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Development of Auto Antigen-specific Regulatory T Cells for Diabetes Immunotherapy

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$CD4^+$ regulatory T cells (Tregs) are essential for normal immune surveillance, and their dysfunction can lead to the development of autoimmune diseases, such as type-1 diabetes (T1D). T1D is a T cell-mediated autoimmune disease characterized by islet β cell destruction, hypoinsulinemia, and severely altered glucose homeostasis. Tregs play a critical role in the development of T1D and participate in peripheral tolerance. Pluripotent stem cells (PSCs) can be utilized to obtain a renewable source of healthy Tregs to treat T1D 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) remain undefined, especially molecular mechanisms that direct differentiation of such Tregs. Auto Ag-specific PSC-Tregs can be programmed to be tissue-associated and infiltrate to local inflamed tissue (*e.g.*, islets) to suppress autoimmune responses after adoptive transfer, thereby avoiding potential overall immunosuppression from non-specific Tregs. Developing auto Ag-specific PSC-Tregs can reduce overall immunosuppression after adoptive transfer by accumulating inflamed islets, which drives forward the use of therapeutic PSC-Tregs for cell-based therapies in T1D.

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Regulatory T cells (T_{regs}) are an integral component of the normal immune system and contribute to the maintenance of peripheral tolerance. T_{regs} can down-regulate immune responses and are essential for immune homeostasis. They can act as key effectors in preventing and treating type-1 diabetes (T1D) (1,2).

Hematopoietic stem cell (HSC)-derived hematopoietic progenitors migrate into the thymus and develop into different types of T cells. The transcription factors Aire (largely expressed in medullary thymic epithelial cells - mTECs) and FoxP3 have key functions in clonal deletion and T_{reg} selection (3). There are links between Aire expression, FoxP3 up-regulation and T_{reg} selection; Aire deficiency affects the negative selection of self-reactive T cells, and FoxP3 controls the development and function of the naturally occurring T_{regs} (nT_{regs}) (4). Our laboratory has shown the development of stable T_{regs} from $CD4^+$ T cells by over-expressing FoxP3 and bcl-xL (5).

Recent advances in the use of large-scale *in vitro* expansion of T_{regs} followed by *in vivo* re-infusion of these cells raises the possibility that this strategy may be successfully utilized for the treatment of T1D. Although polyclonally

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Abbreviations: T_{regs}, regulatory T cells; T1D, type-1 diabetes; IBD, inflammatory bowel diseases; HSC, hematopoietic stem cell; mTECs, medullary thymic epithelial cell; nT_{regs}, naturally occurring T_{regs}; GVHD, graft-versus-host disease; PBMCs, peripheral blood mononuclear cells; T_{effs}, effector T cells; TCR, T cell receptor; CAR, chimeric Ag receptor; LNs, lymph nodes; PSCs, pluripotent stem cells; ESCs, embryonic stem cells; BM, bone marrow; DL, Delta-like; eEF-2, elongation factor-2

expanded populations of T_{regs} exhibit suppressive activity, Ag-specific T_{regs} are more efficient at suppressing local autoimmune disorders such as RA, type-1 diabetes (T1D), inflammatory bowel diseases (IBD), allergic reactions and graft-versus-host disease (GVHD) (6-11). In addition, tissue/organ-associated T_{reg} targeting stabilizes FoxP3 expression and avoids induction of a potentially detrimental systemic immunosuppression (12,13). For T_{reg}-based immunotherapy, *in vitro* generation of tissue/organ (*e.g.*, islets)-associated and non-terminally differentiated effector T_{regs} for *in vivo* re-infusion is an optimal approach. However, current methodologies are limited in terms of the capacity to generate, isolate, and expand a sufficient quantity of such T_{regs} from patients for therapeutic interventions.

There are a number of challenges in T_{reg}-based immunotherapy. *First:* Only low numbers of T_{regs} can be harvested from the peripheral blood mononuclear cells (PBMCs). CD4 and CD25 have been used to isolate T_{regs} for *ex vivo* expansion. $CD4^+CD25^+$ T cells are not homogenous and contain both T_{regs} and conventional effector T cells (T_{effs}). Current expansion protocols activate both T_{regs} and T_{effs}, and because it takes a longer time for T_{regs} to enter the S phase of cell cycle, T_{effs} outgrow T_{regs} (14). In addition, T_{regs} can lose suppressive activity after repetitive stimulation with α-CD3 plus α-CD28 Abs with or without rIL-2 *in vitro*. *Second:* No approach to date has demonstrated the capacity to isolate the entire T_{reg} population with 100% specificity from patients (the current clinical approach). Even FoxP3 or more recently Eos, a transcriptional factor that is considered the gold standard for identification of T_{regs}, is expressed transiently in some activated non-regulatory human T cells (15), highlighting the difficulty in both identifying and isolating a pure T_{reg} population. Adoptive transfer of non-regulatory T_{effs} with T_{regs} has a potential to worsen autoimmune diseases. *Third:* Gene transduction of $CD4^+$ T cells from PBMCs with Ag-specific TCR (16) or chimeric Ag receptor (CAR) (17) and/or TCR with FoxP3 elicits the generation of suppressive T cell populations (7) and overcomes the hurdle of the limited numbers of Ag-specific T cells. However, the engineered T_{regs} express endogenous and exogenous polyclonal TCRs, which reduce their therapeutic potential (the current experimental approach). Also, TCR mispairing is a concern with regards to the safety of TCR gene-transferred T_{regs} for clinical use, because the formation of new heterodimers of TCR can induce immunopathology (18). Therefore, there is a need to improve this strategy and generate monoclonal T_{regs}. *Fourth:* The differentiation state of T_{regs} is inversely related to their capacity to proliferate and persist. The "right" T_{regs} resist terminal differentiation, maintain high replicative potential (*e.g.*, expression of common γc, CD132), are less prone to apoptosis (*e.g.*, low expression of PD-1), and have the ability to respond to homeostatic cytokines (19), which facilitates their survival. In addition, the "right" T_{regs} 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" T_{regs} persist and provide protective immunity. *Fifth:* Because there are too few cells, harvesting sufficient numbers of tissue-associated T_{regs} from PBMCs for TCR gene transduction can be problematic.

Taken together, strong arguments support the development of T_{reg}-based therapies in autoimmune diabetes using engineered T_{regs}. While clinical trials show safety, feasibility, and potential therapeutic activity of T_{reg}-based therapies using this approach, concerns about autoimmunity due to cross-reactivity with healthy tissues remains a major safety issue (20,21). In addition, genetically modified T_{regs} using current approaches are usually intermediate or later effector T_{regs} (22), which only have short-term persistence *in vivo*.

Stem cells have the ability to differentiate into Ag-specific T_{regs} which can be used for cell-based therapies. To date, pluripotent stem cells (PSCs) are the only source available to generate a high number of the "right" T_{regs} (11,23). Human iPSCs can be easily generated from patients' somatic cells by transduction of various transcription factors and exhibit characteristics identical to those of embryonic stem cells (ESCs) (24). 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 (25). Because of the plasticity and the potential for an unlimited capacity for self-renewal, iPSCs have high potential for advancing the field of cell-based therapies.

Our laboratory was the first to show that the development of Ag-specific iPSC-CTLs or iPSC-T_{regs} can be used for cell-based therapies of cancers and autoimmune disorders (11,23,26,27); other groups reported similar results (28-30). We demonstrated that genetically modified iPSCs with Ag-specific T cell receptor (TCR) and the transcriptional factor FoxP3, followed by differentiation driven by Notch signaling can enable iPSCs to pass hematopoietic and T lineage differentiation checkpoints,

resulting in the development of Ag-specific $CD4^+$ T_{regs}. We have developed a novel system to generate stable auto Ag-specific iPSC-T_{regs}. Our ongoing studies will validate this system and provide new insights into the methodologies and mechanistic requirements for efficient development of inflamed tissue-associated iPSC-T_{regs}. Once such strategies become available, there is potential to facilitate the generation of tolerance for autoimmune diabetes. Thus, important advances towards T_{reg}-based immunotherapy in autoimmune disorders are anticipated from the proposed studies.

Signaling mechanisms that direct differentiation of Ag-specific PSC-T_{regs} remain to be determined. PSCs are exposed to a number of signals responsible for their progression. Although the exact signals are not fully understood, part of the mechanism known to be critical for directing T-cell fate occurs *via* Notch signaling, an evolutionarily conserved signaling pathway that regulates cell fate decisions in a number of cell and tissue types. Ligand binding by members of the Jagged or Delta-like (DL) families results in the proteolytic cleavage and release of the intracellular fragment of the Notch heterodimer. Translocation to the nucleus then allows for its regulation of gene expression. Notch-1, specifically, is critical for the establishment of T-cell fate. The loss of function results in the blockade of T cell development and enhanced B cell production, while over-expression results in the blockade of B cell lymphopoiesis and leads to the generation of T cells (31). However, the intracellular signaling pathways by which Notch signaling regulates the differentiation of Ag-specific PSC-T_{regs} remain unknown. PSCs co-cultured on a monolayer of the bone marrow (BM) stromal cell line OP9 cells transfected with the Notch ligand DL1 or 4 exhibit the ability to differentiate into most hematopoietic lineages and T cells (28). Our current studies will determine critical regulations of Hes1 (32), Runx1 (33), survivin (34) and elongation factor-2 (eEF-2) kinase (35) by Notch signaling during the development of auto Ag-specific PSC-T_{regs}.

While auto Ag-specific iPSC-T_{regs} have likely therapeutic effects in T_{reg}-based immunotherapy against autoimmunity, the effectiveness is restricted by the demand to develop a great number of cells by complicated and exclusive *in vitro* differentiation. Furthermore, the extensive period for performing the generation of iPSCs can constraint the practice in personalized treatments. As an alternative, we will implement T_{reg}-based immunotherapy by using the TCR/FoxP3 gene-transduced iPSCs, which have the ability to develop auto

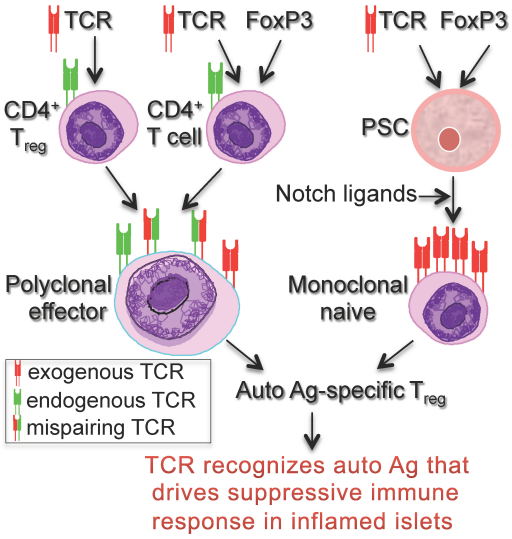

Figure 1. Populations of auto Ag-specific T_{regs} that can be generated for cell-based therapies in T1D. Auto Ag specific T_{regs} can be generated by TCR transduction of $CD4^+$ T_{regs}, TCR/FoxP3-gene transduction of $CD4^+$ T cells, or TCR/FoxP3-gene transduction of PSCs, followed T-cell differentiation driven by Notch ligands.

Ag-specific iPSC-T_{regs} *in vivo* and subsequently control autoimmune diabetes. We will conduct diabetes induction before or after adoptive transfer of the gene-transduced iPSCs. We will then administrate Notch agonists or recombinant cytokines (*e.g.*, rIL-7, rFlt3L) to improve *in vivo* development of auto Ag-specific iPSC-T_{regs}. To avoid the potential tumorigenicity of the gene-transduced cells, we will incorporate a suicide gene, the inducible caspase 9 (36), into the vector as this allows the removal of the transduced T_{regs} by the injection of a bioinert small-molecule dimerizing agent (AP1903) to "shut off" the system.

In summary, a current roadblock to progress in the field is the lack of an efficient system to generate the "right" auto Ag-specific T_{regs} that could be used for cell-based therapies in autoimmune diabetes (Fig. 1). We are using PSC-T_{regs} to address this limitation, allowing derivation of a large number of stable auto Ag-specific PSC-T_{regs} for cell-based therapies. Development of such an approach provides an important step toward personalized therapies for T1D.

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