

# Control of type 1 diabetes by CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells: lessons from mouse models and implications for human disease

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## Summary

In recent years, there has been a revival of the concept of CD4<sup>+</sup> regulatory T (T<sub>reg</sub>) cells as being a central control point in various immune responses, including autoimmune responses and immunity to transplants, allergens, tumours and infectious microbes. The current literature suggests that T<sub>reg</sub> cells are diverse in their phenotype and mechanism(s) of action, and as such, may constitute a myriad of naturally occurring and induced T cell precursors with variable degrees of regulatory potential. In this review, we summarize research from various laboratories, including our own, showing that CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells are critical in the control of type 1 diabetes (T1D) in mouse models and humans. In this review, we also discuss cellular and molecular determinants that impact CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cell development and function and consequential resistance to organ-specific autoimmune disease. Recent advances in the use of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cellular therapy to promote immunological tolerance in the absence of long-term generalized immunosuppression are also presented. Copyright © 2009 John Wiley & Sons, Ltd.

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## Introduction on T1D

The immune system must simultaneously mount effective immunity against invading foreign pathogens while maintaining tolerance to self-antigens. A breach in the mechanisms promoting self-tolerance may result in autoimmune diseases like type 1 diabetes (T1D). T1D is a T cell-mediated disease characterized by the destruction of the endocrine insulin-producing beta ( $\beta$ ) cells of the pancreatic islets of Langerhan's, resulting in plasma glucose dysregulation, persistent hyperglycemia and long-term complications. T1D is a complex disease caused by multiple environmental and genetic risk factors [1]. Evidence for the influence of environmental factors include a global increase of 3% per year and correlative studies associate viral infections, early exposure to cow's milk or gluten to T1D susceptibility [2,3]. Strong clustering within families, such as a sibling recurrence rate of 6% and a monozygotic twin concordance rate of 30–50% suggest that important genetic determinants are involved in T1D susceptibility. Indeed, strong association between several genetic variants and T1D susceptibility has been well documented. The most important genetic association in T1D is found within HLA-DR class II genes [4]. Other established common variants include the *Insulin*, *CTLA-4*, *PTPN22* and *CD25* genes (reviewed in [5]).

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## The NOD mouse model

### Immunoregulatory problems underlie loss to $\beta$ cell tolerance

The NOD (non-obese diabetic) mouse model serves as a prototypic model of human autoimmunity as NOD mice develop spontaneous diabetes, which closely mirrors many features of T1D in humans, such as hyperglycemia and presence of autoantibodies directed against islet cells [6]. NOD mice are genetically prone to be globally immune dysregulated, and as such develop multi-organ autoimmunity including sialitis, thyroiditis, peripheral polyneuropathy and are susceptible to certain experimentally inducible autoimmune diseases such as EAE [7–9]. T1D susceptibility is inherited through multiple genes, with a strong predisposition from genes affecting immune responses to  $\beta$  islet cell antigens. Over 20 diabetogenic or insulin-dependent diabetes (*Idd*) loci have been identified by genomic mapping of congenic NOD strains, which harbour defined genetic intervals from diabetes-resistant mouse strains (C57/BL6), although no single gene is both necessary and sufficient. *Idd* congenic mice represent an important tool in the elucidation of the impact of certain genes on T1D progression, and are invaluable for the identification of checkpoints in the breakdown of self-tolerance in NOD mice. As in humans susceptible to T1D, the *Idd1* locus encodes the disease-prone MHC class II I-A<sup>g7</sup> molecule that remains the strongest genetic contributor to the diabetes susceptibility in NOD animals, as it may be responsible for poor thymic negative selection of self-reactive T cells [10]. Intriguingly, studies have demonstrated that genetic determinants beyond the immune system may account for susceptibility to autoimmune diseases, including increased insulin production, intrinsic resistance of  $\beta$ -islets to CD8<sup>+</sup> T cell-mediated destruction and altered sensory neuronal control over the response of  $\beta$ -islets to the inflammatory infiltrate during the insulinitic phase [11–14].

### Autoreactive T cell responses drive T1D in NOD mice

T1D is T cell-dependent as T cell-deficient NOD animals do not develop T1D. In addition, CD4<sup>+</sup> T cell depletion through monoclonal antibody treatment prevents diabetes onset [15]. The transfer of purified CD4<sup>+</sup> or CD8<sup>+</sup> T cells from diabetic mice into immunodeficient recipients induces T1D [16,17], although the onset and progression to T1D likely requires the combined contributions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells [18]. CD8<sup>+</sup> cytotoxic T cells (CTL) are thought to play a role in effector function and to act early by causing sufficient  $\beta$ -islet destruction to promote robust CD4<sup>+</sup> T cell responses, although some CD8<sup>+</sup> T cell clones were sufficient to destroy  $\beta$  cells without the help of CD4<sup>+</sup> T cells [19,20]. The autoantigens driving this autoimmunity are diverse and include insulin,

glutamate decarboxylase (GAD), insulinoma-associated protein 2 (IA-2) and Hsp60 (reviewed in [21]). Although diabetes cannot be transferred by autoantibodies from new onset diabetic donors, B cells are important in the development of autoreactivity, because B cell depletion by antibody treatment results in protection. Furthermore, a germline mutation that prevents the production of IgM, and consequentially the maturation of B cells, also affords protection, indicating an as-yet undetermined role for B cells in T1D progression [6]. Thus, although the contribution of B cells cannot be ignored, autoreactive T cell responses drive T1D pathogenesis in the NOD mouse.

### Immunoregulatory mechanisms restraining autoreactivity in NOD mice

#### Aberrant T cell regulation predisposes to T1D

NOD mice possess multiple, peripheral immune defects, which collectively contribute to spontaneous multi-organ autoimmunity. NOD mice show a mononuclear immune cell infiltrate in the pancreas at 3–4 weeks of age, often referred to as checkpoint 1. The predominant population consists of CD4<sup>+</sup> T cells, although CD8<sup>+</sup> T cells, B cells, dendritic cells (DC), macrophages and NK cells have been identified in the lesions. Overt diabetes, or checkpoint 2, does not occur until weeks/months later, demonstrating that immunoregulatory networks maintain the autoreactive T cells in check, suggesting that a delicate balance of pathogenic and protective immune responses controls T1D. Insulinitis coincides with reduced T cell proliferation and IL-2 production, which may affect CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T (T<sub>reg</sub>) cell functions. Furthermore, it was purported that pathogenic,  $\beta$ -cell-specific Th1 cell effector functions are favoured in disease pathogenesis, resulting in a skewed Th1 cytokine production, leading to T1D. Interestingly, IL-4<sup>-/-</sup> and IL-10<sup>-/-</sup> NOD mice did not demonstrate an accelerated diabetes phenotype, as onset and incidence resembled that of WT NOD counterparts suggesting that T1D is not caused by a lack of protective Th2 cells and consequent Th1 polarization in the NOD mouse model [22]. However, the cytokine production capability of Th2 cells, which infiltrate the pancreas of diabetic human patients, appears to be impaired as judged by the secretion of either IL-4 or IL-10, demonstrating that caution should be exercised when interpreting animal data for human therapeutic purposes. Defective macrophage and DC maturation and function [23], overexpression of NKG2D and its ligand RAE-1 on NK cells, resulting in compromised NK cell functions [24] and defective NK-T cell and T<sub>reg</sub> cells, whose augmented activity results in the prevention of T1D onset and recurrence [25], have all been suggested as contributors to disease progression in the NOD. Overall, NOD mice possess multiple, peripheral immune defects, which collectively contribute to spontaneous multi-organ autoimmunity.

## CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells: master-switch of peripheral tolerance

Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells have emerged as a dominant mechanism of peripheral self-tolerance. Naturally occurring T<sub>reg</sub> cells arise in the thymus during normal T cell selection through high-affinity recognition of self-antigens, as day 3 thymectomy ablates T<sub>reg</sub> cells and results in autoimmunity [26]. T<sub>reg</sub> cells then migrate to the periphery where they are poised for surveillance of self-antigens. Physical depletion or functional abrogation of CD4<sup>+</sup> T<sub>reg</sub> cells results in the induction of autoimmunity and an increased immunity to tumours, allografts and foreign pathogens, demonstrating that CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells represent a critical immunological switch in the control of immune responses to self and non-self antigens. T<sub>reg</sub> cells are characterized by the constitutive expression of the IL-2R $\alpha$  chain (CD25) and the Foxp3 transcription factor and constitute 1–10% of CD4<sup>+</sup> T cells in mouse and man [27]. Foxp3<sup>-/-</sup> or scurfy mice, which possess a mutation in the Foxp3 gene, succumb to lymphoproliferative disorder and multi-organ specific autoimmunity at an early age, a phenotype that can be reversed by the infusion of T<sub>reg</sub> cells [27–29]. In humans, immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) is a rare disorder characterized by multi-organ autoimmune disease due to a complete ablation of T<sub>reg</sub> cells, which results from mutations in the FOXP3 gene [30]. Murine T cells retrovirally transduced with Foxp3 acquire the T<sub>reg</sub> cell phenotype, as they are profoundly anergic and suppressive *in vitro* and *in vivo* and suppress IBD and T1D in transfer models, suggesting that Foxp3 initiates and supports a gene expression profile that regulates the development and maintenance of T<sub>reg</sub> cells [27,31–35]. Suppressor function is critically dependent upon Foxp3 protein, as T cells transcribing Foxp3, but lacking functional Foxp3 protein lose regulatory activity [36,37].

CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells constitute a polyclonal population with a diverse TCR repertoire skewed towards self-antigens enabling them to effectively patrol the lymphoid organs and subdue the activation of autoreactive T cells [38]. CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells suppress the *in vitro* and *in vivo* proliferative and cytokine responses of several immune cell subsets including T cells, DC, NK, NK-T, B cells, and macrophages. *In vitro*, T<sub>reg</sub> cells exert their suppressive activity in an Ag-non-specific and contact-dependent manner which results in the suppression of IL-2 production in the effector T (T<sub>eff</sub>) cells [39,40]. In some *in vivo* contexts, T<sub>reg</sub> cell functions are dependent on IL-10, as germline ablation or the conditional disruption of IL-10 in T<sub>reg</sub> cells results in spontaneous colitis [41,42]. TGF- $\beta$  has also been implicated in the mechanism of suppression of T<sub>reg</sub> cells in several models of disease [43]. Many other mechanisms have been proposed to operate, alone or in combination, for the control of immune responses and include a role for IL-35 [42], CTLA-4 [44], granzyme/perforin, modulation of intracellular cAMP in

responder cells, and cytokine competition by T<sub>reg</sub> cells, reviewed in [45]. Whether context-dependent factors dictate the use of specific mechanisms or whether different subsets suppress by specific mechanisms in specific sites is unknown [36,37].

## Induced and adaptive T<sub>reg</sub> cells

Recent evidence strongly demonstrates that under unique stimulatory conditions, namely in the presence of TGF- $\beta$ , conventional T cells can be induced in the periphery into functionally distinct adaptive T<sub>reg</sub> cells. Examples include IL-10-producing Tr1 and TGF- $\beta$ -producing Th3 cells, which may express Foxp3 [46]. It has been shown that TGF- $\beta$ -dependent adaptive T<sub>reg</sub> cells develop in the context of anti-CD3 immunotherapy in diabetic NOD mice, in an attempt to regulate T1D [47]. Naïve islet-specific CD4<sup>+</sup>Foxp3<sup>-</sup> T<sub>eff</sub> cells can also be induced into islet-protective CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells, when expanded by DC, in the presence of TGF- $\beta$  [48]. This suggests that promoting the development of induced T<sub>reg</sub> cells by immunotherapy may be feasible in controlling ongoing autoimmunity in human patients.

## Evidence for T<sub>reg</sub> cell function in the control of T1D in NOD

Many studies have implicated T<sub>reg</sub> cells in the control of diabetes onset and progression, and that reduced CD4<sup>+</sup> T<sub>reg</sub> cell frequencies or function in NOD mice, represent a primary predisposing factor to diabetes. Whether thymic development of T<sub>reg</sub> cells is normal in NOD mice has been a contentious issue. Recent studies show that thymic development of T<sub>reg</sub> cells seems to be normal, as thymectomy in NOD mice up to 3 weeks of age results in exacerbated T1D due to a marked reduction in T<sub>reg</sub> cells [49]. Surprisingly, the NOD background proved superior in generating T<sub>reg</sub> cells in the thymus relative to non-autoimmune prone strain C57/BL6, suggesting that central tolerance mechanisms are intact [50]. Furthermore, the frequency and function of single-positive CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells in the thymus of NOD was comparable to diabetes-resistant C57/BL6 mice [51].

Recent studies point to an essential role for CD4<sup>+</sup>Foxp3<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in the maintenance of self-tolerance in NOD mice. Depletion of CD25-expressing T cells or disruption of the B7/CD28 pathway results in a marked acceleration of T1D [52,53]. Recently, Chen *et al.* reported that Foxp3<sup>-/-</sup> NOD mice display an increased incidence and earlier onset of T1D compared to WT NOD mice, although it did not address whether the infusion of T<sub>reg</sub> cells, which rescues from T1D, compensates for the primary deficit in T<sub>reg</sub> cells believed to underlie T1D pathogenesis in these mice, or whether such injection was actually suppressing the global inflammation/pathology that likely arose as a secondary consequence of Foxp3 deficiency [54]. Interestingly, BDC2.5 CD4<sup>+</sup> TCR transgenic

NOD mice, whose T cells express a TCR specific for an unknown pancreatic islet antigen, are characterized by a low incidence of diabetes (10–20% by 20 weeks of age), while recombination-activating gene (RAG)<sup>-/-</sup> BDC2.5 animals that are completely devoid of T<sub>reg</sub> cells, exhibit fulminant diabetes and no lag time between insulinitis and overt diabetes [55]. Infusion of CD4<sup>+</sup> splenic T cells to T<sub>reg</sub> cell-deficient BDC2.5 protects against T1D by suppressing inflammation in the islets [55]. Collectively, this data supports the notion that T<sub>reg</sub> cells exert suppression on diabetes progression in NOD mice.

Several lines of evidence show that T<sub>reg</sub> cells are functionally operative in prediabetic NOD mice. Many studies have reported an expansion of CD25-expressing CD4<sup>+</sup> T cells with apparent regulatory activity in inflamed tissues of adult, prediabetic, and insulinitic NOD mice. It is unclear from these studies whether these CD4<sup>+</sup>CD25<sup>+</sup> T cells are induced T<sub>reg</sub> cells from CD4<sup>+</sup>Foxp3<sup>-</sup> progenitors during pancreatic inflammation, or whether they emerge from the thymus-derived Foxp3<sup>+</sup> T<sub>reg</sub> cell pool. Diabetes transfer is inhibited by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells, with the highest regulatory activity attributed to the CD62L<sup>hi</sup> fraction [56]. Transfer of such cells after long-term *in vitro* expansion has been shown to be protective, although it is unknown whether *in vitro* conditioning of T<sub>reg</sub> cells may have adversely affected their physiological role. NOD mice do eventually develop diabetes, despite the presence of T<sub>reg</sub> cells, suggesting a T<sub>reg</sub> cell-dependent breakdown in self-tolerance.

### Functional waning of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells predisposes to T1D

Although NOD mice harbour T<sub>reg</sub> cells capable of preventing disease progression early in life, pathogenic T<sub>eff</sub> cells proceed to immune-mediated destruction of the  $\beta$ -islet cells. A central unanswered question is whether the onset of diabetes is due to a decline in T<sub>reg</sub> cell functions or from the overriding of such regulation by the uncontrolled activation and expansion of diabetogenic T cells. Intriguingly, several reports noted an increase in T<sub>reg</sub> cells or Foxp3 mRNA expression at the time of disease onset, coinciding with a proliferative burst of T<sub>eff</sub> cells, suggesting that inflammation drives T<sub>reg</sub> cell functions [57,58]. Controversial data exists as to whether a progressive resistance of autoreactive T cells to T<sub>reg</sub> cell-mediated suppression is due to defective T<sub>reg</sub> cell functions or to an age-related decline in T<sub>reg</sub> cell frequency [59,60]. Although one study has suggested a quantitative defect in the NOD contributes to diabetes progression [61], many reports hint to a progressive waning in T<sub>reg</sub> cell functions as a potential mechanism to elucidate diabetes pathogenesis, despite a stable cellular frequency of T<sub>reg</sub> cells [51,58]. Initially, it was found that T<sub>reg</sub> cells isolated from older diabetic NOD females are less suppressive relative to T<sub>reg</sub> cells isolated from younger NOD animals [58,62]. Upon further investigation, criss-cross transfer studies demonstrated a resistance of diabetogenic T cells

to T<sub>reg</sub> cell suppression [58]. In contrast, we demonstrate that T<sub>reg</sub> cells from older or overtly diabetic NOD animals were incapable of preventing diabetes transfer, although the activation status and frequency of T<sub>eff</sub> cells remained unaltered. The possibility that they may be overwhelmed by the T<sub>eff</sub> cell response or by the accumulation of T<sub>reg</sub> cell-resistant autoreactive T cells over time cannot be excluded [63]. Taken together, these data suggest that T<sub>reg</sub> cells of NOD mice have a generalized fitness defect in inflammatory sites, which in turn predisposes to disease onset [64].

Conflicting data in human T1D patients demonstrate decrease T<sub>reg</sub> cell frequency [65], unaltered T<sub>reg</sub> cell frequency with marked decrease in suppressive activity *in vitro* [66] and lastly no differences at all compared to healthy controls [67]. These differences can be explained by several reasons including the inexactitude of the method of T<sub>reg</sub> cell isolation and purification, and the lack of functional tests on islet-specific T<sub>reg</sub> cells in blood. Furthermore, studies in murine models indicate that T<sub>reg</sub> cells exert their function within the target organ undergoing autoimmune attack rather than solely in LN draining sites [68]. Thus, subtle functional differences in the T<sub>reg</sub> cell pool within sites of inflammation may not be adequately reflected in the peripheral blood compartment.

### Mechanism(s) of T<sub>reg</sub> cell function in T1D

T<sub>reg</sub> cells can control or limit the potency of activation of the T<sub>eff</sub> cell pool at various stages such as differentiation and/or proliferation during priming in draining LN, inhibition of IL-2 production, trafficking to the pancreas, or suppression of effector functions *in situ* [63,69]. The presence of T<sub>reg</sub> cells did not block the entry of autoreactive T cells into the LN, but did block their migration to the T-B cell boundary within the LN, suggesting that T<sub>reg</sub> cells impinge the proper migration of autoreactive T cells [70]. Adoptive transfer systems have demonstrated an inverse correlation between the proliferation of T<sub>eff</sub> cells and the amount of T<sub>reg</sub> cells present in draining pancreatic sites [71,72]. In CD28<sup>-/-</sup> or B7<sup>-/-</sup> recipients devoid of T<sub>reg</sub> cells, robust proliferation of antigen-specific BDC2.5 T cells was observed, although this effect has not been observed in the polyclonal repertoire of the NOD and in other models [69]. Reconstitution of T<sub>reg</sub> cells significantly blocked proliferation. Under the conditions where priming of T<sub>eff</sub> cells remained unaffected, the differentiation of Th17 and Th1 cells was markedly hindered by T<sub>reg</sub> cells [51]. Furthermore, the infiltration of T<sub>eff</sub> cells in the target organ was markedly enhanced in the absence of T<sub>reg</sub> cells. CD4<sup>+</sup>CD62L<sup>-</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were much less potent in preventing T1D than the CD62L<sup>hi</sup> fraction, demonstrating that T<sub>reg</sub> cells must home to the draining pancreatic sites in order to exert suppression and maintain tolerance. These data suggest that T<sub>reg</sub> cells may maintain self-tolerance by preventing antigen-specific homing to pancreas and/or

decrease the cycling and differentiation of autoreactive  $T_{eff}$  cells (Figure 1) [68].

## Peripheral homeostasis of $T_{reg}$ cells in NOD mice

### T cell-extrinsic variables impacting $T_{reg}$ cell function in T1D

In the NOD, myeloid DC engulf self-antigen particulates due to normal  $\beta$  cell turnover during islet remodeling, migrate to the draining pancreatic LN and subsequently activate autoreactive T cells [73]. Hence, DC play an active role in the lymphocyte recruitment in the pancreatic islets, as temporary depletion of DC in prediabetic mice was accompanied by the disappearance of lymphocytes in the endocrine tissue of the pancreas, resulting in diabetes protection [74]. A significant body of evidence shows that phenotypic and functional abnormalities of DC underlie the risk of T1D in humans and in the NOD mouse. Recently diagnosed or established T1D human patients have decreased DC numbers in blood relative to healthy subjects. Although T cell numbers remained unaltered in these patients, their activation status may be affected [75]. While DC isolated from NOD mice exhibit a less mature phenotype than control non-autoimmune-prone strains, the correlation of this immature DC phenotype with autoimmunity seems paradoxical considering the central role of DC in steady-state tolerance [76,77]. Interestingly,  $T_{reg}$  cells have a greater requirement for costimulation relative to  $T_{eff}$  cells, as demonstrated by CD28 and B7 knockout studies, which resulted in a marked decrease of  $T_{reg}$  cells [53]. NOD DC, exhibiting lower levels of costimulatory molecules, may not provide the signals for the proper maintenance of the regulatory T cell subset. Thus, alterations in DC maturation or function may indirectly impact  $T_{reg}$  cell homeostasis such that the balance is tipped towards autoimmunity. For example, DC in NOD.B6 *Idd3* diabetes-protected animals exhibit a different maturational status relative to wild-type NOD, and preferentially promote  $T_{reg}$  cell functions resulting in markedly reduced incidence and onset (unpublished observations). Interestingly, a growing body of evidence demonstrates that manipulating DC function may result in diabetes protection, either by skewing the destructive Th1 response to a protective Th2 response or by promoting  $T_{reg}$  cell functions (see Table 1).

Whether DC are targets of  $T_{reg}$  cell-mediated immunosuppression or mere activators of  $T_{reg}$  cell functions remains unknown. The relative contribution of cognate interactions between  $T_{reg}$  cell/DC to the suppressive activity of  $T_{reg}$  cells *in vitro* and *in vivo* remains a highly controversial subject. Our previous work has shown that APC function is not necessary for the effector phase of  $T_{reg}$  suppression [78], although APC-derived signals are clearly essential for their initial activation. In stark contrast, other studies indicate that co-culture of immature

DC with  $T_{reg}$  cells resulted in low levels of costimulation and decreased capacity for stimulation of naïve T cells [79]. The complete ablation of  $T_{reg}$  cells *in vivo* results in the unbridled expansion of  $T_{eff}$  cells, which lead to the excessive activation and maturation of DC. These inflammatory DC thus contribute to fatal autoimmune disorders, suggesting that  $T_{reg}$  cells may exert their suppressive activity *in vivo* through the down-modulation of DC [80]. This suggests that  $T_{reg}$  cells may be modulating DC function by blocking their maturation, preventing the activation of autoreactive  $T_{eff}$  cells and thus promoting self-tolerance.

$T_{reg}$  cells seemingly preferentially interact with DC presenting  $\beta$ -islet antigens, suggesting that these interactions can induce tolerance by enhancing antigen-specific  $T_{reg}$  cell functions. The possibility that  $T_{reg}$  cells alter DC interactions with  $T_{eff}$  cells *in vivo* has been considered in several studies by means of immunofluorescence approaches, although no causal relationship could be made between the frequency of  $T_{reg}$  cell/DC interactions and the loss of  $T_{eff}$  cell interactions with DC [81]. Moreover, this question has been directly addressed in NOD mice by means of 2-photon laser-scanning microscopy of pancreatic LN [69]. In this study, Tang *et al.* elegantly showed that *in vitro* expanded BDC2.5  $T_{reg}$  cells and DC form stable associations, which seemingly correlates with disruption of BDC2.5  $T_{eff}$  cell/DC cellular interactions in panLN, suggesting that  $T_{reg}$  cell/APC interactions may be in part responsible for  $T_{reg}$  cell-mediated protection [69]. However, a direct causal relationship between  $T_{reg}$  cell/DC interactions and suppression of  $T_{eff}$  cells has never been formally made. Lastly, DC are also believed to play a role in  $T_{reg}$  cell thymic development as shown by a recent study demonstrating that TSLP-conditioned DC induce the proliferation and differentiation of  $CD4^+CD8^-CD25^-$  human thymocytes or naïve  $T_{eff}$  cells in NOD into  $CD4^+CD25^+Foxp3^+$   $T_{reg}$  cells [82,83]. Overall, these findings highlight the notion that DC are key players in the development, function and homeostasis of  $T_{reg}$  cells *in vivo*, and strongly suggest that their activation state is a critical determinant of tolerance or autoimmunity.

### Homeostatic functions of DC-derived costimulation

$T_{reg}$  cells can actively expand and mediate antigen-specific suppression after antigen presentation by DC both *in vitro* and *in vivo*, suggesting DC provide requisite signals for the potentiation of  $T_{reg}$  cell functions. In fact, DC have been shown to be the exclusive APC subset capable of potentiating  $T_{reg}$  cell proliferative and suppressive functions both *in vitro* and *in vivo*, which underscores the importance of DC in self-tolerance [84]. Thus, DC are key players in the orchestration of immune responses by playing a dual role in both immunity and self-tolerance [85].

Table 1. Various therapeutic strategies including DC cell-based approaches have been used in the prevention or cure of diabetes in NOD mice.

| State of DC                                                                                                          | Age of NOD mice                                                       | Mechanism                                                                                                      | Diabetes Protection | Reference                                                            |
|----------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|---------------------|----------------------------------------------------------------------|
| Injection of DC transduced to express IL-4                                                                           | At time of insulinitis (4wks) or Invasive insulinitis (beyond 10 wks) | Polarization from pathogenic Th1 to protective TH2 cells                                                       | Yes                 | Feili-Hariri <i>et al.</i> , 2003                                    |
| Injection of DC whose of costimulatory molecules (CD80/CD86) are down-modulated by antisense oligonucleotides        | 5-8wks                                                                | Increase in CD25 <sup>+</sup> CD62L <sup>+</sup> T <sub>reg</sub> cells<br>Th1 cytokine production decreased   | Yes                 | Machen <i>et al.</i> , 2004                                          |
| Injection of $\beta$ -islet (insulin beta9-23 chain, proinsulin C19-A3) pulsed DC                                    | 9wks                                                                  | n.d.                                                                                                           | Yes                 | Lo <i>et al.</i> , 2006                                              |
| Injection of DC derived from pancreatic sites                                                                        | 4wks                                                                  | Suggested enhanced function of regulatory immune cells (although not determined)                               | Yes                 | Clare-Salzler <i>et al.</i> , 1992                                   |
| Injection of BMDC                                                                                                    | At time of insulinitis (4wks)                                         | Polarization from pathogenic Th1 to protective TH2 cells                                                       | Yes                 | Feili-Hariri <i>et al.</i> , 1999, Feili-Hariri <i>et al.</i> , 2002 |
| Myeloid progenitors expanded in the presence of TGF- $\beta$ and prevented to undergo terminal differentiation steps | At time of insulinitis (4wks)                                         | Mechanism unclear (not due to T <sub>reg</sub> cells)                                                          | Yes                 | Steptoe <i>et al.</i> , 2005                                         |
| GM-CSF-induced tolerogenic DC injected into NOD                                                                      | 3wks                                                                  | DC decrease costimulatory molecules<br>DC less apt to activate diabetogenic T cells                            | Yes                 | Gaudreau <i>et al.</i> , 2007                                        |
| GM-CSF overexpression under the control of the rat insulin promoter                                                  | n.a.                                                                  | Enhanced recruitment and antigen presentation of APC to pancreas leading to activation of diabetogenic T cells | No                  | Judkowski <i>et al.</i> , 2004                                       |
| Injection of TSLP-treated DC                                                                                         | 3wks                                                                  | Polarization to protective Th2 response<br>Induced Foxp3 <sup>+</sup> T <sub>reg</sub> cells                   | Yes                 | Besin <i>et al.</i> , 2008                                           |

The importance of costimulation for T<sub>reg</sub> cell function is apparent from the phenotype of mice deficient in B7 and TNF family members. T1D is exacerbated in B7-deficient or CD28-deficient NOD mice due to a profound decrease in T<sub>reg</sub> cells, demonstrating that the CD28/B7 pathway is essential for T<sub>reg</sub> cell homeostasis [53]. B7.1-deficient animals exhibit exacerbated disease due to a concomitant decrease in T<sub>reg</sub> cells and increase in T<sub>eff</sub> cell expansion and survival [86], whereas B7.2-deficient animals are protected due to the hyporesponsiveness of the T<sub>eff</sub> cell pool [87]. Blocking anti-B7.2 mAbs confirm this finding as a reduction in the incidence of disease was noted [88]. However, CD28 is seemingly not required for their effector function, as CD28<sup>-/-</sup> deficient T<sub>reg</sub> cells retain their function *in vitro* and *in vivo* [53]. Furthermore, it has been shown that ICOS, a B7 family member, impacts T<sub>reg</sub> cell functions, as ICOS blockade induces diabetes in the BDC2.5 TCR transgenic animals and promotes changes

in T<sub>reg</sub> cell gene expression profile [89]. Interestingly, germline ablation of CD40, a TNF family member, in NOD animals results in reduced numbers of T<sub>reg</sub> cells, which are not functional when tested *in vitro* [90]. Moreover, CD40L deficiency in NOD mice was shown to hinder the priming of anti-islet CD8<sup>+</sup> T cells and this defect was associated with the inhibition of DC maturation, resulting in T1D [91]. CD40L<sup>+</sup> T cells, activated DC or  $\alpha$ -CD40 monoclonal antibody abrogated this protection suggesting that CD40 signaling releases immature DC from suppression by T<sub>reg</sub> cells, leading to disease development. CD40 is not directly involved in T<sub>reg</sub>-mediated suppression but rather conditions target T cells to receive suppression. These observations suggest a requirement for CD40-CD40L interactions in the suppression of the effector phase of the immune response [92-94]. Overall, costimulation plays a crucial role in self-tolerance, in part, by influencing the development, function or homeostasis of Foxp3<sup>+</sup> T<sub>reg</sub> cells.

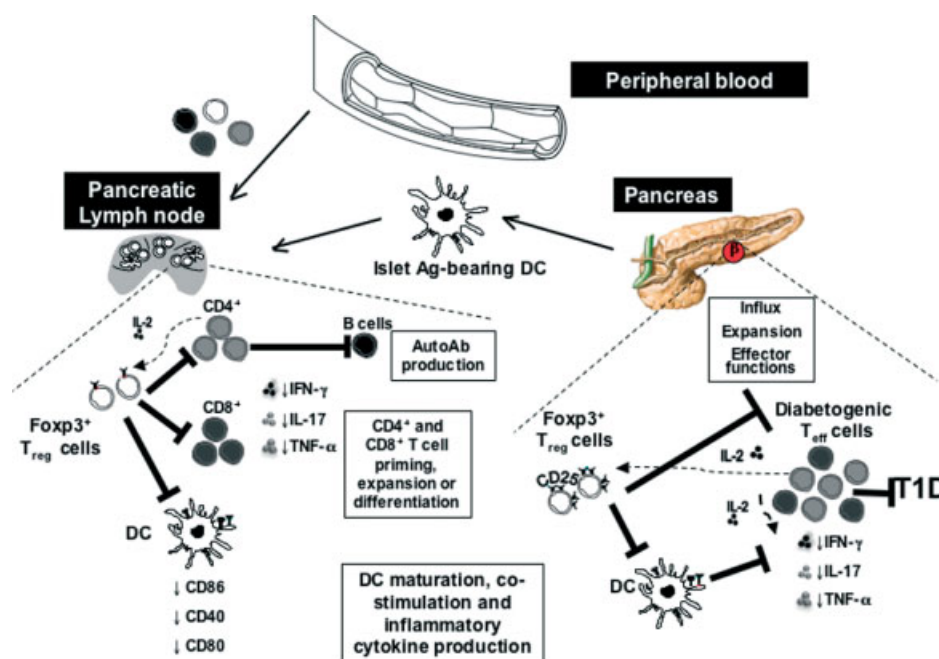


Figure 1. Tissue-derived DC uptake  $\beta$ -islet antigens, traffic to the draining pancLN where they activate islet-specific  $T_{eff}$  and  $T_{reg}$  cells and suppress various aspects of the diabetogenic response including the maturation of DC, the activation, expansion, differentiation and effector functions of  $T_{eff}$  cells, and the activation of autoreactive B cells.  $T_{eff}$  cell-derived IL-2 potentiates the inhibitory functions of  $CD4^{+}Foxp3^{+}$   $T_{reg}$  cells. Quantitative or temporal differences in IL-2 production may precipitate diabetes by negatively affecting  $T_{reg}$  cell functions, as IL-2 is essential for the development, maintenance and metabolic fitness of  $T_{reg}$  cells. Quantitative and qualitative differences in  $T_{reg}$  cells of NOD animals have been purported for the breakdown in self-tolerance mechanisms, which may be attributed to a decline in IL-2 at the time of insulinitis.

### T1D-protective and pro-fitness role of IL-2 in $T_{reg}$ cell development and function

The loss of  $T_{reg}$  functions and accelerated onset in T1D in NOD mice deficient in B7/CD28 and/or CD40/CD40L pathways suggests that pathways may be promoting their survival via induction of anti-apoptotic molecules, cytokines that function as growth, survival or suppressor activity maintenance factors, or induction/maintenance of Foxp3. A significant body of evidence suggests that the reduction of  $T_{reg}$  cell numbers in the above-described costimulation-deficient mice may be secondary to the loss in IL-2 production from effector cells. IL-2 fulfills paradoxical functions as both an initiator and terminator of immune responses by promoting the expansion of naïve T cells, and maintaining the  $T_{reg}$  cell pool, respectively. Evidence suggests that IL-2 is an important signal for the development, peripheral survival and suppressive function of  $T_{reg}$  cells. Indeed, mice deficient in IL-2, IL-2R $\alpha/\beta$  and STAT5A/B succumb to lymphoproliferative disorder and various autoimmune diseases, as they exhibit a marked decrease in  $T_{reg}$  cells, demonstrating that IL-2 signaling is required for  $T_{reg}$  cell development and function [95–99]. These findings begged the further dissection of the importance of IL-2 in  $T_{reg}$  cell functions. From the analysis of GFP-Foxp3 knock-in mice genetically deficient in IL-2 or CD25, IL-2 was shown to not be required for the induction of Foxp3 expression in developing thymocytes, but was nonetheless necessary

for maintaining the expression of genes involved in cell growth and metabolism. These findings indicate that IL-2 may be required for sustaining the homeostasis and competitive fitness of  $T_{reg}$  cells in vivo [100–103]. Interestingly, although IL-2 signaling was not essential to  $T_{reg}$  cell development, signaling through the common  $\gamma$  chain ( $\gamma_c$ ) was shown to be crucial to their development, suggesting that other  $\gamma_c$  cytokines, such as IL-7 and IL-15, can substitute for IL-2 [100].

Paradoxically,  $T_{reg}$  cells are profoundly anergic unless exogenous IL-2 is provided and contributes to the anergic state of  $T_{reg}$  cells [100]. This anergic state can be explained by the action of Foxp3, a transcriptional repressor of IL-2, in  $T_{reg}$  cells, and their failure of chromatin remodeling at the promoter region of the *IL2* gene [104]. As a result,  $T_{reg}$  cells must derive IL-2 from  $T_{eff}$  cells in a paracrine manner in order to sustain their functions. A tightly regulated feedback loop seems to be in place whereby  $T_{reg}$  cells, requiring IL-2 produced by activated  $T_{eff}$  cells for their maintenance and activation, limit the expansion of  $T_{eff}$  cells by shutting down their production of IL-2. When this negative loop is impaired, it may predispose to autoimmunity. Thus, the effect of  $T_{reg}$  cells on  $T_{eff}$  cells is two-fold: the prevention of IL-2 production, and failure to proliferate in response to IL-2 [105].

Strong evidence suggests that inappropriate immunoregulation underlies the pathogenesis of T1D. Interestingly, NOD T cells respond normally to TCR activation until the onset of insulinitis, at which point they become

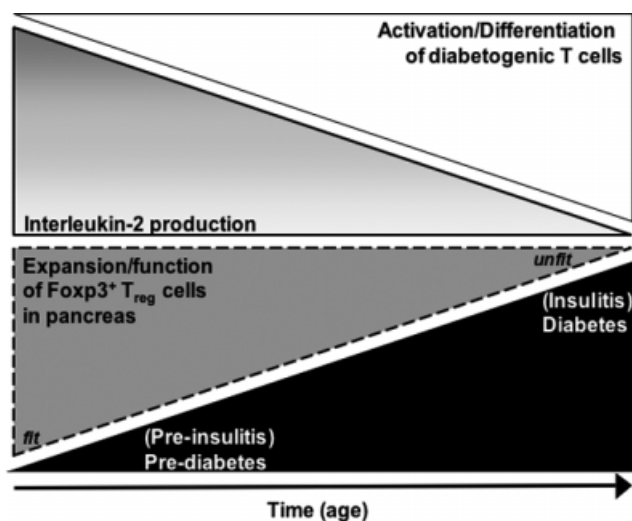


Figure 2. The onset of diabetes coincides with a progression from peri-insulinitis to destructive insulinitis. At the onset of insulinitis, the production of IL-2 declines, resulting in a waning of T<sub>reg</sub> cell functions. Consequently, diabetogenic T cells expand in the pancLN and traffic to the pancreas where they proceed to destroy the insulin-producing  $\beta$ -islets. These autoreactive T cells may also be refractory to T<sub>reg</sub> cell-mediated suppression and override their inhibition. These compounding factors may cooperate to precipitate the onset and progression of autoimmune diabetes.

anergic, and sustain a drastic reduction in IL-2 production. This reduced IL-2 expression and activity in NOD mice may lead to an imbalance in the T<sub>eff</sub> and T<sub>reg</sub> cell populations, abrogating T<sub>reg</sub> cell function and subsequently enabling diabetogenic T cells to make the transition from insulinitis to overt diabetes. Interestingly, this decline in T cell activation and concomitant IL-2 production coincides with a time-dependent decrease in the cycling of T<sub>reg</sub> cells, suggesting that T<sub>reg</sub> cells are sensitive to IL-2 availability in prediabetic NOD mice (Figure 2) [68]. Consistently, we have recently shown that diabetes resistance in NOD.B6 *Idd3* congenic mice correlates with a differential expression of IL-2 relative to wild-type NOD mice, and is associated with a greater proportion of cycling T<sub>reg</sub> cells and enhanced functions in inflamed islets [106]. Furthermore, neutralization studies have shown that IL-2 deficiency precipitates the breakdown of tolerance and the progression of T1D by selectively depleting the T<sub>reg</sub> cell subset [107], and suggests that IL-2 may be essential for controlling diabetogenic T cells by promoting T<sub>reg</sub> cell functions. Consistently, low-dose administration of IL-2 in prediabetic female mice resulted in enhanced frequency of Foxp3<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells, and this regimen resulted in T1D protection [108]. Taken together, variations in IL-2 may affect the balance between islet-specific autoreactive T cells and Foxp3<sup>+</sup> T<sub>reg</sub> cells, and consequently precipitate T1D [106]. While IL-2 is not a susceptibility locus in human T1D, susceptibility to T1D in humans is associated with polymorphisms contained within the *IL-2RA* gene [109], suggesting an important role for IL-2 signaling in human T1D pathogenesis.

## Therapeutic potential of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells

The autoimmune destruction of the pancreas is a dynamic process, which involves multiple players such as diabetogenic T<sub>eff</sub> cells, DC and CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells. Waning of T<sub>reg</sub> cell metabolic fitness and functions may be due to insufficient costimulation and/or supply of IL-2 [52,53,100]. The loss of T<sub>reg</sub> functions in turn may generate a greater proportion of T<sub>eff</sub> cells refractory to suppression and/or with a lower requirement of costimulation for their proper activation and execution of effector functions [51]. Therefore reconstitution of immunological tolerance to pancreatic autoantigens appears to be a reasonable therapeutic avenue for the future.

Although several immune pathways regulate autoimmune responses in NOD mice, T<sub>reg</sub> cells have a central function in the prevention of the induction and pathological consequences of diabetogenic T cell responses towards  $\beta$ -islet cells [63]. Environmental or genetic insults may negatively impact their development or function, and in turn may represent a major factor underlying susceptibility to T1D [1,110]. The ultimate goal in the treatment of T1D is to re-establish tolerance to  $\beta$ -islet specific antigens by potentiating T<sub>reg</sub> cell numbers and/or function *in vivo* or by *in vitro* conditioning prior to infusion into affected individuals at risk or with overt disease [111]. The capacity of DC to shape T cell responses makes them of great clinical interest, as immature DC expressing low levels of costimulation induce anergy or tolerance [112]. In the NOD, CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> T<sub>reg</sub> cells expanded by antigen-loaded DC were able to protect and even restore normoglycemia in overtly diabetic animals [113].  $\beta$ -islet antigen pulsed DC, in the presence of TGF- $\beta$ , can induce islet-protective CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells from naïve islet-specific CD4<sup>+</sup>Foxp3<sup>-</sup> T<sub>eff</sub> cells, suggesting that DC have an impact on the *de novo* generation of T<sub>reg</sub> cells in the periphery [48]. The induced T<sub>reg</sub> cells potentially protected syngeneic islet grafts in overtly diabetic NOD mice, providing a potential therapeutic strategy for the remission of destructive autoimmunity [113,114]. It stands to reason that peptide-pulsed DC could be an antigen-specific therapeutic approach to prevent or reverse autoimmunity by potentiating T<sub>reg</sub> cell functions and inducing tolerance [111]. Enhancing T<sub>reg</sub> cell responses has become an area of intense focus as a means of treating autoimmune diseases like T1D. Reestablishing sufficient amount of T<sub>reg</sub> cells mediated by tolerogenic DC is a feasible therapeutic treatment of autoimmune diseases. The T<sub>reg</sub> cell pool is a rare population that would need to be expanded while maintaining regulatory functions in order to be used in a clinical setting.

However, there are many unresolved issues limiting widespread clinical application of T<sub>reg</sub> cell therapy for human T1D. These include the ill-defined mechanism(s) underlying T<sub>reg</sub> cell-mediated suppression, the divergent observations between *in vitro* versus *in vivo* findings

performed in mice and humans, and the risk of unwarranted suppression towards infectious pathogens and tumours. Furthermore, the unreliable nature of current markers of T<sub>reg</sub> cells in inflammatory contexts often confounds the analysis of many studies. The discovery and validation of more specific biomarkers for T<sub>reg</sub> cells, other than CD25, is imperative, as this will undeniably facilitate our ability to monitor T<sub>reg</sub> cellular frequency and function in the context of T1D, and may enable us to determine the clinical effectiveness of novel therapeutic strategies destined to modulate T<sub>reg</sub> function *in vivo*.

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## Conflict of interest

None declared.

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