

Functional Alterations of Proinflammatory Monocytes by T Regulatory Cells: Implications for the Prevention and Reversal of Type 1 Diabetes

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■ Abstract

The onset and development of type 1 diabetes (T1D) occurs in genetically predisposed individuals, and is attributed to autoimmune destruction of pancreatic β -cells involving a multitude of immune mechanisms. Defects in immune regulation may play a central role in T1D, involving impaired function and communication of both myeloid and lymphoid cells of the innate and adaptive immune compartments. Dendritic cells and regulatory T (Treg) cells are part of this network, which seem to be hampered in their quest to control and regulate tissue-destructive autoimmunity. Recent studies have shown that *in vivo* activated CD16⁺ blood monocytes exhibiting proinflammatory features are present in diabetic subjects. These monocytes may govern T cell-mediated immune responses towards the development of

tissue-destructive Th1 and Th17 subtypes, and give rise to inflammatory macrophages in tissues. Differential effects of cytokines IFN- γ and IL-4 in the development of inflammatory macrophages, and the distinct developmental pathways of proinflammatory or tissue-repair-associated monocytes suggest that controlling the activity of these monocytes could be part of an immune intervention strategy to prevent T1D. Similarly, strategies to target autoantigens to immature, steady-state dendritic cells could guide the immune response away from Th1 and Th17 immune effectors. This review examines potential approaches to this goal by manipulation of myeloid and lymphoid cell regulatory networks in T1D.

Keywords: type 1 diabetes · monocyte · regulatory T cell · autoimmunity · dendritic cell · cytokines · NOD mouse

Introduction

ype 1 diabetes (T1D) is a complex organ-specific autoimmune disease involving defects in immunoregulation. The disease has a relatively strong genetic association, and multiple environmental risk factors are involved in triggering its onset [1]. Different microbes, and chemical and dietary factors have been implicated either individually or in combination in causing

autoimmunity to islet β -cells. In genetically predisposed individuals, such environmental factors are believed to disrupt tolerance mechanisms that normally protect islets from autoimmunity. This may lead to the emergence of diabetogenic T cells, attacking insulin-producing β -cells.

Within the context of this broadly accepted concept, intensive investigations have been made over the past several years to uncover the role played by different subsets of regulating immune

cells. Thymus-derived, natural regulatory T (nTreg) cells, and adaptive Treg (aTreg) cells generated by antigenic stimulation of peripheral CD4⁺ lymphocytes are such subsets. They have emerged as crucial cellular gatekeepers in maintaining the integrity of the immunoregulatory circuits needed to prevent autoimmunity. Lack of these cells is due to mutations in the *foxp3* gene, and results in severe multi-organ autoimmune disease including

autoimmune diabetes both in humans (IPEX syndrome) and in mice (scurfy mice).

Importantly, results gathered from experimental systems showed that the generation of aTreg cells is cross-regulated by dendritic cells (DCs) [2, 3]. DCs are delicate sensors of microbial and inflammatory cues. It is the principal cell type that activates T cells of both regulatory and other subtypes. The challenge in understanding the role of DCs in the generation and activation of aTreg cells results from the remarkable degree of DC plasticity and functional heterogeneity depending on the environment where they develop and mature. Human DCs can be classified in two main subsets. The major subset contains the myeloid DCs (mDCs) that are derived from monocytes [4]. mDCs are typically located in tissues and travel via afferent lymph nodes to draining lymph nodes. They can feature two different activity forms, either low efficiency during steady-state or high efficiency during inflammatory conditions. The other subset, namely plasmacytoid DCs (pDCs), is generated from lymphoid progenitor cells. pDCs produce vast amounts of type 1 interferons (IFN) as an early response to viral infections. They do not express most of the surface markers found on mDCs [5], but they frequently express transcripts of rearranged immunoglobulin molecules. They are found in bone marrow, peripheral organs and lymphoid tissues, where they arrive mostly via high endothelial venules similarly to lymphocytes. In addition to DC and their subtypes, monocytes and macrophages are functionally heterogeneous and bear significant potential to modulate adaptive immune responses during inflammation and tissue repair.

Blood monocytes of T1D subjects exhibit proinflammatory features (increased production of interleukin-1beta (IL-1 β) and IL-6), and are involved in the development of IL-17-producing lymphocytes [6]. Th17 immunity has been associated with autoimmune manifestations both in humans (multiple sclerosis, rheumatoid arthritis) and in corresponding animal models (EAE and experimental arthritides) [7]. In the nonobese diabetic (NOD) mouse, a murine model of T1D, Th17 cells have been found to be implicated in diabetes development, although several studies describe their conversion into Th1 cells before β -cell destruction. Apparently, both Th1 cells and Th17 cells can promote tissue destructive inflammation. Therefore, it is important to regulate both Th1 and Th17 cells in autoimmune diabetes. In the following sections, we discuss some emerging aspects associated with the regulation of Th1/Th17 immunity in T1D by

Abbreviations:

aTreg - adaptive regulatory T cell
 BDC2.5 cell - autoreactive T cell clone isolated from NOD mice
 CDx - cluster of differentiation x protein (protein expressed on cells)
 CD11c - expressed on myeloid cells; adhesion molecule binding to fibrinogen/fibronectin
 CD14 - acts as co-receptor for the detection of bacterial LPS
 CD16 - low affinity Fc receptor found on monocytes and other cells
 CD25 - expressed on activated regulatory T cells
 CD45RA - tyrosinphosphatase, expressed by naïve lymphocytes
 CD62L - expressed on leukocytes; adhesion molecule that binds to CD34
 CD80 - expressed on B cells, DCs and others; co-stimulator, ligand for CD28 and CTLA-4
 CD136 - plasma cell-surface glycoprotein, known as syndecan-1
 DC - dendritic cell
 DEC205 - dendritic cell receptor for endocytosis (CD205)
 EAE - experimental autoimmune encephalomyelitis
 Fc receptor - crystallizable fragment (protein found on monocytes and other immune cells; binding specificity for part of an antibody)
 FoxP3 - forkhead box P3 (transcriptional activator)
 GM-CSF - granulocyte-macrophage colony-stimulating factor
 IFN - interferon
 IPEX - immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (rare disease linked to the dysfunction of FoxP3; leads to dysfunction of regulatory T-cells and the subsequent autoimmunity)
 IL - interleukin
 LPS - lipopolysaccharide
 M-CSF - macrophage colony-stimulating factor
 mDC - myeloid dendritic cell
 MyD88 - myeloid differentiation primary response gene 88
 NLR - NOD-like receptor
 NOD - nonobese diabetic
 nTreg cell - natural regulatory T cell
 PBMC - peripheral blood mononuclear cell
 pDC - plasmacytoid dendritic cell
 PgE2 - prostaglandin E2
 T1D - type 1 diabetes
 TGF- β - transforming growth factor beta
 Th - T helper cell
 TLR - toll-like receptor
 TNF- α - tumor necrosis factor alpha
 Treg cell - regulatory T cell

DCs. Also, we highlight aspects of regulating cells of the monocyte/macrophage lineage within the scope of designing immune intervention strategies to halt or reverse T1D.

Blood monocytes in non-diabetic and diabetic subjects

Human monocytes are heterogeneous, comprising subpopulations at different stages of differentiation and proliferation. They all bear the lipopolysaccharide (LPS) receptor, CD14, and are further classified in two main subsets according to their surface expression of the Fc γ receptor-III CD16 [8]. The major subset contains the phagocytic CD14^{hi}CD16⁻ monocytes that produce high levels of proinflammatory cytokines and tumor necrosis factor (TNF- α) and interleukin-6 (IL-6), following stimulation with LPS. The CD14⁺CD16⁺ subset is heterogeneous and comprises two subpopulations bearing differential levels of surface CD64 (or Fc γ receptor-I). The CD14⁺CD16⁺CD64⁺ subset shares the high phagocytic activity and cytokine (TNF- α and IL-6) secretion property as the CD14^{hi}CD16⁻ monocytes. The CD14⁺CD16⁺CD64⁻ subset appears to resemble the alveolar CD14^{dim}CD16⁺ cells, and primarily release IL-12 following LPS stimulation [9].

Both CD14⁺CD16⁺ and CD14⁺CD16⁻ monocytes can be differentiated into different subpopulations of myeloid dendritic cells (mDCs). When placed in the same standard granulocyte-macrophage colony-stimulating factor (GM-CSF)- and IL-4-conditioned cultures, CD14⁺CD16⁺ monocytes are converted into CD11c⁺CD123⁻ mDCs that express higher levels of the costimulatory molecule, CD80. LPS stimulation can trigger mDCs derived from the two subpopulations of monocytes to secrete comparable levels of IL-1 β and IL-6. However, CD14⁺CD16⁺ monocyte-derived mDCs are found to produce significantly greater amounts of transforming growth factor beta (TGF- β), while higher levels of IL-12 are secreted by mDCs generated from CD14⁺CD16⁻ monocytes [10].

The implication that blood monocytes exist in a proinflammatory state in T1D individuals results from earlier studies which analyzed serum samples from subjects at T1D onset. These samples contained significantly higher levels of monocyte-derived cytokines, such as IL-1 β , IL-6, and TNF- α , than those found in healthy first-degree relatives [11-13]. Direct evidence for the proinflammatory state of blood monocytes in T1D individuals was provided by the observation that monocytes

freshly fractionated from peripheral blood mononuclear cells (PBMCs) of T1D patients expressed genes of these cytokines in the absence of further stimulation [6]. IL-1 β and IL-6 are involved in directing the differentiation of CD4⁺ lymphocytes into IL-17-producing (Th17) cells, which are associated with insulinitis [14]. In this regard, IL-1 β and IL-6 act on CD4⁺CD45RA⁺CD62L⁺CD25⁻ memory cells, and convert them into a Th17 phenotype [6].

mDC differentiation features of blood monocytes in T1D individuals

Several groups have utilized *in vitro* culture systems to evaluate the differentiation of blood monocytes into mDCs in T1D patients. Monocytes derived from PBMCs from patients of different age groups, and at risk children, are found to have a reduced potential to become differentiated into mDCs in GM-CSF and IL-4 conditioned cultures, as compared to those obtained from healthy controls. mDCs generated from diabetic subjects are functionally impaired as judged by their reduced capability to activate autologous T cells, and stimulate allogeneic lymphocytes [15-17]. Although mDCs from diabetic and non-diabetic individuals under similar culture conditions are not phenotypically different, mDCs generated from patients produce significantly higher levels of IL-6 following stimulation with TNF- α [18].

These results could be interpreted in the context that some of the *in vivo* activated proinflammatory monocytes isolated from a cohort of T1D patients did not convert into mDCs in the presence of GM-CSF and IL-4. Therefore, IL-6 detected in the culture supernatant is released by monocytes that have not fully differentiated into mDCs. Elevated levels of IL-6 could potentially result in autoregulation of activated monocytes leading them to differentiate into macrophages rather than DCs [19]. In T1D, the generation of reduced mDC numbers could then occur. This can be attributed to monocytes that do not differentiate normally into mDCs under the influence of elevated level of IL-6 produced by the subpopulation of proinflammatory blood monocytes.

Functional modulation and regulation of proinflammatory monocytes for restoring tolerance in T1D

Abnormalities in blood monocytes can seriously affect DC generation, and eventually can cause

disruption of the immunoregulatory circuit. On this basis, modulation of the monocytes' proinflammatory property could be considered as an essential step for immune intervention in T1D. *In vitro* studies showed that blood monocytes differentiate either into proinflammatory (type 1) or anti-inflammatory (type 2) macrophages [20] when exposed to microbial antigens, interferon-gamma (IFN- γ), or the Th2-associated cytokine IL-4 together with hematopoietic factors such as GM-CSF and M-CSF. The latter are known inducers of proliferation and differentiation of mononuclear phagocytic cells [21, 22].

GM-CSF-cultured monocytes share the IL-12-producing phenotype when stimulated with LPS, and they engage autologous and allogeneic CD4⁺ lymphocytes to trigger their activation. However, monocytes cultured in the presence of a combination of GM-CSF, IL-4, and TNF- α are modulated to differentiate in a different way. In the presence of these cytokines, CD16⁺ and CD16⁻ monocytes are converted into two separate subpopulations of mDCs [10]. The CD16⁺-derived mDCs express higher surface levels of CD80 and CD11c than those derived from their CD16⁻ monocyte counterpart. Upon stimulation with LPS, CD16⁻ monocyte-derived mDCs secrete greater amounts of IL-12, while mDCs generated from CD16⁺ monocytes express higher levels of TGF- β . Human TGF- β is a cytokine that has been shown *in vitro* to be involved in the generation of tolerogenic mDCs that mediate the differentiation of naïve/inactivated human CD4⁺ lymphocytes into Treg cells [23-25].

On the basis of these findings, therapies to promote production and differentiation of CD16⁺ monocytes and repression of CD16⁻ inflammatory monocytes, or therapies to steer differentiation of CD16⁺ monocytes into TGF- β -producing mDCs, could be options for immune intervention in T1D. These monocyte subsets derive from separate developmental pathways, and do not appear to be interchangeable. Therefore, it might be possible to influence their balance during development through modulation of growth factor and cytokine balances. *In vitro* expansion of TGF- β -producing mDCs might be possible through a combination of GM-CSF, IL-4, and TNF- α . The recombinant form of GM-CSF has gained previous regulatory approvals in human trials. Whether a safe dose of the cytokines may act on proinflammatory blood monocytes in T1D subjects to redirect their differentiation into non-pathogenic or IL-10-producing mDCs is unclear. In any case, these criteria would set the benchmark to evaluate the feasibility of this regimen.

M-CSF-exposed monocytes are differentiated into macrophages that express the scavenger receptor, CD136. These CD136⁺ macrophages bear anti-inflammatory features, as shown by their IL-10 secretion property, upon exposure to LPS. Interestingly, M-CSF-modulated monocytes possess regulatory properties as they can interact with CD4⁺ lymphocytes and convert them into CD4⁺D25⁺FOXP3⁺ Treg cells [26]. Furthermore, studies have shown that CD4⁺CD25⁺FOXP3⁺ Treg cells interact with blood monocytes to convert them into macrophages resembling the characteristics of M-CSF-derived macrophages. This event is attributed to IL-10, IL-4, and IL-13 produced by the CD4⁺CD25⁺FOXP3⁺ Treg cells following engagement with the monocytes [27].

In T1D development, autoreactive CD4⁺ cells bearing the Th1 phenotype are implicated in causing islet damage, either directly, or indirectly. Nitric oxide or oxygen radicals released by effector lymphocytes are shown to be toxic to islet β -cells. Furthermore, IL-2 and IFN- γ produced by islet-specific Th1 cells can potentiate the generation of diabetogenic CD8⁺ T cells that infiltrate the pancreatic islets, and attack insulin-secreting β -cells [28, 29]. IL-10 is known to exhibit a key role in suppressing Th1 responses [30]. The cytokine is also primarily involved in the induction of adaptive Treg cells from peripheral CD4⁺ lymphocytes [31].

Within the context of this information, it is not clear whether serum M-CSF level is elevated in T1D patients. The range of M-CSF measured in the serum samples of normal human subjects is between 700-900 units per ml [32]. In cancer trials, infusion of recombinant M-CSF at a dose that does not cause hepatic toxicity, led to the expansion of circulatory phagocytes [33-36]. These reports may serve as a reference for the rationale of evaluating M-CSF-based therapy in the immune intervention of T1D. In developing a possible therapy, it will be important to clarify whether administration of a permissible dose of the cytokine is capable of directly modulating the proinflammatory function of the monocytes in T1D subjects. In addition, it will be necessary to clarify if the cytokine can guide the differentiation pathway of the blood monocytes to become IL-10-producing macrophages able to promote the generation of Treg cells in restoring tolerance.

Various experimental systems showed that there is a cross-regulatory relationship between DCs and Treg cells [2, 3]. Observations by Tiemesan *et al.* revealed that cross-regulation also occurs between anti-inflammatory macrophages and

Treg cells. In fact, macrophages in the gut lamina propria of mice were recently shown to induce Foxp3-expressing Treg cells and to suppress the induction of Th17 cells by neighboring DCs [37]. Production of immunomodulatory cytokines such as IL-10 and TGF- β by Treg cells, in turn, suppresses monocyte maturation and production of TNF- α , IFN- γ and IL-6 [38]. This suggests that Treg cells are also instrumental in controlling pro-inflammatory monocytes in T1D individuals. Figure 1 summarizes the discussed approaches based on modulation of the proinflammatory monocytes with cytokines to steer their differentiation into non-pathogenic or regulatory macrophages/DCs.

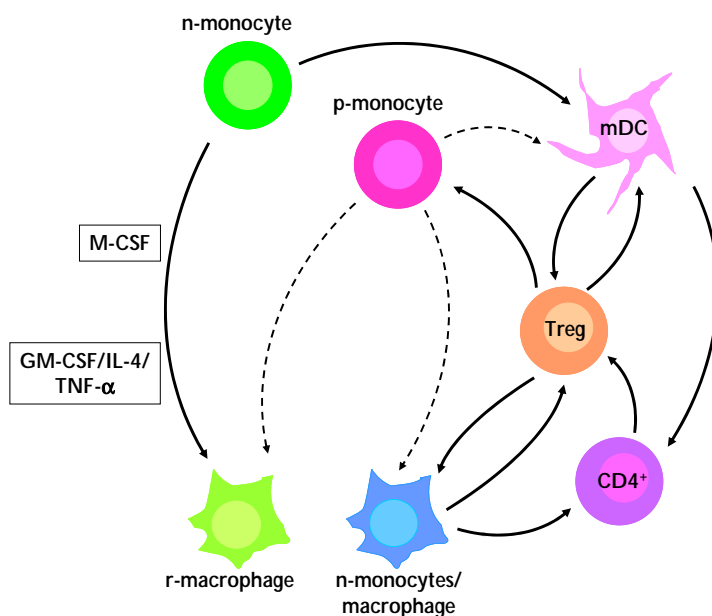


Figure 1. Conceptual approaches to redirect the differentiation of proinflammatory blood monocytes in T1D subjects. Normal blood monocytes (n-monocytes) cultured in the presence of GM-CSF and IL-4 would differentiate into CD11c⁺ mDCs. Subpopulations of the normal blood monocytes upon exposure to a combination of GM-CSF, IL-4, and TNF- α , or M-CSF are shown to differentiate and acquire TGF- β - and IL-10-expressing phenotypes, respectively. The regulatory monocytes/macrophages (r-monocytes/macrophages) and DCs generated could then cross-regulate the functional activity of T regulatory (Treg) cells to maintain the integrity of the immunoregulatory circuits and to prevent T1D development. However, it is unclear whether the proinflammatory monocytes found in T1D subjects could be steered to differentiate along these pathways (indicated by dashed lines). If they do, the information would be useful to devise strategies for immune intervention in T1D.

Cross-talk between microbes and myeloid cells: relevance for diabetes?

Monocytes as well as other myeloid cells can recognize a variety of microbe-associated molecular patterns. They respond to microbial stimuli via their pattern recognition receptors such as toll-like receptors (TLRs) and NOD-like receptors (NLRs). Thus, microbes, or the collection of different microbial species, lining the surface of mucosal epithelium in gastrointestinal, respiratory, and genitourinary tracts are an important environmental factor and affect the inflammatory vs. tolerogenic balance in these tissue compartments.

NOD mice develop diabetes most reliably when they are kept in an environment with a low microbial load [39]. MyD88 is a key element in innate immune signaling via TLRs, and is supposedly involved in the cross-talk of innate immune cells with microbes lining mucosal surfaces. Recently, it was discovered that NOD mice deficient of MyD88 harbor gut microbiota which differs from wild type (i.e. MyD88 competent) NOD mice and confers partial protection against diabetes development [40]. The idea that the cross-talk between gut microbes and the immune system is disturbed in diabetes is also supported by the observations that NOD mice kept under conditions allowing high diabetes incidence develop an inflammatory milieu in the gut soon after weaning [41], and show aberrancies in the antigen-presenting function of innate B lymphocytes [42]. These observations are dependent on the nutrition and type of gut microbiota in the mouse colony. This suggests a role for microbe-associated factors in regulation of diabetogenesis, as was also found in the MyD88-deficient NOD mice.

In the human setting, aberrant immunologic activity in the small intestine in children with T1D, and microbial colonization of the gut in infants has been implicated in the subsequent regulation of immune responses elicited against food an-

tigens [43, 44]. In infants suffering from atopy, administration of lactobacilli after birth was shown to reduce symptoms for an extended follow-up period. It is not clear whether regulation of immune responses to autoantigens such as insulin could be modulated by microbial adjuvants (or probiotics). However, the findings imply that administration of selected probiotic bacteria (perhaps as part of autoantigen feeding regimens) to induce Treg cells may be an effective and safe regimen that should be investigated for its applicability in the immune intervention of T1D.

Induction of Treg cells by targeted presentation of autoantigen to dendritic cells

Inflammatory stimuli derived from tissue or secreted by various leukocyte subtypes (e.g. natural killer cells) present in the lymph nodes shape the local milieu during activation of antigen-specific T cells. Inflammatory stimuli can be created by IL-12, IFN- γ , IL-1 β , and IL-6. These cytokines induce maturation and activation of DCs, and favor differentiation of responding T cells into Th1, and/or Th17 subsets. In the absence of inflammatory stimuli, DCs remain in a "steady-state", display immature phenotype, and typically induce T cell tolerance instead of productive immune response (reviewed in Mueller, 2010 [45]).

Peripheral tissue antigens such as islet antigens are normally presented by DCs in the absence of inflammatory stimuli. This typically leads to either deletion of antigen-specific T cells or to induction of Treg cells (reviewed in Steinman and Nussenzweig, 2002 [46]). DCs cultured in the presence of immunosuppressive agents

such as corticosteroids, 1,25-dihydroxyvitamin D, or prostaglandin E2 (PgE2) are rendered immature. Adoptive transfer of such DCs may be exploited to induce tolerance in transplantation medicine and in autoimmune diseases (reviewed in Morelli and Thomson, 2007 [47]). However, it is also possible to target antigen directly to steady-state DCs *in vivo* by taking advantage of their expression of the endocytosis receptor DEC205 (CD205). Coupling of antigen to a monoclonal antibody against DEC205 can target it to the surface of immature DC. Initially, the feasibility of this approach was shown by studying peripheral deletion of CD8 T cells specific for a model antigen, ovalbumin [48]. More recently, the role of DEC205⁺ DCs in induction of Foxp3 Treg cells was established [49]. Due to the selective role of DEC205⁺ DC in Treg cell induction, it is an attractive idea to target autoantigens to DEC205⁺ DCs to induce *de novo* Foxp3⁺ Treg cells.

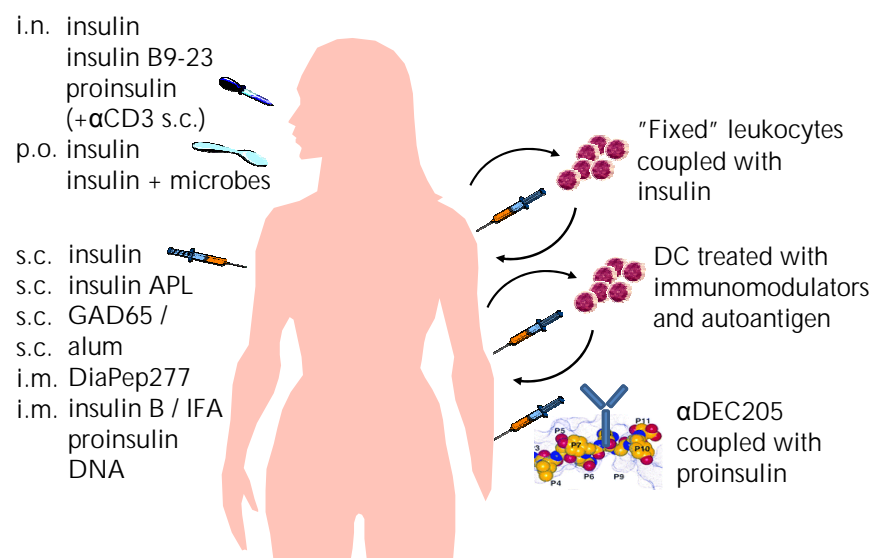


Figure 2. Approaches of antigen-specific immunotherapy for T1D. Education of the immune system to tolerate islet autoantigens by inducing passive (anergy of pathogenic leukocytes) or active (promotion of regulatory T cells) tolerance is a long sought treatment for T1D. Alum: aluminium dihydroxide. IFA: incomplete Freund's adjuvant. APL: altered peptide ligand. DiaPep277: a peptide derived from heat shock protein 60. Dosing regimens: i.n. (intranasal), p.o. (peroral), s.c. (subcutaneous), i.m. (intramuscular). α CD3: modified antibody against T-lymphocyte CD3 receptor. "Fixed" leukocytes: ethylenecarbodiimide-treated, killed leukocytes to which autoantigen is covalently attached. DC: DC differentiated *in vitro* from blood monocytes in the presence of immunomodulators such as corticosteroids, prostaglandin E2 or vitamin D. α DEC205: antibody against dendritic cell endocytosis receptor DEC205. Data from both animal models and clinical trials are included (see text for references and details).

In this issue of *The Review of Diabetic Studies*, Petzold and coworkers report their seminal studies in the NOD mouse model to induce islet-specific Treg cells via *in vivo* targeting of autoantigen to immature DCs [50]. Previously, they showed that coupling antigen to fusion antibodies against the DEC205 endocytic receptor on steady-state DCs induces Treg cells *in vivo* [51]. This strategy had potential benefits in preventing disease progression. Now they went a step further. They took the approach of coupling autoantigen (proinsulin or a mimotope of BDC2.5 cells) similarly to anti-DEC205 antibody, and studied the feasibility of inducing islet-specific Foxp3 Treg cells during ongoing autoimmunity [50]. It showed that BDC2.5-derived Foxp3 Treg cells persisted over time in mice receiving injections of autoantigen-mimotope coupled to DEC205. Also, the anti-DEC-205-mediated targeting of whole proinsulin in prediabetic NOD mice substantially reduced the incidence of diabetes. As the number of mice studied was small, these findings on the potential efficacy in suppressing diabetes await confirmation by subsequent studies. However, while other regimens of administering autoantigen under tolerance-promoting conditions do not yield more convincing results than we currently have, targeting autoantigen to DEC205⁺ DC remains an interesting approach that deserves further study. A sum-

mary of potential approaches of inducing antigen-specific tolerance in T1D (including approaches mainly tested in other autoimmune diseases and transplantation medicine) is depicted in Figure 2.

Conclusions

The findings that *in vivo* activated blood monocytes displaying proinflammatory features are present in the circulation of T1D patients calls for measures to control their activities and to restore a balanced immunity in prediabetes. Monocytes clearly have their role in balancing effector and regulatory T cell induction.

Yet, efforts to utilize DCs to restore immune tolerance must also be continued. The work by Petzold *et al.* together with the increasing insight into peripheral tolerance mechanisms demonstrate the potential of DC modulation to induce Treg cells [45, 47, 50, 51]. We expect a continued research interest in testing these and conceptually similar approaches (reviewed in Luo *et al.*, 2010 [52]) for their applicability to regulate autoimmunity in animal models of type 1 diabetes and in clinical trials involving human subjects at risk of developing type 1 diabetes.

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