

## Replenishing Peripheral CD4<sup>+</sup> Regulatory T Cells: A Possible Immune-Intervention Strategy in Type 1 Diabetes?

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### ■ Abstract

Controlling the diabetogenic activity of peripheral islet antigen-specific T cells is essential to halt the progression of autoimmunity that leads to the development of type 1 diabetes mellitus (T1DM). Over the past years, evidence has been gathered to suggest that the dysfunction of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells, and the interleukin-10 (IL10) - secreting type 1 regulatory T (Tr1) cells are associated with disease onset in diabetic patients. Although CD4<sup>+</sup>CD25<sup>+</sup> Treg cells develop as a distinct lineage of T cells in the thymus, results from recent studies have shown that they can

also arise independently from the peripheral pool of conventional CD4<sup>+</sup> lymphocytes. These observations have led to the development of various methods to convert peripheral CD4<sup>+</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup> Treg and Tr1 cells *in vitro* or to induce the development and expansion of Treg cell subsets *in vivo*. This article reviews the progress made in Treg cell recruitment *in vivo* that involves the potential for the prevention or even reversal of T1DM.

**Keywords:** Treg cells · peripheral CD4 cells · type 1 diabetes · inoculum

### Introduction

Type 1 diabetes mellitus (T1DM) is an organ-specific autoimmune disease characterized by the progressive destruction of pancreatic islet  $\beta$ -cells by both diabetogenic CD4 and CD8 T cells [1, 2]. The onset of diabetes is associated with multiple factors, including those attributed to the genetic constitution of susceptible individuals as well as the environmental factors they are exposed to. When considering the prevention of T1DM, approaches to control genetic and environmental factors are not available. Immunotherapy is an option to halt the ongoing pathogenic activity of autoreactive T cells. The autoimmune basis of T1DM is caused by the loss of immunological tolerance to pancreatic islet antigens. The evidence for this stems from the observation that autoantibodies are reactive to glutamic acid decarboxylase (GAD) and tyrosine phosphatase-like protein IA-2 expressed in islet

cells as well as insulin and its precursor proinsulin secreted by islet  $\beta$ -cells, which are detected in the serum of diabetic subjects [3, 4]. Furthermore, autoreactive T cells reactive to these antigens are present in these patients [5-7].

In healthy individuals tolerance to self-antigens is maintained by the elimination of islet antigen-reactive T cells through negative selection in the thymus (central tolerance). Effective deletion of these T cells through central tolerance requires the engagement of their high affinity T cell receptor (TCR)  $\alpha\beta$  with the islet antigen-MHC (major histocompatibility complex) molecules presented by medullary thymic epithelial cells [8]. Proinsulin/insulin, IA-2, and GAD65 genes are transcribed in the human thymus [9, 10]. Studies conducted with thymic proinsulin gene transcription have revealed that its level of expression in the thymus can critically affect the elimination of proinsulin-reactive T cells. Insulin gene expression is linked to

polymorphism of a variable number of tandem repeats (VNTR) positioned upstream of the insulin promoter. Class I alleles with short VNTR containing 28-44 times of such repeats confer high risk to T1DM development, while class III alleles with 138-159 times of the VNTR is associated with disease protection [11]. Apparently, lower thymic insulin expression is associated with individuals carrying class I and not class III alleles [10, 12]. Consequently, insufficient presentation of islet antigen peptides to respective high affinity TCR $\alpha\beta$ -bearing T cells would lead to their less effective elimination in the thymus, thus allowing them to escape into the periphery. This phenomenon could account for the presence of peripheral HLA-DR4-restricted T cells that react to proinsulin and IA-2 epitopes and secrete the proinflammatory cytokine IFN- $\gamma$  in significantly higher amounts in diabetic than normal individuals [13]. GAD65-reactive T cells have similarly been reported to exist in peripheral blood mononuclear cells (PBMC) of diabetics [14]. Islet antigen-reactive T cells are shown to exist as memory cells in the periphery; as such, they are readily activated upon encounter with the respective autoantigens which allows them to exert their diabetogenic activity [15].

### Relationship between Treg cell subsets and T1DM development in human subjects

Controlling peripheral islet antigen-reactive T cells is undoubtedly a critical factor in maintaining immune homeostasis for the prevention of T1DM development. Clonal deletion and anergization as well as suppression by Treg cells have been identified to be the mechanisms involved in maintaining peripheral tolerance. Whether these mechanisms are needed to operate in concert to control autoimmunity is not fully known. While results obtained from TCR transgenic mouse models have suggested that bone marrow-derived antigen presenting cells (APC) are responsible for triggering the initial stimulation of autoreactive T cells [16-18], different subsets of Treg cells have been shown to suppress the activity of diabetogenic T cells in the periphery. One such subset detectable in the peripheral blood of human subjects is among the CD4<sup>+</sup> population of T lymphocytes. This type carries the IL-2 receptor  $\alpha$ -chain, CD25, as well as other molecular markers such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR), and the transcription factor Forkhead box P3 (FoxP3). These cells are acquired through their differentiation in the thymus [19-21]. Unlike their CD4<sup>+</sup>CD25<sup>-</sup> counterparts, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, de-

spite bearing high affinity TCR $\alpha\beta$ , are programmed to bypass negative selection during central tolerance induction resulting in their release into the periphery [22]. The role of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in regulating immune homeostasis is shown by their ability to suppress TCR $\alpha\beta$ -triggered proliferation and IL-2 production of autologous CD4<sup>+</sup>CD25<sup>-</sup> T cells by cell-to-cell contact [23-25]. Analysis of Treg cell specificities revealed that these cells utilize a broad range of TCRV $\beta$  repertoire to recognize both self and foreign antigens in association with MHC molecules [26]. The number of peripheral CD4<sup>+</sup>CD25<sup>+</sup> T cells is kept relatively constant at approximately 2-5% of the total CD4<sup>+</sup> cell repertoire in human subjects [25].

In autoimmune diabetes, early studies reported that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells are present at significantly lower frequencies in diabetic than in non-diabetic individuals [27]. On the other hand, subsequent studies showed that the number of Treg cells significantly increases with age in type 1 diabetic as well as normal subjects. This phenomenon is responsible for the occurrence of a balanced relation of Treg cells in both population groups [28, 29]. However, the cytokine secretion profiles of Treg cells in diabetic subjects seem to differ from normal ranges. This can be considered as the cause for the defective activity in suppressing autologous effector T cells *in vitro*, as observed in both studies [28, 29]. Interestingly, Lindley *et al.* found that adult type 1 diabetes individuals of recent onset have a decreased production of IL-2, but an increased IFN- $\gamma$  and TGF- $\beta$ 1 secretion compared to those who are healthy. Thus, Treg cells in diabetics seem to drift towards an inflammatory phenotype [28]. In contrast, Brusko and co-workers found that in diabetic patients all three cytokines are secreted at a lower amount compared to healthy persons of the same age [29]. There are apparently differences in the cytokine profile of Treg cells in diabetic patients at different ages and diabetes duration. Despite the controversy on the Treg cell cytokine profile, observations made from these studies strongly suggest that proper Treg cell function is essential to control autoimmunity and T1DM development.

Another imbalance of a T cell subset, namely the periphery-derived Tr1 cells, could be observed between patients and controls. Tr1 cells are characterized predominantly by their IL-10 secretion. Arif *et al.* found that T cell responses to the naturally processed IA-2 and proinsulin peptides are associated with IL-10 secretion more frequently in non-diabetics than in those who progress to clinical disease [13]. On the other hand, IFN- $\gamma$ -secreting CD4<sup>+</sup> T cells, which rec-

ognize peptides naturally processed from IA-2 antigen and proinsulin, are found in a significantly higher proportion in newly diagnosed T1DM patients carrying HLA-DR-encoded alleles than in non-diabetics [30]. Consequently, a higher frequency of autoreactive T cells but a lower frequency of suppressive Tr1 cells occur in patients when compared to healthy individuals.

### Generation of human Treg cell subsets from the pool of peripheral CD4<sup>+</sup> lymphocytes as an option to encompass tolerance

Given that impaired and different functions of CD4<sup>+</sup>CD25<sup>+</sup> and Tr1 cells bear a positive association with T1DM, replenishing the peripheral pool of functional Treg cells would be a possible option to control the disease. In this regard, several approaches have been tested experimentally to explore how peripheral human CD4<sup>+</sup> cells may be converted into Treg cells. Initial studies have found that simultaneous stimulation of the TCR $\alpha\beta$ -associated molecule, CD3, and the primary costimulatory molecule, CD28, on CD4<sup>+</sup>CD25<sup>-</sup> T cells with mitogenic anti-CD3 and anti-CD28 antibodies can result in changing the cells to acquire the CD4<sup>+</sup>CD25<sup>+</sup> phenotype with the expression of FoxP3, a specific marker associated with the CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Generated population(s) of CD4<sup>+</sup>CD25<sup>+</sup> cells can mediate cell contact-dependent inhibition of CD4<sup>+</sup>CD25<sup>-</sup> T lymphocyte proliferation in response to anti-CD3/28- and mitogen-triggered stimulation [31]. Repeated stimulation of TCR $\alpha\beta$  on CD4<sup>+</sup> T cells with an immunodominant epitope encompassing the amino acid sequence 73-90 of proinsulin presented by the HLA-DR4 molecules on autologous APCs has also been used to induce FoxP3-expressing lymphocytes with IL-10 secretion property [30].

Another protocol describes that CD4<sup>+</sup> Treg cells expressing higher levels of CTLA-4 can be generated from the non-regulatory pool of CD4<sup>+</sup> T lymphocytes when they are co-cultured with IL-10-treated dendritic cells. Treg cells generated by this means inhibit antigen-specific proliferative responses of syngeneic CD4<sup>+</sup> T cells via cell-contact involving a CTLA-dependent mechanism [32].

Other methods have shown that the modulation of surface receptors expressed on CD4<sup>+</sup> T cells are effective in inducing Treg cells. Based on observations that immune responses to bacterial infections can lead to the generation of Treg cells [33], it has been suggested that the simultaneous modulation of the complement regulatory protein CD46 with either a recombinant M

protein of *Streptococcus pyogenes* or anti-CD46 monoclonal antibodies, together with anti-CD3 monoclonal antibodies can induce the conversion of CD4<sup>+</sup> T lymphocytes into Treg cells. These Treg cells are able to inhibit proliferative responses of autologous CD4<sup>+</sup> cells through IL-10 secretion. It has also been observed that the cells can induce apoptotic death of T cells via cell-to-cell contact which involves granzyme/perforin-mediated mechanisms [34].

Another way to induce Treg cells is the simultaneous modulation of the RO and RB isoforms of CD45 that are associated with CD4<sup>+</sup> lymphocyte activation. Exposure of human CD45RO/RB<sup>low</sup> T cells, which belong to the central pool of memory T cells, to a chimeric antibody directed against the RO and RB molecules results in their conversion into Tr1 and not CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. This conversion has been observed by means of their enhanced secretion of IL-10 [35]. Another study has found that ligation of CD47 by a peptide encompassing the C-terminal domain of its natural anti-inflammatory ligand, thrombospondin-1 (TSP), is able to induce naïve or memory CD4<sup>+</sup> T cells to become Treg cells expressing the CTLA-4<sup>+</sup>OX40<sup>+</sup>GITR<sup>+</sup>Foxp3<sup>+</sup> phenotype characteristic of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Cross-linking of CD47 with specific monoclonal antibodies can produce a similar effect. The generated CD4<sup>+</sup>CD25<sup>+</sup> Treg cells suppressed proliferation and IFN- $\gamma$  secretion of autologous CD4<sup>+</sup> T cells in a cell contact-dependent manner [36]. TSP has been reported to be a potent stimulator of TGF- $\beta$  production [37], thus TSP-CD47 engagement-mediated conversion of CD4<sup>+</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup> Treg cells could be TGF- $\beta$ -driven. Interestingly, a recent report has described that *in vitro* incubation of human CD4<sup>+</sup> T cells with copolymer-1 (COP-1, Copaxone<sup>TM</sup>), which is a random polymer comprising of the amino acid sequence EKAY found in the myelin basic protein (MSP), can lead to their FoxP3 expression through the IFN- $\gamma$ -mediated pathway. The generated FoxP3<sup>+</sup> cells exhibited suppressor activity as shown by their capability to block anti-CD3/28-triggered proliferation of autologous CD4<sup>+</sup> T cells [38]. Although a human study with T1DM patients has not been conducted, it is interesting to note from this report that subjects with MS given COP-1 administration are found to have a significantly higher number of FoxP3-expressing T cells compared to individuals who have not received the treatment.

In the context of the various *in vitro* protocols that have been described to enable Treg cells to be generated from non-regulatory CD4<sup>+</sup> T cells, two separate studies have recently reported that peripheral CD4<sup>+</sup>

lymphocytes can actually be converted into Tr1-like cells through their interaction with activated CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. The secondary (converted) Treg cells are functionally different from primary CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in that they exert their suppressor activity against the proliferation of autologous CD4<sup>+</sup> T cells through secretion of either IL-10 or TGF- $\beta$  [39, 40]. Given that similar protocols are used in both studies, these findings imply that different subsets of Treg cells can be derived from the peripheral pool of CD4<sup>+</sup> lymphocytes through interaction with the instructing master CD4<sup>+</sup>CD25<sup>+</sup> Treg cells.

Collectively, the use of various *in vitro* culture methods shows clearly that peripheral CD4<sup>+</sup> lymphocytes can be converted into CD4<sup>+</sup>CD25<sup>+</sup>/CD4<sup>+</sup>CD25<sup>-</sup>-like and Tr1/Tr1-like Treg cells under different experimental conditions. In the context that fresh functional Treg cells would be needed to replenish dysfunctional cells in diabetic patients, it still needs to be clarified whether this phenomenon can also be achieved *in vivo* through lymphocytes from T1DM patients.

### ***In vivo* induction and generation of peripheral Treg cells**

The observations that both human CD4<sup>+</sup>CD25<sup>+</sup> T cells and Tr1 regulatory cells can be converted from the peripheral pool of CD4<sup>+</sup> T cells could lead to the development of two separate approaches for the immune-intervention of T1DM. The first is the *in vitro* generation of Treg cells from peripheral blood-derived T cells of an individual, and their subsequent adoptive transfer back to the same individual. This approach has already been discussed [41]. The second option could be the design of efficient protocols for the treatment of the diagnosed patients. The latter requires the formulation of inoculum preparations to induce Treg cells *in vivo* capable of suppressing existing and emerging diabetogenic T cells. Attempts to develop such an intervention have so far been limited. Based on earlier epidemiological observations that vitamin D<sub>3</sub> intake correlates with a significantly decreased risk of T1DM development in human subjects [42], a 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> analog, Ro 26-2198, has been tested for its non-specific induction of Treg cells in NOD mice. 8-12 wk old NOD mice orally fed with Ro 26-2198 are found to have an increased occurrence of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells compared to control animals. The analysis of the CD4<sup>+</sup>CD25<sup>+</sup> Treg cell population generated *in vivo* revealed that they accumulate in the pancreatic lymph nodes during the treatment period

where they exert suppressor activity on IA-2-specific T cells [43]. This observation is accompanied by a greater resistance to T1DM development in the Ro 26-2198-administered animals. COP-1 is another reagent that has shown itself to be able to induce Treg cells via administration, which has been discussed in the preceding section. Given that COP-1 is able to induce the generation of FoxP3-expressing CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in mice and MS patients [38], it ought to be interesting to verify if the polymer may work out to be effective in the treatment of T1DM.

Observations made from several experimental investigations have suggested that antigen administration could be effective in inducing Treg cells for the prevention of T1DM. An earlier study reported that intravenous injection of GAD65 into 12-week-old NOD mice is effective in conferring resistance against diabetes. The protection is attributed to tolerance induction of the existing islet antigen-specific T cells, as judged by their reduced proliferation when exposed to islet antigens. Functional analysis revealed that GAD65 vaccinated mice generated CD4<sup>+</sup> Treg cells with the IL-4-producing phenotype [44]. Since other cytokines have not been determined, it is still of interest if CD4<sup>+</sup> Treg cells with other properties corresponding to those of the CD4<sup>+</sup>CD25<sup>+</sup> and/or Tr1 phenotype may also be induced in this way.

The results obtained from another study have suggested that young NOD mice fed with insulin conjugated to a candidate mucosal adjuvant, cholera toxin B-subunit, exhibited delayed onset of T1DM as compared to control animals. Protection is associated with the induction of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells that accumulate in the pancreatic lymph nodes [45]. It was recently reported that the injection of a MHC class II-restricted influenza hemagglutinin epitope conjugated to an immature dendritic cell-specific monoclonal antibody, DEC-205, is capable of protecting INS-HA/TCR-HA mice from developing spontaneous T1DM [46]. Disease resistance is attributed to an increased generation of IL-10-producing Treg cells that express FoxP3 [47]. The free HA 107-119 peptide itself, to which the cells are specific, is capable of inducing naïve T cells to become CD4<sup>+</sup>CD25<sup>+</sup> Treg cells when it is allowed to be delivered at subimmunogenic doses in a continuous fashion via an implanted mini-osmotic pump in TCR-HA-RAG-2<sup>-/-</sup> or BALB/c mice. Immunized BALB/c mice are unable to elicit CD4<sup>+</sup> T cell responses upon exposure to the immunogenic inoculum of IFA-formulated HA protein [48]. These findings imply that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells induced by an epitope-containing peptide could exert broad suppression

against the production of immune responses by the whole protein. Given this approach has potential clinical application, it remains to be established whether the delivery of suitable islet antigens may similarly lead to the induction of Treg cells able to suppress the activity of diabetogenic T cells.

## Conclusion

Increasing evidence showing that CD4<sup>+</sup>CD25<sup>+</sup> and Tr1 regulatory cells can be induced from the peripheral pool of CD4<sup>+</sup> T cells indicates that the therapeutic propagation of Treg cells is relevant in two aspects for the future development of an effective, preventive or

curative treatment of T1DM. First, the culture methods that have been used for the generation of the two types of Treg cells from normal human CD4<sup>+</sup> lymphocytes need to be tested for their applicability to T1DM patients. This information is necessary for the development of therapeutic strategies involving adoptive transfer of autologous Treg cells into individual diabetic patients. Second, the use of non-specific and antigen-specific approaches to induce Treg cell generation *in vivo* is attractive in terms of their broad, preventive and cost-effective clinical application. The effectiveness of such protocols should be further assessed in future preclinical studies.

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