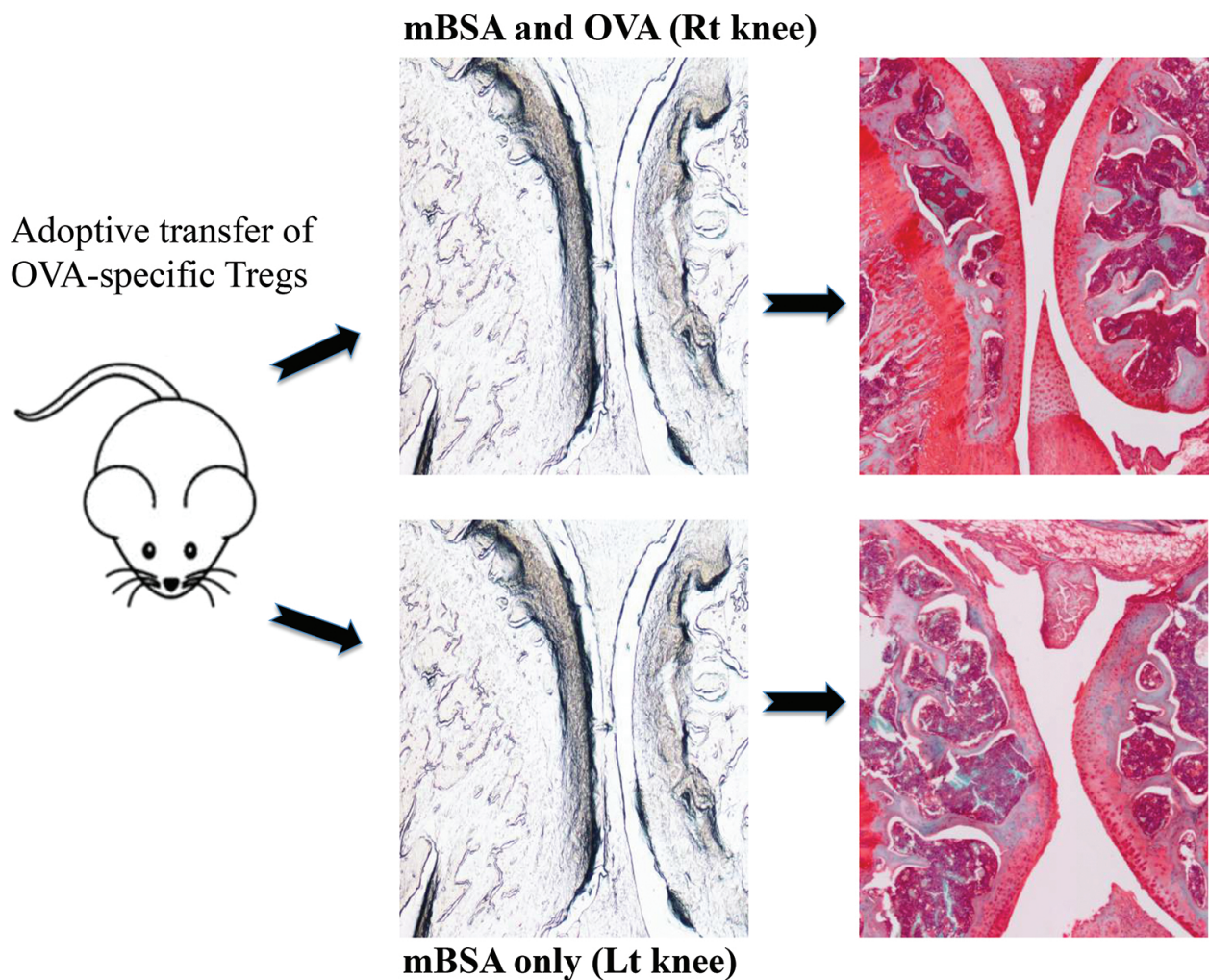


Figure 1. AIA in mice. Mice were immunized with methylated BSA (mBSA) followed by intra-articular knee re-challenge with mBSA to induce T cell-mediated tissue damage. In each animal, one knee (right, Rt) was injected with mBSA and OVA, and the contralateral control knee (left, Lt) was injected with mBSA only. As a result, the Ag-specific Tregs just recognized the OVA Ag in the Rt knee. Conversely, the arthritis-inducing T cells recognized the mBSA Ag in both knees. The data presented that while OVA was present (Rt knee), the transferred iPSC-derived Tregs essentially reduced the inflammatory knee swelling, however, did not protect the control Lt knee in which only mBSA was injected.

vitro. In addition, adoptive transfer of Ag-specific iPSC-Tregs significantly suppressed the development of autoimmunity in murine models.

Adoptive transfer of OVA-specific iPSC-Tregs in a well-established mouse model of Ag-induced arthritis (AIA) inhibited the development of arthritis [11]. In this murine model, arthritis was induced by intra-articular injection of methylated bovine serum albumin (mBSA) into the knee (Rt). To direct the transferred cells to the knees, OVA was injected into one knee and phosphate-buffered saline (PBS) was injected into the contralateral knee (Lt). Arthritis was characterized by swelling of the synovium and damage of the cartilage around the joints leading to the joint destruction (**Figure 1**). OVA-injected knees were protected from developing arthritis, where PBS injected the knee developed severe arthritis (**Figure 2**). Particularly, OVA-specific iPSC-Tregs infiltrate into the knee joints and maintain the Treg phenotype *in vivo* (**Figure 3**). These results indicate that genetically modified iPSC-derived Tregs are tissue-associated and are able to suppress autoimmune arthritis.

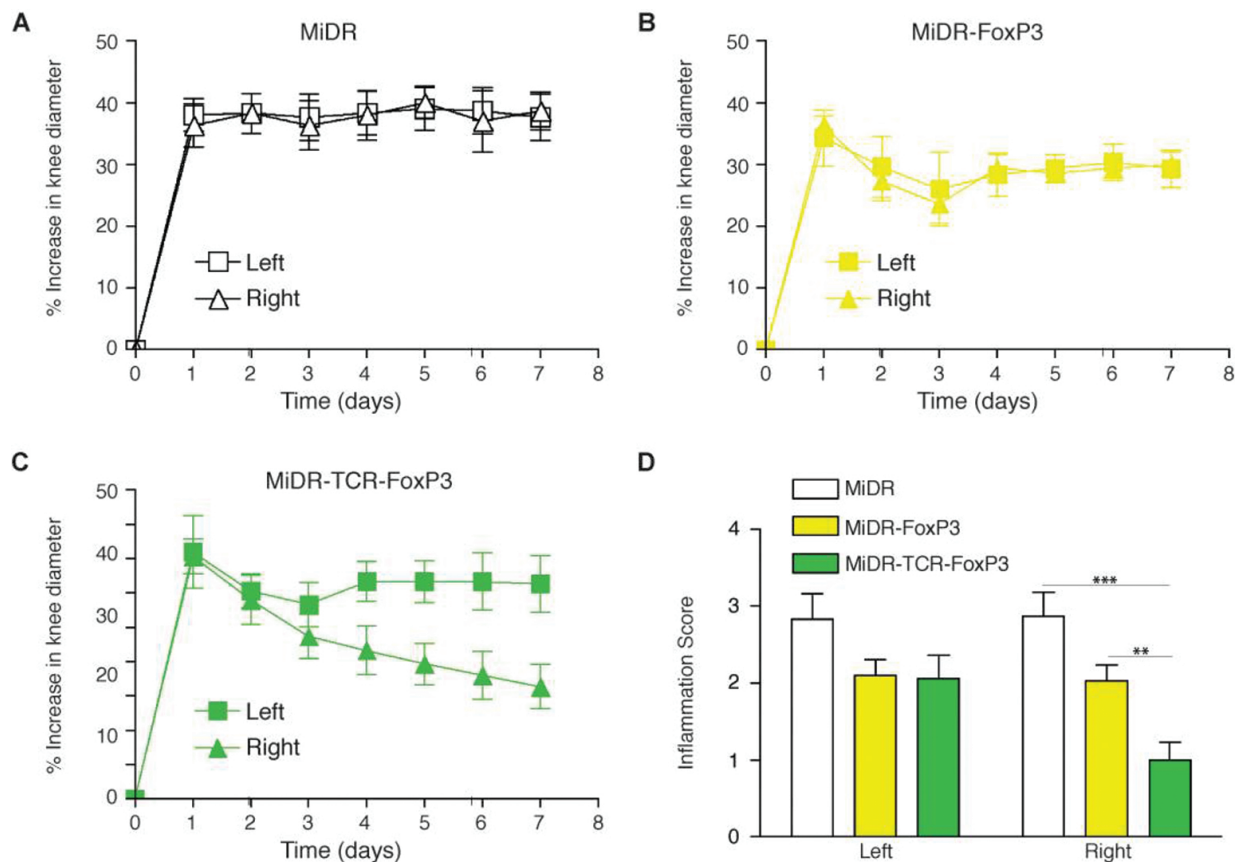


Figure 2. Ag-specific iPSC-Tregs ameliorate AIA in mice. Murine iPSCs transduced with the retroviral construct MiDR, MiDR-FoxP3, or MiDR-TCR-FoxP3 and were cocultured on the OP9-DL1/DL4/I-Ab cells. On day 7, the gene-transduced cells (3×10^6 /mouse) were *i.v.* adoptively transferred into C57BL/6 mice that were induced AIA 2 weeks later after the cell transfer. On the following day of arthritis induction, arthritis severity was monitored by the measurement of knee diameter. (**A–C**) % increase in knee diameter. (**D**) The mean scoring on day 7 for both knees from five mice. Data are represented as the mean \pm s.d. from three independent experiments (** $p < 0.01$, *** $p < 0.001$, two-way ANOVA).

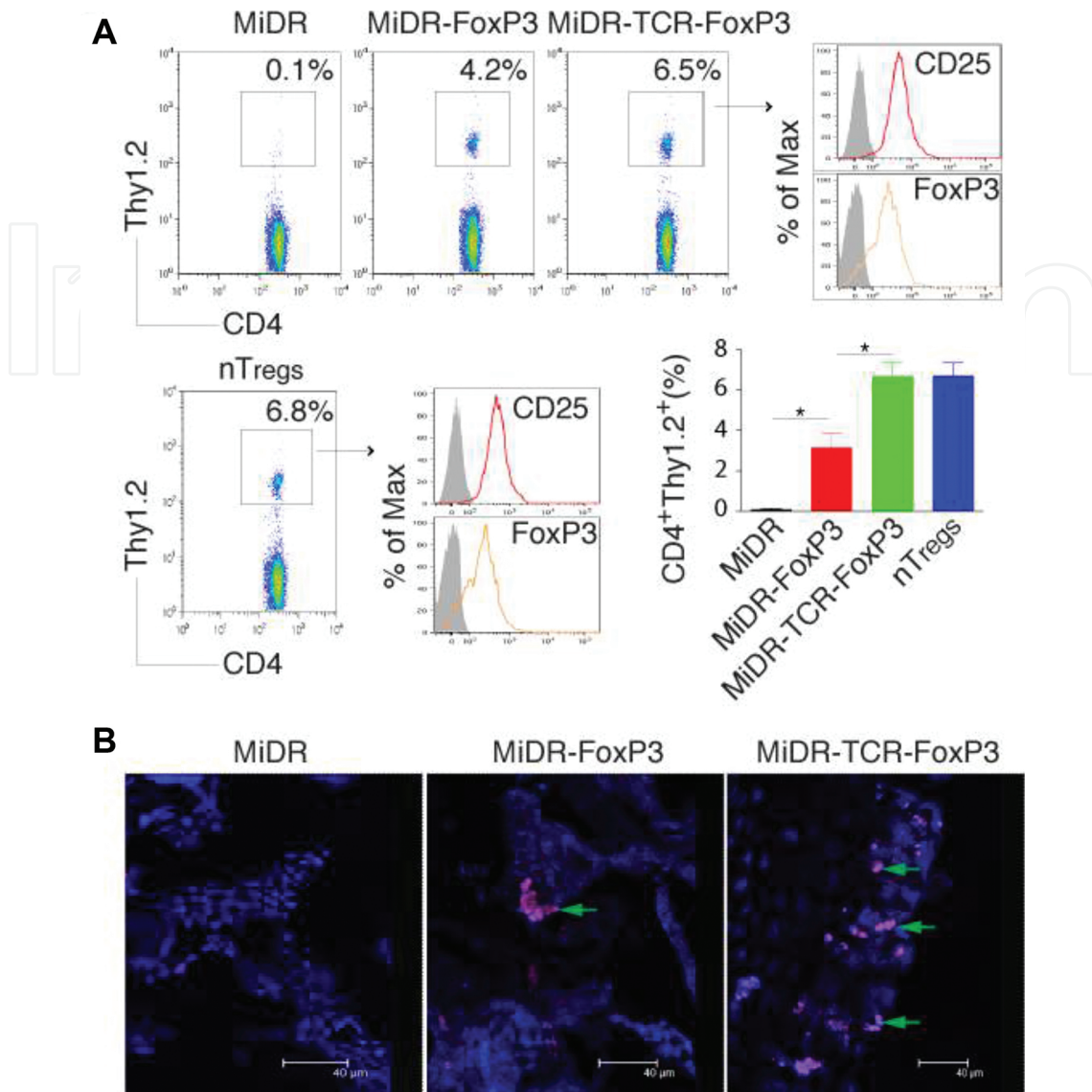


Figure 3. Ag-specific iPSC-Tregs infiltrate into the knee joints and maintain the Treg phenotype *in vivo*. OVA-specific iPSC-Tregs or nTregs (CD4⁺CD25⁺) from OT-II TCR transgenic mice (Thy1.2⁺) were *i.v.* adoptively transferred into C57BL/6 congenic mice (Thy1.1⁺) with AIA. (A) Six weeks later, the popliteal lymph nodes (LNs) from the inflammatory right side were analyzed for CD4⁺Thy1.2⁺ cells. The mean \pm s.d. from three independent experiments is shown ($* p < 0.05$, one-way ANOVA). (B) On days 7–14 after arthritis induction, knees were removed and stained for immunohistology with FoxP3. The FoxP3⁺ cells (–) are indicated.

Adoptive transfer of OVA-specific iPSC-Tregs in a well-established mouse model of autoimmune diabetes suppressed the development of diabetes. T1D is driven by self-reactive T cells that infiltrate the pancreatic islets of Langerhans and induce the destruction of beta cells and the loss of insulin production. This gradually causes pancreas to be unable to control blood glucose levels. During the development of diabetes, the pancreatic islets release the beta cell component that is occupied by immature DCs (IDC) in pancreatic islets. IDC carried the beta cell component to the draining pancreatic lymph node, process the Ag, and present to CD4⁺ T cells. T cell priming in lymph node leads to the expansion of circulating autoreactive T cells. Following clonal expansion, autoreactive T cells express a number of adhesion molecules,

including ICAM 1, the intercellular adhesion molecule 1, and LFA1, the lymphocyte function-associated Ag 1 [92]. This allows the effector cells to home to the pancreatic islets. Once they are in the pancreas, they activate inflammatory cells and causing insulinitis (**Figure 4**). PSC-derived Ag-specific Tregs have been used to cell-based therapies of autoimmune diabetes in a murine model, RIP-mOVA \times OT-I TCR F1 double transgenic mice. Mice will develop autoimmune diabetes (blood glucose levels >250 mg/dl) when challenged with vaccinia viruses expressing OVA (VV-OVA). Adoptive transfer of OVA-specific iPSC-Tregs significantly affected clinical outcome (**Figure 5**) by secreting IL-10 and TGF- β in the pancreas and reducing the expression of ICAM 1. Particularly, adoptive transfer of OVA-specific iPSC-Tregs reduces the number of inflammatory cells and protects beta cell destruction in the pancreas (**Figure 6**). These results also suggest that genetically modified iPSC-derived Tregs are tissue-associated and are able to suppress autoimmune diabetes.

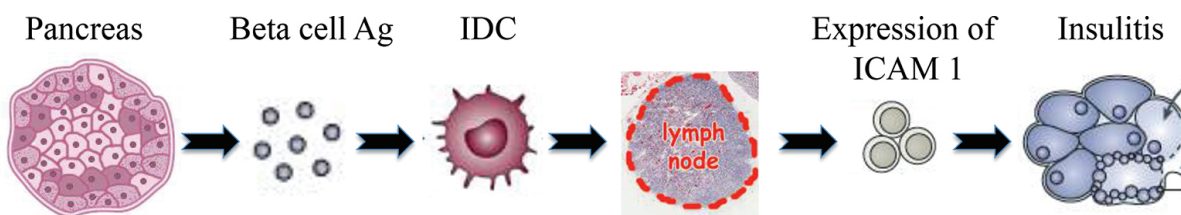


Figure 4. Insulinitis in autoimmune arthritis. During the development of diabetes, the pancreatic islets release the beta cell component that is taken-up by IDC in pancreatic islets. IDC carried the beta cell component to the draining pancreatic lymph node, process the Ag, and present to CD4⁺ T cells. T-cell priming in lymph nodes leads to the expansion of circulating autoreactive T cells. Following clonal expansion, autoreactive T cells express a number of adhesion molecules, including ICAM 1, which allows the effector cells to home to the pancreatic islets. Once they are in the pancreas, they activate inflammatory cells and causing insulinitis.

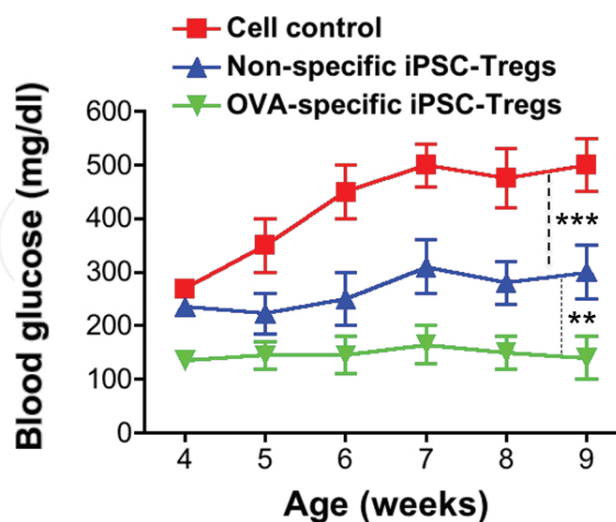


Figure 5. Ag-specific iPSC-Tregs ameliorate autoimmune diabetes in mice. 3-week-old RIP-mOVA \times OT-I F1 transgenic mice ($n = 5$ /group) were *i.p.* injected with VV-OVA viruses and adoptively transferred with OVA-specific iPSC-Tregs, non-specific iPSC-Tregs, or iPSC control. In the following weeks, the blood glucose levels were monitored by measurement of blood glucose (***) $p < 0.001$, ** $p < 0.01$, one-way ANOVA).

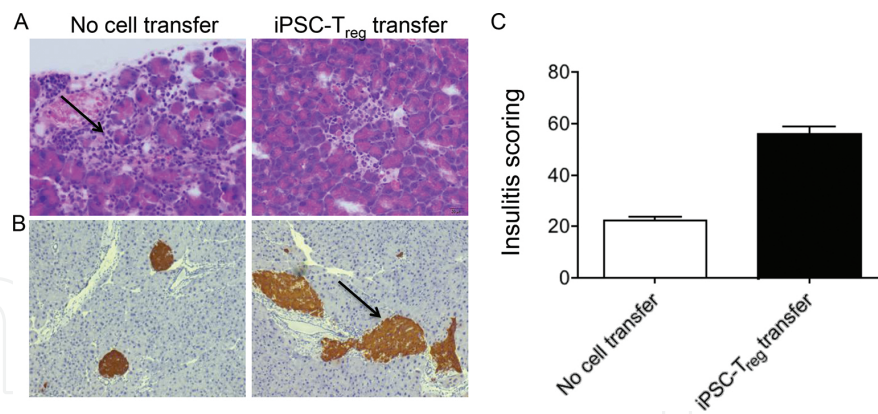


Figure 6. Adoptive transfer of Ag-specific iPSC-Tregs reduces the number of inflammatory cells and protects beta cell destruction in the pancreas. At week 2 following T1D induction, the pancreases were removed, sectioned, and stained with HE or insulin immunofluorescence. (A) Representative photomicrographs of HE staining. The cellular infiltrations (-) of inflammatory cells are indicated. (B) Representative photomicrographs by insulin immunofluorescent staining. Insulin-secreting cells (-) are indicated. (C) Quantitation of beta cell colonies (insulinitis scoring) in each group.

8. Future perspectives

T-cell-mediated suppression in immunologic tolerance still remains an exciting area of active research in immunology. It has already been established that a unique Treg population is engaged in the maintenance of immunologic self-tolerance. The natural development of such Tregs will be the crucial for mediating the self-tolerance. However, Tregs are hard to define phenotypically due to the lack of characteristic surface markers. Investigating the function and development of Tregs will contribute to the understanding of immunologic self-tolerance and shed light on the acquisition of autoimmune disorders. Published evidence showed that human Tregs constitutively express high levels of FoxP3 and that mutations in *FOXP3* results in severe autoimmunity. This demonstrates that the expression of this transcription factor has a key role in Treg function. Moreover, genetic modification of stem cells with FoxP3 for the differentiation of Ag-specific Tregs can pave a way for new strategies for the treatment or prevention of autoimmune diseases. However, preclinical data supporting the safety and efficacy of gene therapy approaches is required to allow the transition from the bench to the clinic.

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