Running title: Treg in autoimmune diabetes

Title: Regulatory T cells in autoimmune diabetes: mechanisms of action and translational potential

Authors: Vitalijs Ovcinnikovs[1], Lucy SK Walker[1]

Affiliations: [1]Institute of Immunity & Transplantation, University College London Division of Infection & Immunity, Royal Free Campus, London, NW3 2PF, UK.

Abstract:

Since the discovery of specialized T cells with regulatory function, harnessing the power of these cells to ameliorate autoimmunity has been a major goal. Here we collate the evidence that regulatory T cells (Treg) can inhibit Type 1 Diabetes in animal models and humans. We discuss the anatomical sites and molecular mechanisms of Treg suppressive function in the Type 1 Diabetes setting, citing evidence that Treg can function in both the pancreatic lymph nodes and within the pancreatic lesion. Involvement of the CTLA-4 pathway, as well as TGF-β and IL-2 deprivation will be considered. Finally we summarize current efforts to manipulate Treg therapeutically in individuals with Type 1 Diabetes. The translation of this research area from bench to bedside is still in its infancy, but the remarkable therapeutic potential of successfully manipulating Treg populations is clear to see.

Keywords: Type 1 Diabetes, Treg, CTLA-4, IL-2, immunotherapy, immune regulation

1. Introduction

Type 1 Diabetes (T1D), also known as early-onset or juvenile diabetes, is an autoimmune disease, caused by the destruction of insulin producing beta-cells in the pancreatic islets of Langerhans. It manifests as a development of inflammatory infiltrates in the pancreas accompanied by a loss of blood glucose homeostasis, eventually leading to life-long dependency on exogenous insulin for the affected individual. T1D is considered a result of breakdown of tolerance to self-antigens of the pancreas, however the precise disease-causing mechanisms are multifactorial. The ~50% concordance in identical twins suggests a significant environmental influence in addition to the strong genetic links implicating mechanisms of central and peripheral immune tolerance[1]. Considerable insight into the genetics of T1D has derived from analysis of the insulin dependent diabetes (*idd)* loci of non-obese diabetic mice (NOD), an established animal model of T1D research. Importantly, human genome-wide association studies reveal striking similarity in genetic susceptibility regions between species[2].

Development of central tolerance takes place in the thymus, where T cell precursors are selected on the strength of their T cell receptor (TCR) interaction with peptide presented on the major histocompatibility complex (MHC) proteins (HLA in humans). Thymocytes that express TCR with high affinity for self-peptides are eliminated via the process of negative selection, which prevents highly autoreactive T cells from arising. In some cases, self-reactive T cells can differentiate into regulatory T cells (Tregs). The process of central tolerance is, however, imperfect and allows some autoreactive T cells to escape into the periphery[3,4]. Fortunately, in healthy individuals the autoreactive conventional T cell (Tconv) pool is effectively controlled by peripheral tolerance, most notably via Treg populations, whose suppressive mechanisms, orchestrated by the master transcription factor FoxP3, serve to prevent autoimmunity from arising[5].

1.1 Genetic control of autoimmunity

Given that HLA molecules determine what T cells can "see", it is not surprising that alleles at the HLA locus represent a major genetic susceptibility to T1D and indeed influence susceptibility to multiple autoimmune diseases[6]. In the case of T1D, variations in HLAII-DQ and -DR loci, affecting specific peptide-binding pockets, may negatively affect self-antigen presentation in the thymus, thus affecting the selection of the T cell repertoire[7,8]. For instance, insulin-specific T cells in NOD mice recognize a suboptimal MHC-binding register of insulin that is insufficient to trigger apoptosis due to weak affinity[9-11]. A similar HLA-DQ8 restricted insulin-specific population of T cells has been identified in humans[12]. In addition to T1D, the abnormal topology of TCR binding that leads to suboptimal MHC interactions also features in multiple sclerosis and its mouse models suggesting that altered antigen presentation may be a common factor in autoimmunity[13,14]. In addition, variation in the magnitude of self-antigen expression may contribute to autoimmune susceptibility. For instance, the IDDM2 polymorphism, which maps to the promoter region of the insulin gene has been linked to reduced insulin mRNA in the thymus, possibly influencing central tolerance development via impaired deletion of insulin specific T cells; or peripheral tolerance via reduced development of Treg[15,16].

PTPN22, a non-receptor tyrosine phosphatase implemented in TCR signaling, is also strongly associated with susceptibility to autoimmune disease[17,18]. Several studies have suggested that the R620W variant of this gene lowers TCR signaling and allows for more autoreactive T cells to escape thymic selection that would otherwise develop into Treg[19,20]. Maine[21] and Brownlie[22] report an increase in thymic Treg output in PTPN22 deficient mice, pointing to a possible shift in selection thresholds. Conversely, others report PTPN22-R620W as a gain-of-function variant that is unable to bind Csk, a negative regulator of TCR activation. Thus by reducing the threshold of T cells activation R620W is responsible for the overactive responses from lower-affinity TCR T cells in the periphery[23]. A mouse model of the human R620W variant supports this by showing enhanced TCR signaling as a cause of increased effector and memory T cell generation alongside general features of systemic autoimmunity, whilst the Treg function remains unaffected[24]. Work from the Zamoyska lab demonstrated that the increase in T cell activation in PTPN22 knockout mice was due to enhanced responses to

low affinity antigens in the periphery, indicating that PTPN22 acts as a brake on responses to weak antigens[25]. Dampening of weak-to-medium TCR signals by phosphatases has recently been shown to be critical for selection of weakly self-reactive clones to occur in the thymus[26]. It is therefore possible that controlled responses to weak antigens may play a role in both thymic selection and later activation in the periphery.

Several mutations within the interleukin-(IL-)2 pathway are also found to associate strongly with T1D, including IL-2 itself as well as IL-2R-α (CD25) and IL-2R-β (CD25 and CD122) receptor susceptibility loci [1,27,28]. IL-2 is central to the development and homeostasis of Treg that constitutively express CD25, the α-chain of the high affinity IL-2 receptor. At higher doses it acts as a growth factor for effector CD4 and CD8 cells, therefore sensing and removing IL-2 from the local environment is an important mechanism of Treg[29]. Whether the contribution of IL-2 related polymorphisms is causative for T1D is unclear. This is due to low disease penetrance despite common occurrence of these polymorphisms in humans[30]. In NOD mice, who harbor all of these mutations, low IL-2 levels, reduced CD25 and increased apoptosis is observed on Treg within the pancreatic lesion, suggesting altered Treg homeostasis. Furthermore, the absence of IL-2, as an inhibitor of Tfh development, might also lead to the predominant T follicular helper (Tfh) cell phenotype found in T1D diabetes [31,32].

Lastly, cytotoxic T lymphocyte antigen 4 (CTLA-4), a major suppressive mechanism of Treg cells has been identified as a susceptibility locus in T1D[33]. CTLA-4 plays a central role in preventing T cell activation by depriving them of costimulatory ligands CD80 and CD86 (B7) from the antigen presenting cells (APC)[34]. The role of costimulation is to dramatically enhance the TCR-MHCII signal and amplify T cell activation, leading to subsequent clonal expansion and differentiation aided by the cytokine environment. Importantly, B7/CD28 pathway is carefully balanced by CTLA-4, a close homologue of CD28 with greater affinity to its ligands that does not relay a costimulatory signal[35]. CTLA-4 is dramatically upregulated upon TCR-MHCII binding on Tconv and to an even greater extent on Treg[36]. Levels of CTLA-4 expression in humans[37] as well as various mouse isoforms have been connected to T1D susceptibility[38,39].

Taken together, the genetic association data now implicates considerable numbers of loci in contributing to autoimmune susceptibility. Collectively, these data firmly indicate a role for T cells and increasingly point towards variability in immune tolerance mechanisms that are required to keep self-reactive T cells under control. From an immunological perspective it is increasingly clear that such self-tolerance is in large part maintained by specialized populations of regulatory T cells (Treg).

1.2 Control of autoimmune diabetes by regulatory T cells

Multiple lines of evidence support a key role for regulatory T cell in suppressing the development of Type 1 Diabetes. Perhaps most striking is the observation that T1D manifests in approximately 80% of individuals suffering from IPEX due to deficient Treg development[40]. This suggests a non-redundant role for Foxp3-expressing Treg in preventing islet autoimmunity in humans. Substantial evidence from mouse models also supports this conclusion. BDC2.5 TCR (specific for an islet autoantigen) transgenic NOD mice rendered Treg deficient by the scurfy mutation developed rapid and aggressive diabetes, with 100% incidence by 21 days post birth[41]. Similarly, punctual ablation of Treg in BDC2.5/NOD mice, using the transgenic diphtheria toxin receptor system, leads to fulminant diabetes within 3 days[42]. Consistent with this idea, diabetes caused by adoptive transfer of pancreas-specific conventional T cells can be controlled by co-injection of $CD25^+$ Tregs[43].

In T1D patients, despite initial reports to the contrary[44], it appears that the frequency of Treg in peripheral blood is probably unchanged compared to healthy controls[45-47], and Tregs from patients typically exhibit normal suppressive capability *in vitro*[48]. Nevertheless, careful longitudinal analysis during the 12 months following T1D onset suggested a transient decrease in Treg function that manifested between 2 weeks and 6 months post diagnosis and had resolved by 9 months[49]. Some evidence also suggests that certain individuals with

longstanding T1D may exhibit reduced Treg function[50], for example as a result of alterations in the capacity of their Treg to respond to IL-2[51,52].

Thus, Treg appear to be an important defense against the development of autoimmune diabetes in both mice and humans. Understanding the mechanisms by which Treg control autoimmunity offers an opportunity to gain insight into the molecular control of disease development and ultimately to potentially develop better treatments.

2. Sites of Treg action in T1D

An important question concerning the regulation of autoimmune diabetes by Treg is that of anatomical location: do Treg exhibit their suppressive effects in the pancreas-draining lymph node or within the pancreas itself? Addressing this issue has implications for our understanding of the biological processes that naturally inhibit diabetes in healthy individuals, as well as highlighting migration considerations that are pertinent to therapeutic Treg manipulation.

2.1 Pancreatic LN

The priming and activation of naïve T cells occurs in the draining lymph node where antigen-bearing dendritic cells migrate from the tissue. When islet-specific BDC2.5 TCR transgenic CD4 T cells were adoptively transferred into NOD recipients, they proliferated specifically in the pancreatic lymph node (PanLN), and this occurred at timepoints significantly earlier than insulitis could be detected[53]. Transgenic antigens (e.g. Ovalbumin, Hen Egg Lysozyme) expressed under the insulin promoter are also presented to T cells in the PanLN[53,54]. Elegant studies using intrapancreatic injection of CFSE-labelled cells[55] or mice transgenically expressing GFP under the control of the insulin promoter[56] have permitted the dendritic cells trafficking antigen to the PanLN to be visualized, indicating that they bear a CD11c+CD11b+CD8α- phenotype. The importance of T cell activation that takes place in the PanLN is illustrated by its excision, which leads to protection from disease in NOD mice[57,58].

The interaction between T cells and dendritic cells (DCs) within lymph nodes has been demonstrated *in vivo* by numerous studies using two-photon microscopy[59-61]. There, T cells actively scan the DC network in search of their cognate antigen and upon recognition exhibit arrest and form stable interactions with DCs[62]. Celli *et al*.[63] estimate that a 6hr duration of the arrest phase is required to trigger proliferation and clonal expansion. The ability of multiple transient interactions (swarming) to initiate T cell proliferation has also been reported[64], and abundance and quality of antigen [65] as well as the state of DC maturation[66] are key factors in determining the size and nature of the T cell response.

Tadokoro *et al*.[67] demonstrate that the arrest duration of Tconv upon recognition of antigen delivered by immunization is reduced two-fold when Treg are also present. This phenomenon of Treg influencing the nature of T cell:DC interactions is also observed in response to self-antigen as demonstrated by Tang *et al*[68]. In this study, the authors demonstrated that islet specific (BDC2.5) Treg formed stable interactions with the APCs in the PanLN, whilst denying stable contact formation by BDC2.5 Tconv and promoting swarming behavior. This was associated with reduced Tconv proliferation. Importantly, Treg did not form contacts with Tconv directly and did not appear to compete for antigen on the antigen-bearing DC as BDC2.5 TCR Treg could suppress conventional T cells specific for a different antigen (BDC12-4.1 TCR Tconv). This carries an important implication for therapeutic use of Treg since it implies that a single specificity Treg population can suppress self-reactive T cell clones of multiple epitope specificities provided they are interacting with antigen in the same microenvironment.Using a model involving diabetogenic TCR transgenic CD8 T cells (8.3-CD8), Serra *et al.* generated evidence that Treg were able to modulate the phenotype of APC in the PanLN of NOD mice and suppress CD8 T cell responses[69]. The effect of Treg in this system was to lower expression of costimulatory ligands on the PanLN-resident dendritic cells, particularly CD80, as well as altering expression of CD40 and CD11c.

In vitro assays of Treg function traditionally measure the inhibition of conventional T cell proliferation, a readout of suppression which is robust and dose dependent. However the effects of Treg *in vivo* in diabetes models have been less clear. Indeed our own experiments

investigating the consequences of Treg in an adoptive transfer model of diabetes revealed a surprisingly modest effect on Tconv proliferation and IL-2 production, but a dramatic inhibition of Tconv IFN-γ production and islet infiltration[43]. Decreased IFN-γ production was linked with impaired upregulation of the chemokine receptor CXCR3[43], consistent with a potential role for this chemokine receptor in mediating pancreas entry. In other models, the presence of Treg robustly inhibited the proliferation of Tconv in the PanLN[68]. These conflicting observations were largely reconciled by the findings of Tang *et al*.[68] who reported that if Treg were transferred two days prior to Tconv, they were able to completely abrogate Tconv proliferation in the PanLN. In contrast, when transferred at the same time, proliferation was unaffected but IFN-γ production was markedly reduced. Thus the balance of Treg:Tconv within the local microenvironment likely dictates whether Treg suppression targets Tconv cytokines alone or also shuts down Tconv proliferation. Interestingly Treg appear to selectively inhibit IFN-γ rather than orchestrating blanket inhibition of Th1 differentiation since induction of Tbet and TNFα production are spared[70]. Thus, the actions of Treg in the pancreatic LN can involve both effects on Tconv proliferation and on effector cytokine production.

A role for Treg suppression within the draining LN is consistent with the observation that CCR7-/- Treg, that lack the capacity to home to LN, fail to suppress contact hypersensitivity or IBD despite effectively migrating to inflamed skin or gut respectively[71]. However, evidence from T1D models suggests that the draining LN is not the only site of Treg action as will be discussed below.

2.2 Pancreatic Islets of Langerhans

Treg are known to be present within the infiltrated pancreas in mouse models of T1D raising the possibility that they may also act at the site of the autoimmune attack (Figure 1). In BDC2.5/NOD mice, $CD4^+CD25^+CD69^-$ Treg are found alongside conventional CD4 cells within the lesion even before the onset of disease. Infiltrating T cells appear to arrive at around 2-3 weeks of age, more than 2 months before the onset of disease[72]. Indeed live imaging of diabetogenic CD8 T cells suggests that they enter the lesion via postcapilliary

vessels directly adjacent to the islet site that appear to become "leaky" during the onset of prediabetes[73].

[Insert Figure 1 Here]

Whether such "leakiness" permits entry of bystander T cells to the islet has been a contentious issue, with some studies suggesting islet entry is tightly restricted to antigen-specific T cells[74,75]. However, more recently it has been suggested that T cell infiltration is beta-cell antigen specific in the early stages but progresses to become more open to cells with wider specificity after the onset of islet inflammation, with IFN-γ signaling and induced expression of CXCR3 chemokines playing a key role in the recruitment of bystander T cells[76]. Recent work using the Kaede transgenic mouse model to track fluorescently labeled immune cells from a non-draining LN supports the open status of the diabetic lesion at all stages of disease with many infiltrating T cells bearing a naïve phenotype[77]. Treg were also shown to access the pancreatic infiltrate in this model, although they exhibited less active migration dynamics both in terms of exiting the labeled LN and appearing in the pancreas.

The presence of Treg in the pancreas of humans with T1D is far harder to assess. A significant factor here is that insulitis is mainly thought to be present close to T1D diagnosis[78] such that even pancreas tissue obtained from individuals with "new onset" disease may exhibit very little lymphocytic infiltration. Welcome developments in early disease detection and management have all but eradicated fatal ketoacidosis at T1D presentation. However, prior to these developments, a small number of unfortunate cases meant that pancreas tissue was available to study very close to the time of T1D diagnosis. In one such instance, tissue was obtained from a 12 year old girl who died within 24h of diagnosis and had insulitis in 24% of islets. Most of the infiltrating T cells were positive for HLA-DR, suggestive of activation, and a proportion expressed CD25 raising the possibility that they were Treg[79]. Willcox and colleagues further demonstrated the presence of FoxP3+ cells within the inflammed islets of recent onset T1D patients, although these cells were understandably rare given the scarcity of lymphocytes in general[80]. In this regard it should be noted that the highest frequency of

FoxP3+ cells within infiltrated islets was observed prior to the onset of diabetes in NOD mice[31], a timeframe that is understandable difficult to study in humans.

Assuming then that Treg are present within the pancreatic infiltrate in mice and humans with T1D, do they elicit suppressive function at this site? Evidence suggesting this is the case comes from careful comparative analysis of diabetes development in either the presence or absence of Treg in the BDC2.5/NOD model. Abrogation of Treg development by the scurfy mutation did not alter the activation of T cells in the pancreatic LN in this model, but instead had a profound impact on the nature of the insulitis rendering it immediately aggressive[41]. These data suggest that Treg operate within the pancreas itself to moderate the capacity of conventional T cells to penetrate the islet cell mass and elicit damage to the beta-cells. A similar conclusion was reached in experiments where Treg were targeted for deletion by expression of the diphtheria toxin receptor under the control of the FoxP3-promoter: within hours of diphtheria toxin-mediated Treg depletion, the islet resident cells mounted a dramatic response, including large amounts of IFN-γ production by islet-resident NK cells[42].

Further support for the idea of intra-pancreatic regulation comes from the observation that the capacity of cyclophosphamide treatment to promote diabetes was associated with a sustained depletion of Treg in the pancreas, but not the PanLN of NOD mice[81]. In addition, progression to diabetes in NOD mice was associated with a decrease in the Treg:Teff ratio in the pancreas, and not in the PanLN, suggesting a loss of regulation *in situ*[31]. During disease development, islet-infiltrating Treg displayed a decreased expression of CD25, coupled with lower expression of FoxP3 and survival factor Bcl-2, suggesting the decline in frequency is due to increased apoptosis. This, in turn can be a result of defective IL-2 production, a cytokine known to be essential for Treg homeostasis.

Additional insight has been provided by Mahne *et al*.[82], who demonstrated that Treg reduced the accumulation of islet-specific CD8 T cells. Furthermore, Treg abrogated the production of IFN-γ by both CD8 and CD4 T cells through the reduction of mTOR pathway activation by depriving effector cells of IL-2. Local APC phenotype was also modestly affected in these

experiments, with decreased expression of CD86 and CD40, although T cell:DC interactions did not appear to be disrupted[82].

Thus considerable evidence suggests that Treg are present within inflamed islets and are able to exert suppressive function at this location.

3. Mechanisms of Treg suppression in T1D

The importance of regulatory T cells in preventing autoimmunity is extremely well demonstrated in the scurfy mice[83,84] that have a frame shift mutation in FoxP3, the master transcription factor responsible for the Treg programme[85-87]. These mice succumb to an early death from a lymphoproliferative syndrome with multi-organ inflammation. To achieve regulation, Treg are believed to employ a broad array of suppressive mechanisms, including use of soluble mediators or cell-to-cell contact[88,89]. Some of these have been demonstrated to contribute to immune regulation in a T1D autoimmune setting.

3.1 Role of CTLA-4

CTLA-4 is an essential negative regulator of T cell activation, deficiency of which causes lethal autoimmunity in mice[90,91]. CTLA-4 binds to the same ligands as the T cell costimulatory receptor CD28 (CD80 and CD86, previously called B7-1 and B7-2 respectively). Several mechanisms explaining the action of CTLA-4 have been proposed. Early models focused on the idea that CTLA-4 delivers a negative signal when bound to its ligands. However, evidence for this model is incomplete[92], and within this framework there is currently no explanation of how CTLA-4 can suppress Tconv, but not Treg cells that express it at even higher levels. Furthermore, mouse bone marrow chimaeras that produce a mixture of wild-type T cells and ctla4-/- T cells are healthy, and show no evidence of lymphoproliferation or autoimmune tissue infiltration[93,94]. The elegant bone marrow chimaera experiment serves as strong evidence for an extrinsic mode of CTLA-4 action, in which CTLA-4 on wild-type T cells

effectively controls the activation of *ctla4-/-* cells[93,94]. This idea is consistent with a role of CTLA-4 in regulatory T cells since Treg are the archetypal mediators of cell extrinsic regulation[95]. It has been known for many years that CTLA-4 can compete with CD28 for its ligands and thus diminish costimulation and prevent T cell activation[96]. Indeed, costimulation of CD28 by CD80 and CD86 is the driver of pathology in *ctla4-/-* mice, as *ctla/cd28* double knockout or *ctla4/cd80/cd86* triple knockout animals show no evidence of T cell overactivation[97,98]. Thus, the biological role of CTLA-4 appears to be to regulate signals delivered through CD28. Interestingly, in numerous studies downregulation of CD80 and CD86 expression on APCs has been observed as the consequence of CTLA-4 action[34,69,99-103]. More recently, Qureshi *et al.*[104] have demonstrated that CTLA-4 can physically remove B7 from the surface of an APC, internalise and drive it down the endocytic compartment for eventual degradation by the process termed trans-endocytosis.

CTLA-4 is clearly necessary for the maintenance of tolerance to islet antigens (Figure 2), as *ctla4-/-* BDC2.5 TCR mice develop diabetes at higher incidence and an accelerated rate compared to their CTLA-4 sufficient counterparts[105]. Diabetes is also enhanced in mice treated with a blocking anti-CTLA-4 antibody[106]. Furthermore, CTLA-4 deficient DO11 TCR transgenic T cells exhibit an increased capacity to induce diabetes in an adoptive transfer model compared with CTLA-4 sufficient T cells[107]. Whether CTLA-4 acts in the Treg compartment to suppress diabetes appears to depend on the particular *in vivo* model under study. In one TCR transgenic adoptive transfer system, Treg-expressed CTLA-4 was crucial for suppression of diabetes caused by adoptive transfer of islet autoantigen specific CD25- T cells, as, unlike wild type Treg, CTLA-4 deficient Treg failed to control the disease[101]. However in another system, TCR transgenic Treg lacking CTLA-4 were as effective as their CTLA-4-expressing counterparts at inhibiting diabetes[108]. Importantly, in the latter model the recipient animals were CD28-/-NOD mice, meaning that the pathogenic immune response was not driven by CD28 costimulation. If CTLA-4 serves to regulate CD28 engagement, CTLA-4 function would not be predicted to control a CD28-independent response. It therefore makes sense that CTLA-4 was not required for regulation in this setting and that alternative Treg mechanisms were at play. In this regard, it is known that Treg from CTLA-4 deficient

mice express higher levels of TGF-β than wildtype Treg[101,109] and that this can partially compensate for the lack of CTLA-4 in *in vitro* suppression assays[109].

[Insert Figure 2 here]

Whether CTLA-4 serves as a Treg mechanism to regulate T1D in humans is yet to be revealed. However heterozygous mutations in CTLA-4, resulting in defective Treg function, have recently been described in humans[110,111] and notably two studies suggest that T1D is amongst the autoimmune manifestations associated with this condition[111,112].

3.2 Role of cytokine deprivation

Treg express high levels of CD25 (IL-2R-α) which in conjunction with IL-2R-β and the common-γ chain forms the high affinity IL-2 receptor. It has therefore been suggested that one facet of Treg suppression may be to consume IL-2 locally, thereby depriving conventional T cells of this well recognized growth factor[113,114]. This notion was challenged by the observation that Treg from CD25 deficient mice, isolated on the basis of FoxP3-GFP expression, showed intact suppressive function *in vitro*[115]. Nevertheless, evidence that local cytokine deprivation can contribute to Treg function has been steadily growing. Pandiyan *et al.*[116] demonstrated that Treg were able to consume IL-2 produced by conventional T cells, taking advantage of an IL-2-GFP reporter to preclude the possibility that the Treg were instead inhibiting IL-2 transcription. Deprivation of IL-2 was associated with a fraction of the Tconv undergoing apoptosis by a pathway involving the Bcl2 family proteins Bad and Bim. The capacity of Treg to deplete their local microenvironment of IL-2 could potentially support bystander suppression of Tconv with distinct specificities that are present in the vicinity. In models of cytokine deprivation, the spatial separation of Treg and Tconv becomes a significant factor in determining the degree of suppression[117] as the cells embark on a "tug-of-war" over local IL-2[118]. Conclusions about the contact-dependence of Treg suppression based on transwell assays, where Treg and Tconv are separated by distances of an order of

100 times the diameter of a lymphocyte, may not be sound considering that IL-2 competition may require a proximity in the region of 1-2 cell diameters[119].

Assuming then that cytokine deprivation can represent one mechanism of action of Treg, is this mechanism at play in the regulation of diabetes? In this regard, the loss of CD25 expression on islet-infiltrating Treg that precedes the onset of diabetes in NOD mice[31] could render them less able to sequester IL-2 from conventional T cells in addition to cutting them off from critical survival signals. Furthermore, within 8h of Treg depletion in BDC2.5/NOD mice, a signature of IL-2-induced genes was detectable in the islet resident NK cells and flow cytometry revealed a striking increase in STAT5 phosphorylation[120]. This suggests that the capacity of Treg to limit bioavailability of IL-2 within the islet lesion is a key factor controlling the immune response at this site. Consistent with the findings of Pandiyan *et al.*, this study found that the capacity of CD4 T cells to synthesize IL-2 at early timepoints was not altered by Treg, implying that IL-2 consumption rather than transcriptional inhibition was responsible[120]. Thus Treg appear to regulate ongoing anti-islet responses by competing for local IL-2: the defects in IL-2 signaling associated with T1D[51,52,121-123] may be relevant to this in ways yet to be elucidated.

If sequestering IL-2 represents a mechanism by which Treg control anti-islet T cell responses, then factors that alter IL-2 production may have an impact on immune control. In this regard, data from mouse models have suggested that intra-islet T cells produce the cytokine IL-21[32,124,125]. Interestingly IL-21 was able to substitute for the lack of IL-2 in conventional T cells but not Treg[126] , thereby selectively impairing the regulatory arm of the immune system. Furthermore, IL-21 increased CD25 expression in Tconv rendering them better able to compete for residual IL-2. Therefore factors that decrease T cell production of IL-2 have the capacity to interfere with immune regulation by impairing Treg homeostasis and interfering with their capacity to dominantly sequester this cytokine.

3.3 Role of TGF-β

It is now established that Treg are able to use TGF-β to suppress conventional T cell responses in some settings[127-129]. Indeed high expression of the integrin αvβ8 on effector Tregs, enabling them to activate latent TGF-β, has recently shown to be important for their capacity to regulate active inflammation[130]. In the setting of diabetes, local TGF-β may act to positively regulate Treg homeostasis within the islet lesion[131]. The contribution of TGF-β to Treg control of diabetes has been tested in adoptive transfer models: diabetes induced by adoptive transfer of NOD splenocytes to NOD-SCID recipients was inhibited by transfer of CD25+ Treg, however protection was abrogated in the presence of anti-TGF-β antibody[132]. In a different diabetes model, it was shown islet-reactive CD8 T cells that were unable to receive TGF-β signals were refractory to control by Treg[133]. On the other hand, data obtained from the BDC2.5/NOD diabetes model indicated that TGF-β neutralisation failed to recapitulate the effects of Treg depletion[120], despite internal controls indicating that the antibody was bioactive. This emphasizes the challenge of pinpointing which particular suppressive mechanism is dominant at any one time in a cell type (Treg) that is a "jack of all trades"[89]. Assuming that many suppressive mechanisms will be at play simultaneously (CTLA-4 function, IL-2 deprivation, inhibitory cytokines), blocking one will only abrogate Treg function if the others fail to compensate. Small differences in the particular diabetes model under study, or the timing of an experimental intervention, may lead to differences in the cellular composition of the lesion, and shift the balance between which regulatory mechanism is dominant. In short, blocking an individual suppressive mechanism and looking for Treg failure is a blunt tool for surmising whether that mechanism is in use. Nevertheless, on balance the evidence suggests that TGF-β can contribute to regulation of the anti-islet immune response.

4. Therapeutic manipulation of Treg in T1D

4.1 Therapeutic Treg expansion

The current understanding of etiology of autoimmune disease and the molecular pathways that underlie the maintenance or breakdown of immune tolerance offer the potential for a range of therapeutic interventions. Such interventions can broadly act to dampen immune activation, promote immune tolerance or, perhaps for the largest effect, utilize a combination of both. With respect to Treg this can be achieved by altering the Treg:Tconv ratio through boosting Treg numbers or enhancing their function (Figure 3).

[Insert Figure 3 with the 4.1 section, left or right hand side of text]

4.1.2 Anti-CD3

Monoclonal antibodies have shown promise in depleting pathogenic T cells and creating a favourable Treg:Tconv ratio. FcR non-binding anti-CD3-ε treatment promoted tolerance in NOD mice by selectively depleting pathogenic T cells, whilst sparing the Treg population[134]. In addition, the intervention appeared to induce the generation or expansion of Treg that were capable of suppressing in a TGF-β-dependent manner[132]. In an experiment involving the treatment of overtly diabetic mice, co-administration of a blocking anti-CTLA-4 Ab abrogated the disease remission induced by anti-CD3 treatment[135]: this could suggest a role for CTLA-4 dependent Treg function, or could reflect the fact anti-CD3 induced tolerance mechanisms cannot compensate for the lack of the CTLA-4 pathway. In a humanized mouse model anti-CD3 was shown to trigger IL-10 production from a subset of T cells that migrated to the gut[136].

The exact route by which anti-CD3 can differentially impacts effector T cells and Treg is still unknown. Valle *et al.* demonstrate that *in vivo* anti-CD3 effects correlate with heterogeneity of CD3 expression levels and therefore lower expressing Treg are preferentially spared[137]. It has also been shown that anti-CD3 treatment downregulates IL-7R on Tconv while upregulating it on Treg, suggesting a model in which IL-7 signals may contribute to the maintenance of Treg homeostasis[138].

Following the success of this therapy in animal models, the anti-CD3 treatment has also been tested in clinical trials of human T1D. A single treatment course of anti-CD3-ε (Teplizumab) in newly diagnosed individuals led to significant improvements in C-peptide response 2 years post-treatment[139]. The improvement correlated with a reduction of IFN-γ production by CD8 T cells as well as higher levels of IL-10. In a previous trial, however, anti-CD3 was found to cause increased adverse effects with elevated dosing and did not prevent the decline in C-peptide production, although there was a trend for the fall in production to be slower[140]. Glycosylation modified anti-CD3-ε (Otelixizumab) at a total dose of 48-65 mg preserved beta-cell function and reduced insulin requirements maintaining the effect at 3 years post-trial[141]. Reactivation of EBV was a prominent side effect that is resolved by using lower drug dosing, which may come at the expense of efficacy[142,143].

Thus, anti-CD3 antibodies have shown considerable promise in modulating the diabetogenic immune response in mouse models and T1D patients, with the actions of Treg most likely contributing the protective mechanisms. However challenges remain regarding the choice of endpoints for clinical trials (with composite endpoints proving problematic[144]) and the selection of doses which preserve efficacy while avoiding adverse effects.

4.1.2 Low dose IL-2

It is well known that IL-2 is a critical survival factor for regulatory T cells. This is exemplified by the lymphoproliferation and autoimmunity observed after blockade[145] or genetic deficiency[146,147] of IL-2 in mice. Harnessing the IL-2 pathway to promote Treg homeostasis therefore offers huge therapeutic potential[148]. For instance in diabetic mice, low dose IL-2 promoted homeostasis of Treg in the pancreas and it was further effective at preventing and reversing T1D[31,149,150].

In humans, low-dose IL-2 has been trialled in GvHD patients in a dose escalation study that showed protective effect of Treg at lower doses. Furthermore, patients demonstrated

increased thymic output and proliferation of Treg with improved resistance to apoptosis[151]. The results were in line with the role of IL-2 for Treg development and homeostasis. A similar boost in Treg function was found in patients with autoimmune HCV vasculitis[152]; and low-dose IL-2 was generally well tolerated[151].

With regards to diabetes, the therapeutic expansion of Treg by IL-2 has also been tested in clinical trials. In the first trial, IL-2 was used in conjunction with rapamycin, the mTOR inhibitor known to inhibit the activation of effector T cells[153]. Although an increase in Treg number was observed and persisted for some time after the trial, a drop in C peptide was seen, signalling a deleterious effect of the treatment. Moreover, an expansion in NK cell population was also noted in addition to marked eosinophilia. In a second IL-2 only dose-range estimation trial, no negative effect on beta-cell function was observed, pointing to the deleterious contribution of rapamycin in the previous study[154,155]. Interestingly, further studies in the NOD model suggested that although rapamycin augmented IL-2-induced Treg expansion it impaired the capacity of those Treg to suppress T cell cytokine production[156]. Subsequent immunomonitoring of the second trial revealed a dose range of 0.3‑1 MIU/day that does not initiate the NK cell transcriptional profile whilst expanding and activating Treg, as measured by enhanced GITR, CD25 and CTLA-4 expression [157].

Overall, despite the caution warranted by the prospect of IL-2 altering additional immune cell populations, harnessing the IL-2 pathway to promote Treg homeostasis appears a promising strategy in T1D and other autoimmune diseases. A meticulous approach to dose range estimation will be important for success, and relevant dose response trials are already underway[158]. The modification of IL-2 to alter its half-life and binding preferences also offers exciting opportunities to maximise the immune-regulatory effects of this therapy[159].

4.2 Therapeutic Treg induction

In addition to *ex vivo* Treg expansion and subsequent reinfusion, attempts have been made to influence tolerance directly *in vivo* in an antigen-dependent fashion. Tolerance to an

antigen can be established at mucosal sites, most notably, the gut, via the induction of Treg from naïve conventional T cells[160]. In NOD mice, oral[161], nasal and subcutaneous[162] routes of insulin administration all showed promise in disease protection. The following human trials have, despite initial encouraging results[163] largely presented with negative results[164-166]. Nevertheless, TrialNet oral insulin T1D prevention study is currently underway and with more detailed immunological analysis may shed light on the translational challenges[167].

In humans, where insulin injection is a standard therapy to control blood glucose levels, patients typically enter a short remission phase, shortly after the start of treatment. It has been suggested that this may be associated with the induction of insulin-specific Treg[168]. Attempts to mimic this phenomenon in NOD mice showed early promise, where administration of exogenous insulin has led to protection[169] or delayed onset of T1D[170], suggesting a potential benefit of insulin immunization. Once again, translation has been difficult, as intramuscular insulin in adjuvant was ineffective at preserving the C peptide response, however, Treg development was observed and these newly induced cells persisted two years post-study[171]. Immunization with the 65kd isoform of glutamic acid decarboxylase (GAD), another major T1D antigen, showed mixed results, with a potential preservation of stimulated C-peptide level, but no difference were observed in other measurements when compared to the placebo group[172-174].

Despite limited success of Treg induction approaches, new methods are still being researched. For instance, in a recent study by the Roncaloro group a novel approach at Treg induction was tested[175]. By targeting non-integrating lentiviral vector-mediated expression of insulin B chain 9-23 to hepatocytes, induction of both B9-23 specific Tconv and Treg was achieved in NOD mice. The Treg were able to inhibit islet infiltration and in combination with a single suboptimal dose of anti-CD3 this was able to reverse T1D. This study suggests that an antigen-specific non-integrating gene therapy that offers low risk of genotoxicity[176] could be combined with other immunomodulatory approaches to potentially become an effective T1D treatment.

4.3 Treg Cell therapy

A different approach to harness the multitude of tolerance mechanisms offered by regulatory T cells is by treating patients with *in vitro*-expanded Treg cells. Similar to therapeutic Treg expansion *in vivo*, the goal is to modulate the deleterious immune response to islet auto-antigens without causing global immunosuppression and increased susceptibility to infection or cancer. Furthermore, the tolerogenic environment created by such therapy could conceivably be long-lasting without the need for future cell reinfusions or for lifetime drug treatment.

In mice, ample evidence demonstrates that adoptively transferred Tregs are able to effectively prevent[43,177-179] and even reverse[180,181] autoimmune diabetes. Although antigen-specific Treg are more potent than polyclonal Treg populations, there are indications that functional suppression can extend beyond the TCR specificity of the injected Treg population[180]. Accordingly, injection of BDC2.5 TCR transgenic Treg, which recognize a single islet antigen, is sufficient to control a polyclonal autoimmune response in NOD mice, that presumably encompasses multiple antigenic targets[179,180]. This is consistent with the idea of "infectious tolerance" as proposed by Waldmann and colleagues[182] whereby "linked suppression" can be generated against antigens presented by the same APC[183]. In the case of T1D, this is epitomized by BDC2.5 Treg exhibiting the capacity to suppress the proliferation of 4.1 TCR transgenic T cells that are located in the same draining pancreatic lymph node but presumably recognize a different islet antigen[68].

A certain constraint when it comes to humans is the difficulty of obtaining sufficient numbers of cells from peripheral blood. Isolation can be performed on $CD4^+CD127^{lo}CD25^+$ surface markers, which enriches for the $FoxP3^+$ positive cells to > 95% purity[45]. The following expansion with anti-CD3- and anti-CD28-coated bead stimulation in the presence of high concentrations of IL-2 results in a 3000-fold increase in cell numbers after 2 weeks and a 90% FoxP3 purity with a stable Treg phenotype[48]. Clinical grade protocols have been developed and are utilized in human trials[184].

A second issue relates to the stability of adoptively transferred Treg populations. While the FoxP3$^+$ subset is relatively stable, Treg display a degree of phenotypic plasticity under the influence of inflammatory conditions[185]. Reprogramming of FoxP3$^+$ BDC2.5 T cells was observed in the NOD setting by Zhou *et al.*[186] in mice that permanently expressed a YFP reporter following FoxP3 expression. The frequency of 'exTreg' cells, that had once expressed FoxP3 but were not currently doing so, was small, however this population was slightly larger in the pancreas of diabetic mice. FoxP3 expression is sustained by methylation and is reliant on the presence of IL-2 signalling[187,188]. One study has suggested that IL-6 can re-methylate a suppressive CpG motif and abrogate FoxP3 expression[189]. It is therefore possible that under conditions of low IL-2 and increased pro-inflammatory cytokines, Treg can acquire plasticity through the loss of FoxP3 expression. Data from Battaglia group offers partial supports for the notion of Treg plasticity in T1D by demonstrating that cells isolated from human PanLNs include a population of epigenetically imprinted TSDR$^+$ "former" Treg that are FoxP3 negative[190]. FoxP3 is also known to be upregulated in activated T cells, and this could offer an alternative explanation for the induction of reporter expression.

A number of human trials investigating the safety and efficacy of Treg cell therapy have been initiated or completed (summarized in [191].) Safety results appear encouraging, as no adverse off-target suppression or increased risk of cancer has been reported in either T1D or GvHD trials[192-194]. Moreover, a large proportion of injected cells persisted for over 12 months *in vivo*[184]. In a study with 10 T1D children that received polyclonal expanded Treg within 2 months of diagnosis preliminary suggestions of efficacy were seen[195]. Four months following therapy, C peptide levels had declined in the control group but remained significantly higher in recipients of Treg cells[195]. Eight out of the 10 recipients of Treg cells were still in partial remission (informally referred to as the honeymoon phase) compared with only four out of the 10 patients in the comparison group. Follow up at the one year timepoint (with the recruitment of 2 additional patients to the Treg arm) showed that eight out of 12 recipients of Treg cells were still in partial remission with two children insulin-independent [196]. In contrast, only 2 out of 10 patients from the untreated control group remained in partial remission and this group

exhibited lower C-peptide levels and a higher insulin requirement compared to those treated with Treg cells. The purity of injected Treg cells was high in this trial, with FoxP3 >90% being one of the release criteria for the Treg preparation so it is possible that this, combined with the timing of intervention so close to diagnosis created favorable conditions for efficacy. However it should be noted that the length of the honeymoon phase is notoriously variable so caution must be used in interpreting the results of trials involving small numbers of participants. Moreover, it appears the rate of decline in C-peptide levels is not constant over the 2 years following T1D diagnosis, further complicating interpretation of such data[197]. Nevertheless, the fact that Treg therapy had a detectable impact on circulating Treg numbers in the short-term and did not raise safety issues is highly encouraging.

To improve the efficacy of Treg therapy in T1D, further benefit can potentially be gained from the use of antigen-specific Treg[180]. Several approaches exist. First, antigen-specific Treg could theoretically be expanded from polyclonal populations *in vitro* by stimulation with recombinant MHC class II presenting islet peptide mimotope as demonstrated in mice [198]. This approach however has the disadvantage of very low cell numbers. Alternatively, induced Treg can be generated by culturing bulk antigen-specific CD4 T cells in the presence of polarizing conditions (i.e. TGFβ and vitamin D)[199]. Here, however, lineage stability may become an issue, and this could potentially increase the pool of diabetogenic T cells thereby posing a safety concern. Another approach, is to redirect antigen specificity by introducing transgenic TCRs[200]. Their dual specificity may, however, represent a caveat, risking off-target action due to the endogenous TCR, suboptimal homing to target tissues or hampered activity due to insufficient CD3 availability[201]. To address these possible issues, chimeric antigen receptors (CAR) may be used instead, taking advantage of added activation or homing signals as well as suicide triggers as an invaluable safety mechanism should adverse reactions take place[202].

5. Concluding Remarks

Despite initial problems in defining the Treg phenotype, the field has progressed rapidly and is now at the point where clinical application has become a realistic possibility[203]. The capacity of Treg to prevent or even reverse autoimmune diabetes has been amply demonstrated in mouse models, highlighting the exciting potential that harnessing the power of these cells offers. The ability of Treg to suppress CD4 and CD8 T cell responses, as well as B cell responses[204-206] and even innate immunity[207] means that they are well placed to regulate the complex multi-cellular events that lead to T1D. There is evidence to support the view that Treg can act in both the pancreas and in its draining lymph nodes, suggesting the prospect of inhibiting both early initiating events and the ongoing immune-mediated damage. Whether the balance of suppressive mechanisms employed by Treg differs between these two sites remains open to speculation.

There are several areas worthy of consideration regarding the translation of Treg-directed therapies to the clinic. For example, it is important to consider factors that may counteract the activity of Treg. Using the DO11xripOVA model Clough *et al.*[208] showed that Tconv from diabetic mice were less sensitive to Treg suppression than Tconv from non-diabetic animals. This result is consistent with reports from the NOD model[209], where age-dependent development of Tconv resistance to Treg suppression was observed[178,210]. The Tconv resistance in the DO11xripOVA diabetes model was correlated with elevated levels of the cytokine IL-21 in the PanLN. In humans, IL-21 can also interfere with Treg supression[211], and there is some evidence that Tconv from the peripheral blood of T1D patients exhibit resistance to Treg supression[50,212]. Intriguingly, it recently transpired that Tfh cells, which produce IL-21 and can co-express IFN-γ and TNFα, are overrepresented in the effector T cell pool in DO11xripOVA mice and peripheral blood of T1D patients [32,213]. Thus it is possible that IL-21 or other mediators[214] could counteract the suppressive effects of Treg in T1D. Although there is evidence that suppression can be restored if Treg are present in high enough numbers[208], an additional strategy might to accompany Treg cell therapy with the simultaneous blockade of pathways shown to interfere with suppression.

Another consideration pertinent to immunotherapy aimed at expanding Treg *in vivo* is the subtle difference in CD25 expression between mouse and human systems. Although the broad principles are conserved between species (Treg being CD25hi and naive T cells expressing the lowest levels), the distinction between CD25 levels on Treg versus other T cell populations is far clearer in mice than in humans. A key factor here is likely to be the presence of a larger (CD25+) memory T cell population in humans, who have faced years of immunological challenge unlike mice that are typically maintained in a more restricted antigenic environment. One consequence of this is that IL-2 immunotherapy is more likely to act selectively on Treg in mouse models. Thus careful dose response analyses are required to establish the optimal delivery strategy for IL-2 immunotherapy in humans[158].

Finally, while we have focused on CD25+FoxP3+ Treg in this review, it is clear that additional regulatory populations also exist (reviewed in [215]). For example, IL-10 can potently regulate diabetes in BDC2.2/NOD mice, and its main cellular source does not appear to be CD4+CD25+ Treg[216]. There is evidence that the balance between production of IL-10 and pro-inflammatory cytokines is also relevant to T1D disease pathogenesis in humans[217,218]. Thus, strategies aimed at augmenting or inducing additional types of regulatory populations may also be worthwhile.

Overall, it is an exciting time for immunotherapy in the setting of Type 1 Diabetes and therapeutic manipulation of Treg populations is gathering momentum as a credible strategy. Increasing refinements are likely to result in the combination of this approach with additional interventions in order to maximize therapeutic benefit.

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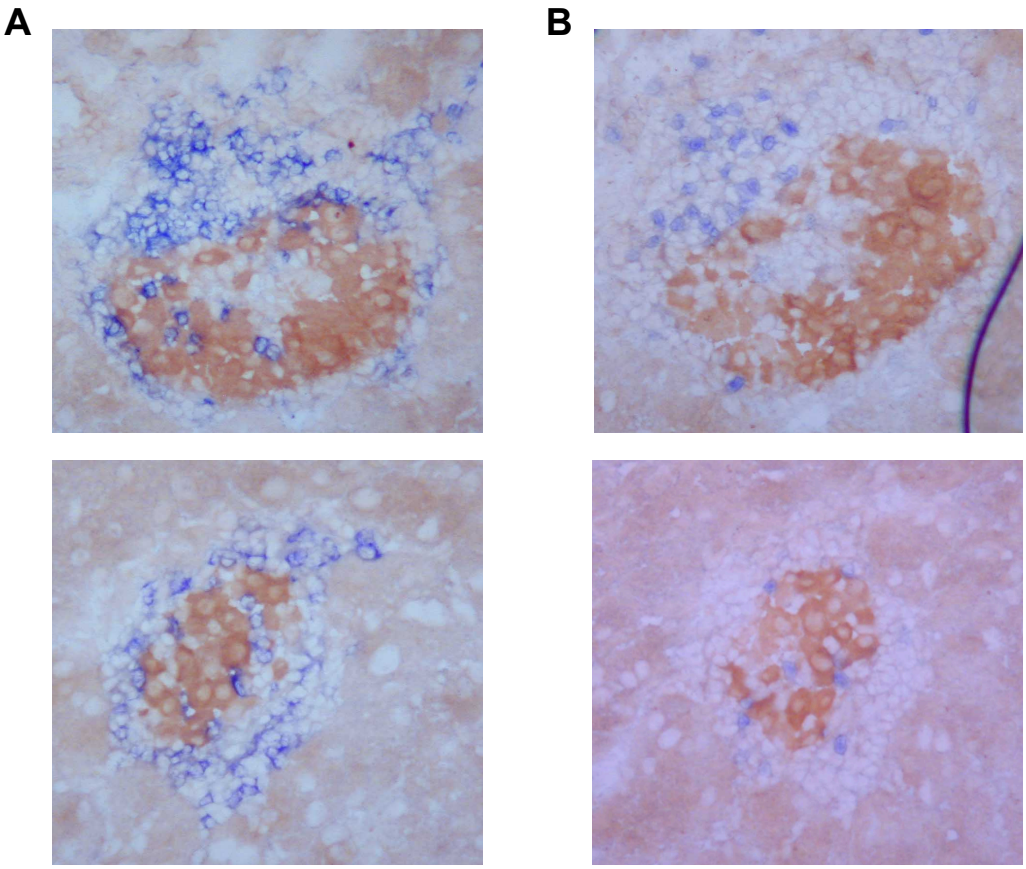

Figure 1. Treg are present in the pancreatic islet infiltrate of diabetic mice. Images show serial sections from frozen acetone-fixed pancreas of diabetic DO11 x rip-OVA mice. (A) CD4+ T cells (blue) infiltrating the islet of Langerhans marked by insulin producing (brown) beta-cells. (B) FoxP3+ Treg cells (blue) are present within the infiltrate. Insulin producing beta cells are stained in brown.

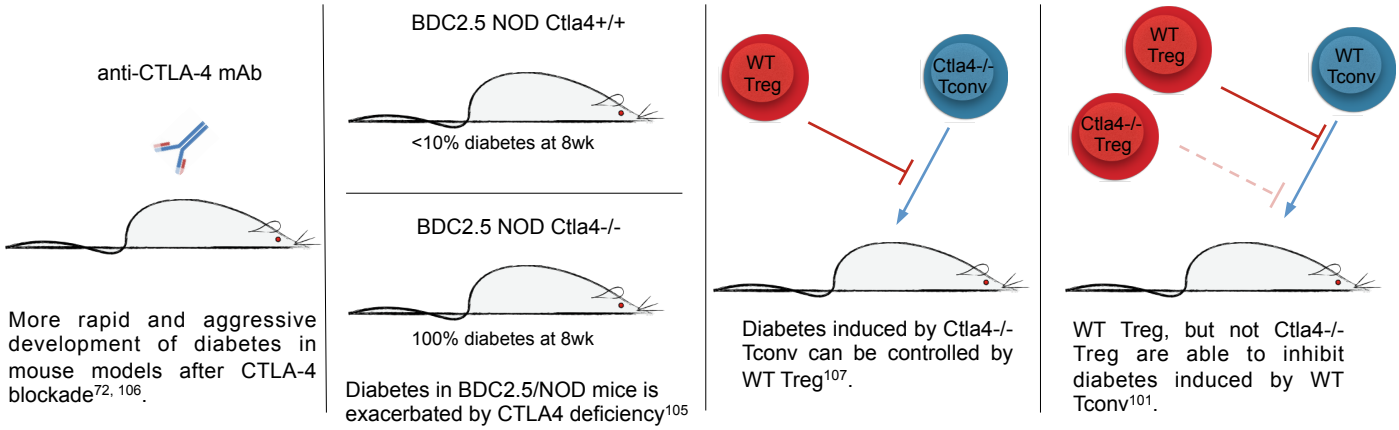

Figure 2. Summary of the evidence that supports a role for the CTLA-4 pathway in the regulation of autoimmune diabetes. This was demonstrated in a series of studies that utilized antibody blockade, genetic deficiency and adoptive transfer methods.

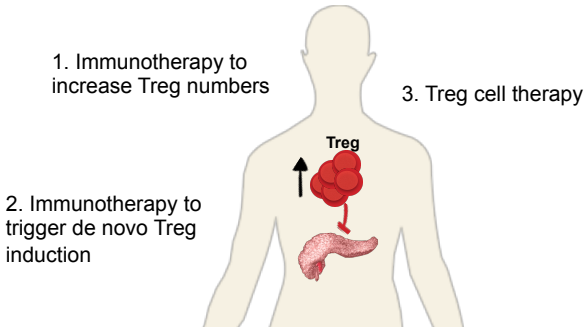

Figure 3. Therapeutic approaches centered around the enhancement of the Treg population for the treatment of type 1 diabetes, with the goal of inhibiting autoimmune destruction of the pancreas.