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IL-2 as a therapeutic target for the restoration of Foxp3+ regulatory T cell function in organ-specific autoimmunity: implications in pathophysiology and translation to human disease

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Abstract

Peripheral immune tolerance requires a finely controlled balance between tolerance to self-antigens and protective immunity against enteric and invading pathogens. Self-reactive T cells sometimes escape thymic clonal deletion, and can subsequently provoke autoimmune diseases such as type 1 diabetes (T1D) unless they are controlled by a network of tolerance mechanisms in the periphery, including CD4+ regulatory T cells (T_{reg}) cells. CD4+ Treg cells are characterized by the constitutive expression of the IL-2Rα chain (CD25) and preferentially express the forkhead winged helix transcriptional regulator Foxp3. These cells have been shown to possess immunosuppressive properties towards various immune cell subsets and their defects are thought to contribute to many autoimmune disorders. Strong evidence shows that IL-2 is one of the important stimulatory signals for the development, function and fitness of Treg cells. The non-obese diabetic (NOD) mouse model, a prototypic model of spontaneous autoimmunity, mimics many features of human T1 D. Using this model, the contribution of the IL-2-IL-2R pathway to the development of T1 D and other autoimmune disorders has been extensively studied. In the past years, strong genetic and molecular evidence has indicated an essential role for the IL-2/IL-2R pathway in autoimmune disorders. Thus, the major role of IL-2 is to maintain immune tolerance by promoting Treg cell development, functional fitness and stability. Here we first summarize the genetic and experimental evidence demonstrating a role for IL-2 in autoimmunity, mainly through the study of the NOD mouse model, and analyze the cellular and molecular mechanisms of its action on Treg cells. We then move on to describe how this data can be translated to applications for human autoimmune diseases by using IL-2 as a therapeutic agent to restore Treg cell fitness, numbers and functions.

Introduction

Peripheral immune tolerance requires a finely controlled balance between maintaining tolerance to self-antigens and mounting protective immunity against enteric and invading pathogens [1]. Self-reactive T cells sometimes escape thymic clonal deletion, and can subsequently provoke autoimmune diseases such as type 1 diabetes (T1D) unless they are controlled by a network of tolerance mechanisms in the periphery, including CD4+ regulatory T cells (T_{reg}) cells [2]. These cells constitute 1-10% of thymic and peripheral CD4+ T cells in humans and mice, and arise during normal thymic lymphocyte development. T_{reg} cells are characterized by the constitutive expression of the IL-2Rα chain (CD25) and preferentially express Foxp3, a forkhead winged helix transcriptional regulator, which is critical for their development and function [3]. CD4+ T_{reg} cells have been shown to possess immunosuppressive properties towards various immune cell subsets, although the mechanism varies according to the genetic background of the host, microflora and target tissue. As such, T_{reg} depletion, or alterations of the *foxp3* gene, as seen in *Scurfy* mice or IPEX patients, results in a loss of T_{reg} cells, and catastrophic multi-organ autoimmunity [4,5]. Hence Treg cell homeostasis and function

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is necessary to the maintenance of peripheral tolerance, and their defect leads to organ-specific autoimmune disorders such as T1 D.

The non-obese diabetic (NOD) mice are a prototypic model of human autoimmunity as they spontaneously develop multi-organ autoimmune diseases including T1D [6]. T1 D is a chronic autoimmune disease that results in the destruction of the insulin-producing beta (β) cells of pancreatic islets of Langerhans, resulting in insulin deficiency and persistent hyperglycemia. Development of diabetes in NOD mice comprises several stages: a non-pathogenic peri-islet immune infiltration appears by 3-4 weeks (*checkpoint 1*). Following a clinically silent period, a progressive T cell-dependent destruction of the β islet cells occurs around 12 weeks of age (*checkpoint 2*) [7]. Coincident with the checkpoint 1 to 2 transition, a switch between regulatory and pro-inflammatory cytokine production occurs: prior to β cell death, a period of Th2-dominated (IL-4/IL-10), non-destructive insulitis is observed, followed by a destructive phase during which inflammatory cytokines such as IFNγ, TNF-α and IL-17 are produced. This step-wise progression, as well as cytokine switch, has led to the conclusion that waning immunoregulatory mechanisms were involved in T1 D pathogenesis [8-10]. Indeed, studies suggest that reduced $CD4^+$ T_{reg} cell frequencies or function represent a primary predisposing factor to T1 D. Transfer of CD25-depleted splenocytes into NOD. *scid* hosts leads to a quicker onset of T1 D than total splenocytes [11]. A disruption of foxp3, B7/CD28 or CD40/CD40L pathways in NOD mice alters the thymic development and peripheral homeostasis of T_{reg} cells, and leads to an accelerated T1 D onset compared to WT NOD mice [12,13]. Thus, T1 D onset/progression may be triggered by a reduction in $Foxp3^+$ T_{reg} cell numbers and/or functions.

Strong evidence shows that IL-2, as well as other common gamma chain (γc; also known as CD132) signaling, are important stimulatory signals for the development, function and fitness of nTreg cells. Its signaling cascade is initiated by the binding of IL-2 to its trimeric IL-2 receptor (IL-2R) which consists of the α-chain (IL-2Rα; also known as CD25), the β-chain (IL-2Rβ; also known as CD122) and the γc chain. All three subunits contribute towards IL-2 binding, but only IL-2Rγ and the γc are required for signal transduction. The IL-2Rβ and the γc are also components of other cytokine receptors, expressed by many cell types and tissue, whereas the IL-2Rα expression is mostly restricted to activated T cells, and Treg cells [14].

In recent years, strong genetic and molecular evidence has shown that the IL-2/IL-2R pathway promotes Treg cells development and functional fitness, and functional variations of this pathway can promote susceptibility to autoimmunity. Here, we review these recent findings and explore the role of the Treg/IL-2 axis in the pathophysiology of organ-specific autoimmune disorders such as T1 D the functional potential of IL-2 and its possible implication as a therapeutic agent in the context of autoimmunity.

Genetic evidence for a role of IL-2 in autoimmunity

The IL-2-IL-2R pathway plays an essential role in the development of T1 D and other autoimmune disorders in humans and mice. IL-2 is well-described to promote activated T cell proliferation, survival and differentiation [15]. However, mice deficient for IL-2, IL-2Rα (CD25) or IL-2Rβ (CD122) die prematurely from a severe, multi-organ, autoimmune and lymphoproliferative disorder [16]. Similarly, rare genetic disorders due to mutations of the *il2*, *cd25* or *stat5a/b* genes lead to autoimmune syndromes [17-19], emphasizing the importance of IL-2 in the maintenance of self-tolerance [16].

Il2 allelic variation (Idd3) and resistance to autoimmunity in NOD mice

T1 D susceptibility is inherited through multiple genes, with a strong predisposition for those affecting T cell responses to γ cells [7]. At present, genomic mapping studies of congenic NOD mice have identified 20 insulin-dependent regions (Idd) that influence either the onset insulitis, progression to overt T1 D, or both [20]. No single gene is both necessary and sufficient for T1 D susceptibility. Of particular interest is the Idd3 locus which was mapped to a 0.15-cM interval on the proximal mouse chromosome 3 between the microsatellite markers D3Nds55 and D3Nds40b [20-22]. Fine mapping studies show that the Idd3 locus encompasses several genes of potential immune relevance, notably: *Il-2*, testis nuclear RNA-binding protein (*Tenr*), *Il-21*, Centrin 4 (*Cetn4*) and Fibroblast growth factor 2 (*Fgf2*) [20], although the *IL-2* gene is the strongest and primary candidate gene for protection in NOD mice congenic for the C57BL/6 *Idd3* locus [20,22]. NOD mice introgressed with the protective Idd3 allele from C57BL/6 display a reduced onset and severity of T1 D, as well as reduced susceptibility to other organ-specific autoimmune disorders, such as experimental autoimmune encephalomyelitis (EAE) and autoimmune ovarian dysgenesis [23]. Yamanouchi *et al.* showed that expression of protective Idd3 alleles in $CD8^+$ T cells results in a 2-fold increase in IL-2 transcription and protein production compared to susceptible alleles [22]. The protection conferred by the Idd3 C57BL/6 allele can be explained by the presence of 46 SNPs upstream of the minimal promoter of the *IL-2* gene that can alter the transcriptional activity of this gene compared to NOD mice [22]. Moreover, polymorphisms in *il2* exon 1 cause multiple amino acid

changes that have been proposed to be responsible for a differential glycosylation pattern [24]. As such, the presence of a proline rather than a serine at position 6 of the mature IL-2 protein, is associated with an increased glycosylation and prolongation of the IL-2 half life [24]. However, NOD.CZECH Idd3 mice, whose IL-2 glycosylation pattern is identical to that of wild-type NOD mice, is resistant to T1 D, suggesting that glycosylation differences, on their own, do not account for T1 D protection in NOD.B6 Idd3 mice [22]. Candidate-gene approaches have also demonstrated a role for the Idd3 locus in human celiac disease and RA [25], as well as in T1D [26,27]. Interestingly, neither the Idd3 locus, nor any of the candidate genes enclosed therein (*il-2, il-21*), have been identified as correlating with T1 D in the recent genome-wide association studies (GWAS).

cd25 genetic polymorphisms are associated with human T1 D

Genetic evidence linking the IL-2/IL-2RA pathway to the predisposition of human autoimmunity, and in particular T1 D, has also emerged in recent years. First, Vella *et al*. observed that SNPs in the *cd25* gene indeed correlate with T1 D in a large European cohort. However, this quite large interval also encompasses other immune-relevant genes such as IL15RA, and the authors could not pin-point the causal variant with the locus [28]. The genetic interval was significantly narrowed down thanks to the power of GWAS performed on large cohorts around the world. As such, two sets of SNPs have been identified in the 5' and 3' vicinity of the promoter of *IL-2RA* [29-31]. The molecular and functional consequences of these SNPs remain to be characterized, however they seemingly do not cause splicing variations, nor do they directly affect the five known promoter regulatory regions of CD25 [31]. Some insights could come from the observation that levels of the soluble form of CD25 (sIL-2-RA) are slightly lower in the serum of patients carrying predisposing alleles [31], although the functional relevance of sIL-2-RA is ill-defined. Indeed, sIL-2RA seems to be able to partially block signaling downstream of IL-2 *in vitro*, all-the-while enhancing T cell activation and proliferation [32], a finding reminiscent of the recent observation on the impact of IL-2/anti-IL-2 mAb complexes (discussed below).

A study by Qu *et al*. observed an allelic imbalance of the CD25 SNP variants whereby the susceptibility haplotype correlates with lower CD25 mRNA in lymphoblastoid cell lines [33]. In accordance, it was simultaneously shown that CD4^{+} T cells of the memory subset display higher surface expression levels of CD25 in patients harboring a predisposing allele [34]. CD25 SNPs have been suggested to affect the onset and progression to T1 D.

Indeed, a study of late-onset T1 D in a Finnish cohort suggested that the predisposing SNPs originally described by Lowe *et al*. also correlate with the age of onset, and do so as strongly as the HLA-DQ2/DQ8 predisposing haplotype [35]. Furthermore, the predisposing haplotype of CD25 SNPs described by Qu *et al*. [29] was found to correlate with acute-onset diabetes, but not slow-onset or fulminant, in a Japanese cohort [36].

Role of IL-2 in stabilizing Foxp3^{+} Treg cells homeostasis in T1 D progression
Defective Treg cell fitness and survival in target organ as a trigger of autoimmunity
Several lines of evidence point to a critical role of the IL-2/IL-2R pathway in Treg cell development, function and homeostasis in human and murine autoimmunity. First, we and others have asked whether a possible quantitative or qualitative deficiency in Foxp3^{+}CD4^{+} Treg cells contributes to the onset and establishment of autoimmune diabetes in NOD mice [8]. We showed that thymic and peripheral CD4^{+}CD25^{+} T cells are fully functional *in vitro* and *in vivo* in both normal NOD mice and the BDC2.5 antigen-specific model of T1D [8]. Furthermore, Treg cells do not affect the priming or expansion of antigen-specific diabetogenic T cells in pancreatic lymph nodes, but regulate late events of diabetogenesis by localizing in the pancreas where they suppress the accumulation and function of effector Th1 and Th17 cells [8]. Interestingly, the function of Treg cells, while fully operative in neonatal mice, declines progressively with age [8]. The proportion of Foxp3^{+} Treg cells in secondary lymphoid tissues is similar in the NOD mice relative to T1D-resistant C57BL/6 mice While T1 D progression is not attributed to systemic fluctuations in CD4^{+}Foxp3^{+} Treg cell numbers, there is a paradoxical increase of Treg cells in the pancLN at T1 D onset [8]. Interestingly, the transition from peri-insulitis (checkpoint 1) stage to T1 D onset (checkpoint2) is associated with a progressive loss of CD4^{+}Foxp3^{+} Treg cells in pancreas, but not in the pancLN, which in turn perturbs the Treg/Teff cell balance and allows the triggering of Teff cell pathogenicity in inflamed islets [8]. Moreover, intra-islet Treg cells expressed reduced amounts of CD25 and Bcl-2 relative to Treg cells in the pLN, suggesting that the Treg/Teff cell imbalance was due a defect in intra-islet Treg survival [10]. Collectively, these studies suggest that T1 D onset is associated with a loss of Treg cells numbers or/and function [37-42]. Several findings suggest that IL-2/IL-2R signaling is necessary for the peripheral maintenance and fitness of Treg cells. In Fontenot *et al*., the analysis of Foxp3-GFP reporter knock-in mice genetically deficient for IL-2 or IL-2R (CD25) revealed that IL-2 signaling is not required for the induction of Foxp3 expression in

thymocytes. These findings were further confirmed by demonstrating that Treg cell development is independent of IL-2, while this cytokine is essential for survival of Treg cells [43]. Moreover, although IL-2$^{-/-}$ or IL-2R$^{-/-}$ mice display reduced numbers of Treg cells *in vivo*, their suppressive function *in vitro* remains unaffected [44]. Nonetheless, gene expression analysis showed that IL-2 signaling was required for the maintenance of the expression of the genes involved in the regulation of cell growth and metabolism [22]. Hence, IL-2 has a critical role in the homeostasis and competitive fitness of Treg cells [3]. Interestingly, the adoptive transfer of WT Treg cells either in IL-2$^{-/-}$ or IL-2R$^{-/-}$ mice can only prevent autoimmunity in IL-2R$^{-/-}$, and not IL-2$^{-/-}$, mice [16,45]. These results indicate that the lack of Treg cells in IL-2$^{-/-}$ and IL-2R$^{-/-}$ mice contributes to the autoimmune phenotype and that IL-2 maintains self tolerance by increasing the number of Treg cells present in the peripheral organs [46].

Similarly, T cell-specific deletion of STAT5a/b leads to reduced Treg cell numbers [47]. Antov *et al.* demonstrated that adoptive transfer of C57BL/6 background WT mice CD4$^+$CD25$^+$ Treg cells into STAT5$^{-/-}$, mice was sufficient to prevent the development of splenomegaly and autoimmunity, demonstrating that disease symptoms in STAT5 mice are due to defective Treg cells [48]. Another player in the IL-2 signaling cascades is the Jak3 kinase. Jak3$^{-/-}$ mice display symptoms of autoimmunity and accumulation of auto-reactive T cells in the lymphoid organs [48]. It has been shown that the frequency of CD25$^+$CD4$^+$ Treg cells in the spleen of Jak3$^{-/-}$ mice was similar to that in IL-2$^{-/-}$ and IL-2$\beta^{-/-}$ mice, and was reduced compared to the C57BL/6 background WT mice [48]. Altogether, these findings indicate that Jak3 and STAT5a/b signals are required to maintain normal numbers of Treg cells in peripheral lymphoid organs and maintain self-tolerance downstream of IL-2/IL-2R signaling. Overall, IL-2 may not be absolutely required for the thymic generation of Treg cells but is a critical contributor of peripheral tolerance by maintaining a fit Treg cell pool.

IL-2 restores the Treg/Teff balance in T1 D

The importance of IL-2 in the maintenance of Treg cell homeostasis and suppression in T1 D has been suggested by IL-2 neutralization studies [49]. Administration of an IL-2-neutralizing antibody into neonatal NOD mice precipitated T1 D development by selectively depleting the Treg cell subset, reinforcing the importance of IL-2 in promoting Treg cell functions [49]. Similarly, a recent study by Tang *et al.* showed that CD4$^+$ Teff from islets of NOD mice were selectively impaired to produce IL-2, consistent with s report documenting the appearance of TCR hyporesponsive T cells coincident with the development of insulitis [10].

Conversely, low dose administration of IL-2 in prediabetic NOD mice restored CD25 expression and survival in intra-islet T$_{reg}$ cells, increase of the overall frequency of Foxp3$^+$CD25$^+$ Treg cells in islets and led to T1 D prevention [50]. Overall, these results show that an IL-2 deficiency contributes to intra-islet T$_{reg}$ cell dysfunction and progressive loss of self-tolerance in the islets.

As discussed above, the increased transcriptional activity of protective Idd3 alleles translates into higher levels of IL-2 production by auto-reactive CD8$^+$ T cells in response to antigenic stimulation and, controls the size of the Treg cell pool in the pancreatic lymph nodes of NOD mice [10,22] These results show that *IL-2* gene variation may affect the balance between islet-specific auto-reactive T cells and Foxp3$^+$ Treg cells, and consequently precipitate T1 D. In Sgouroudis *et al.*, we asked if *Il2* allelic variation potentiates Foxp3$^+$ T$_{reg}$ cell-mediated regulation of T1D [9]. NOD.*Idd3*^{B6} mice show a markedly reduced incidence and delayed T1 D onset compared to control NOD mice. This resistance is associated with significantly reduced insulitis scores and frequencies of IFN-γ, TNF-α and IL-17 secreting autoreactive CD4$^+$ T cells, and correlates with increased IL-2 gene expression and protein production in antigen-activated CD4$^+$ T$_{eff}$ cells [9]. The *Idd3*^{B6} allele favors the suppressive functions of T$_{reg}$ cells *in vitro*, and this increased T$_{reg}$ cell function, in contrast to controls, restrains the expansion and effector functions of CD4$^+$ T$_{eff}$ cells more efficiently *in vivo* [9]. Interestingly, T1 D resistance in *Idd3*^{B6} mice correlates with the ability of protective *Il2* allelic variants to promote the expansion of T$_{reg}$ cells directly within islets undergoing autoimmune attack [9,51]. Thus, T1D-protective IL2 allelic variants impinge the development of γ-islet autoimmunity by bolstering the IL-2 production of pathogenic CD4$^+$ Teff cells, and in turn, driving the functional homeostasis of CD4$^+$Foxp3$^+$ T$_{reg}$ cells in the target organ.

Treg lineage commitment and stability of Foxp3 expression

IL-2 is important in instructing Treg lineage commitment. Apart from thymic-derived Treg cells, induced Treg cells can acquire Foxp3 expression following T cell activation in the periphery, a process that is facilitated by IL-2 [52]. For example, TGF-γ1 induction of Foxp3-expressing Treg cells *in vitro* is highly dependent on IL-2. Recent evidence also points to the functional plasticity of Foxp3$^+$ T$_{reg}$ cells in which Foxp3 expression and suppressive activity can be modulated in pre-committed Foxp3$^+$ Treg cells depending on the inflammatory milieu. This is evidenced by a recent study by Zhou *et al.* which points out that a loss of Foxp3 expression within T$_{reg}$ cells has been described as a

critical event which can break self-tolerance and trigger autoimmunity [53]. The ensuing unstable Foxp3$^+$ Treg cells acquire a pathogenic phenotype, as reflected by the production of pathogenic cytokines such as IFN- γ and IL-17, and contribute to the onset of T1D [53]. These results suggest that an IL-2 functional deficiency in the target organ may disturb the positive feedback loop that controls Foxp3 stability, such that T$_{reg}$ cells convert to Teff cells with a high diabetic potential. Moreover, Komatsu *et al.* noted that Foxp3$^+$ cells with low CD25 expression lose more Foxp3 expression and become effector T cells, where cells with high CD25 expression are more resistant to such a conversion [54]. These findings have important implications for the role of Foxp3 in Treg cell lineage commitment, suggesting a role of IL-2 as a key player in Treg cell plasticity and heterogeneity. These studies also shape our thinking as some human trials have been initiated that use Treg cells-based immunotherapy.

Molecular basis underlying IL-2 mediated Treg cell homeostasis

Recent evidence shows that microRNAs (miRNA) can play an important role in the regulation of immunological responses by influencing Foxp3 stability [55-57]. As such, it has been shown that when DICER, a molecule critical to the function of miRNA, is deleted, Treg cells down-regulate Foxp3 expression, adopt an effector-like phenotype, and mice rapidly develop a fatal systemic autoimmune disease resembling the Scurfy syndrome [58]. More specifically, miRNA155 is preferentially expressed in Foxp3$^+$ cells, and a miR155 deficiency results in an increased suppressor of cytokine signaling 1 (SOCS1) activity in Treg cells, which has been previously described as a negative regulator of the IL-2 signaling. Furthermore, miR155-deficient Treg cells display a low proliferative capacity in response to limiting amounts of IL-2, whereas high amounts of IL-2 lead to normal levels of STAT5 phosphorylation [55]. Hence miRNA155 is required for Treg cell fitness in contexts of differential IL-2 levels in contexts of homeostasis and inflammation. Therefore, the waning of Treg cells, and ensuing breakdown in the self tolerance, could depend on the *in situ* IL-2 environment. These data all together suggest that Treg cell stability and their responsiveness to the IL-2 can be controlled by different miRNA therefore opening new avenues for potential therapeutic targets for the prevention and treatment of autoimmune disorders.

IL-2 may also directly impact the survival of Foxp3$^+$ Treg cells by promoting the expression of CD25 and Bcl2, a critical anti-apoptotic gene in T cells. Indeed, Tang *et al.* have shown that progression from peri-insulitis to destructive insulitis in the NOD mice correlates with intra-islet Treg cells expressing decreased levels of CD25 and Bcl2. These data suggest that Treg cells decrease in number by apoptosis due to a deficiency of IL-2 in inflammatory sites [10]. Hence, IL-2 may function as critical an anti-apoptotic factor for Treg cells.

Evidence of Treg deficiencies in human T1 D

It is unclear whether a quantitative or qualitative Treg cells defect contributes to human T1 D pathogenesis. Indeed, some studies claim a numerical defect [59], others a functional one [38,60], some none at all [61,62]. Defining Treg cells in human is much more challenging than in mouse due to the lack of stringency of FOXP3 expression as a marker of Treg cells. Indeed, in humans, FOXP3 is expressed by activated Teff cells [63], and forced or natural expression of FOXP3 does not always correlate with a regulatory function [2,64](our unpublished data).

The association between IL-2 and Treg cells in humans has also presented with more challenges than in murine work, due to the lack of reliable phenotypic markers discriminating human Treg from Teff cell populations. *In vitro* studies have shown the absolute necessity of IL-2 for the maintenance of FOXP3 expression and maintenance of the suppressive phenotype in Treg-enriched CD4$^+$CD25$^+$ cells [65,66]. Accordingly, it was further shown that Treg-enriched CD4$^+$CD25$^+$ cells isolated from diabetic subjects displayed a concomitant defect in IL-2 signaling and a difficulty to maintain FOXP3 expression levels even in the presence of IL-2 [67]. This study does not, however, address whether a potential loss of suppressive function correlates with FOXP3 loss. Interestingly, the lack of suppression of auto-reactive T-cells from peripheral blood of subjects after the clinical onset of T1 D is due an increased apoptosis in Treg cells, possibly mediated by deprivation of growth signals such as IL-2 [68]. Hence, IL-2 has a potential critical role in the fitness and/or lineage maintenance of human Treg cells, which is likely one of the major mechanisms by which the IL-2/IL-2-RA pathway impacts T1 D resistance in humans.

Autoantigen-driven Treg cell defects in organ-specific autoimmunity?

An important aspect in our understanding of the pathogenesis of autoimmunity is that potential immune defects may only be apparent when and if they affect autoantigen-specific fractions within Teff or Treg cell compartments. Indeed, the onset of organ-specific autoimmune disorders such as T1 D, MS and RA, can be interpreted in two ways: 1) a cell-autonomous, genetically-driven, defect exists in autoantigen-specific Treg cells, in turn leaving the activities of autoantigen-specific Teff unchecked in a given organ. The local inflammatory micro-environment or the degree of functional Treg ablation are contributing factors which may unveil this Treg defect, and in turn, mark the transition to

overt autoimmunity; and 2) the autoantigen-specific Treg cell pool remain unaffected but genetic variation influences immune selection and/or activation of antigen-specific, pathogenic T cells, leading to a breakdown of self tolerance in a given organ. These two scenarios are of course non mutually-exclusive in individual subjects.

In the implications of such considerations lies the relevance of studies examining defects on a global population of Treg cells obtained from the peripheral blood, as opposed to examining the defects solely in the antigen-specific subset of T cells, and Treg cells in particular. Indeed, only islet-specific T cells can enter the pancreas to contribute to diabetes [69]. Additionally, the T cells found in the blood, whether it be in their repertoire, function and state of activation, may not accurately reflect the status and behavior of their counterparts localized in the target organ.

In this latter regard, there is experimental evidence that the blood carries at least a fraction of those cells with undeniable pathogenic potential. As such, it has been shown that beta islet cell-specific CD8+ T cells can be found in the blood of mice, that constitute a predictive marker of onset [70-72]. Furthermore, the number of islet-specific CD4+ T cells increases in the blood of pre-diabetic mice in correlation with increased infiltration of pancreas, however, their repertoire, unlike CD8+ cells, was found to be more restricted in the islet than in the blood [73]. The authors also point out that when taken in blood, antigen-specific CD4+ T cells are less pathogenic, whereby when adoptively transferred, recipients do not develop disease unless the cells were obtained from islets [74]. Thus, caution is required when interpreting functional data obtained from peripheral blood.

In humans, islet-specific T cells are found in the blood of normal subjects, but are slightly more prevalent in T1 D patients or at-risk subjects [75,76]. Interestingly, only in at risk and T1 D patients does this subset exhibit markers of prior activation, namely the memory marker CD45RO [77,78]. Given the extremely low abundance of T1 D autoantigen-specific cells in the blood, combined with the very low frequency of Treg cells, it has not been elucidated yet whether or not quantitative or qualitative defects in T1 D auto-Ag specific Treg cells can be detected in the blood. Thus, observations from the blood, if not mimic, at least reflect events ongoing at the specific site of inflammation. Whether or not those events that are translated into the blood encompass autoantigen-specific Treg cell defects remains to be determined.

Modulation of the IL-2/IL-2R pathway for therapeutic purposes

Given the strong link between IL-2 and autoimmunity, it seems appealing to consider the use of IL-2 as a therapeutic tool for T1 D. However, this might prove quite challenging, as IL-2 is first and foremost a T cell growth factor, and as such, has strong proliferative effects on all T cells, including pathogenic CD4+ and CD8+ Teff cells. For the past decade, IL-2 has been used in the treatment of several diseases where the immune system necessitates strengthening of the activated T cell pool. As such, IL-2 is a frequent therapy in the treatment of solid tumors, mainly melanoma and renal cancer. In such cases, high doses of IL-2 are injected frequently leading to tumour regression in only about 10% of patients, and devastating side effects. While Teff cells were believed to be the primary target of treatment in treated patients, a 4-fold increase in suppressive CD4+CD25+FOXP3+ cells was described although immune responses in patients for whom IL-2 treatment had worked were not analyzed [79]. Hence the main hurdle to human IL-2 immunotherapy for T1 D is to obtain an efficient and timely targeting of activated Treg versus Teff cells during distinct phases of T1 D progression. Several studies report the use of several strategies to modulate IL-2 signals and ultimately impact the Teff/Treg balance *in vivo*:

Low dose IL-2 prophylaxis therapy
Treg cells differ from their Teff counterparts in their IL-2 signaling pathways. Indeed, Treg cells are able to form the highest affinity receptor complex for IL-2, due to their constitutive expression of CD25, making them especially sensitive to very low levels of IL-2, in a fashion that seems to be relatively independent of the IL-2Rγ chain [80]. This supports the rationale of examining the potential of low-dose IL-2 as a "Treg-only enhancing treatment". Low-dose IL-2 has been used for several years to facilitate hematopoietic stem cell transplantation (HSCT). Studies in such patients indicate that Treg cells do increase in response to the treatment, and that this effect seems to be increased with prolonged time of treatment [66]. Of note is the fact that this effect correlates with a medically positive outcome, i.e. absence of graft rejection and GVHD. Accordingly, a low-dose IL-2 regimen diminishes the magnitude and frequency of CTL responses to a peptide vaccine against melanoma [81]. These observations are consistent with a recent report showing that administration of low-dose IL-2 promoted Treg cell survival and protected mice from developing diabetes in NOD mice [10].

Anti-IL-2 blockade in vivo
One explanation for the initially observed need for high doses of IL-2 in the treatment of cancer might have originated from the very short half-life of purified IL-2 after injection (3-5 min in mice) [82]. However, high-dose IL-2 leads to a devastating syndrome resembling septic shock. Hence, several avenues have been explored in order to stabilize the molecule *in vivo*, allowing for

lower doses to reach sufficient therapeutic potency. As such, fusion with a carrier protein such as gelatin, BSA or even an irrelevant immunoglobulin chain have successfully prolonged IL-2 half life and reduced the side effects [82].

The undesired emergence of Treg cells has been pointed out as a potential culprit for treatment failure in cancer. Thus, focus has been put on modulating the affinity of IL-2 for its receptor complexes. Indeed, if IL-2 could be made to have a greater affinity for IL-2Rγ than IL-2Rα, the preferential bias of Treg cells in receiving IL-2 signaling would be cancelled out. As such, targeted mutations of the IL-2/IL-2RA binding sites have shown promising results [82].

More recently, a novel therapeutic tool has emerged that enables both higher stability, and selective cellular targeting of IL-2 *in vivo*. Indeed, binding of IL-2 to its receptor complexes could also be modulated by coupling IL-2 with different anti-IL-2 monoclonal antibodies (mAb). By varying the clone of the mAb, IL-2 can be targeted preferentially towards either CD25 or CD122 [82,83]. These complexes, when "stimulating", show a therapeutic effect *in vivo* in mice [84]. However their exact mechanism of action remains unclear. Recently, it was shown that the effect of the stimulating IL-2/anti-IL-2 mAb complex treatment is recapitulated by a conjoint prolongation of IL-2 half-life and a blockade of CD25. Moreover, the effect of IL-2/anti-IL-2 mAb does not depend on FcRs [85].

Combination therapy with rapamycin

Another way of selectively targeting Treg cells could be the use of pharmacological agents that selectively modulate biochemical pathways in Teff or Treg cells. Rapamycin (Sirolimus) is a commonly used immunosuppressive drug which targets the cytosolic protein FK-binding protein 12 (FKBP12) and downstream mTOR pathway, and in turn inhibits IL-2 responsiveness in activated T cells [86]. Investigations into its mechanism of action have highlighted that Treg cells respond differently than Teff. Indeed, upon rapamycin treatment, Treg cells upregulate anti-apoptotic, and down-regulate pro-apoptotic molecules [87,88], in turn altering the Teff/Treg balance. Interestingly, the same anti-apoptotic molecules were increased downstream of IL-2 signaling [88]. Moreover, rapamycin treatment in humans seems not to affect the phenotype of Treg cells *in vivo*, and leads to an increase of their functionality [89]. These findings have prompted research into the use of combining IL-2 and rapamycin therapies. In NOD mice, IL-2 synergizes with the therapeutic effects of sirolimus on T1 D development, leading to a reduction in disease incidence of about 80%. The effect was further confirmed to improve islet graft survival in diabetic mice [90], although the cellular mechanisms underlying this protection have yet to be examined.

In humans, exposing CD4$^+$ T cells to both IL-2 and rapamycin *in vitro* leads to an increase in the cellular frequency of FOXP3$^+$ T cells, originating from nTreg cells and *de novo* induced Treg cells [91]. Clinical trials are currently underway to assess the effects and benefits of this double therapy.

Combination therapy with cellular infusion

The idea of cellular therapy has also been examined. The major challenge in this case is the very low abundance of Treg cells. The possibility of expanding and/or differentiating Treg cells *in vitro* prior to re-infusing them into patients is currently the focus of several clinical trials. One major limitation to such therapy could be the lack of stability if these "artificial" Treg cells. Indeed, FOXP3$^+$ Treg cells have been shown to fluctuate in their phenotype, function, and FOXP3 expression levels upon introduction in various murine models. Subsequently, studies have highlighted the instability and heterogeneity of the Treg transcriptional signature. Hence, the risk of loss of function of massively injected Treg population, and their subsequent likely conversion into pathogenic T cells, casts doubts over the future of Treg immunotherapy. Interestingly, IL-2 has been shown to play a major role in the stabilization of the FOXP3$^+$ Treg phenotype and function [53]. Hence, IL-2 therapy could, in combination with Treg infusion, represent a plausible alternative. Indeed, low dose IL-2 in addition to donor CD4$^+$ T cell infusion has shown to significantly improve medical outcome in HSCT by increasing Treg expansion *in vivo* [92].

Alternatively, administration of selective demethylation agents and histone protein deacetylases could be considered in order to enhance Treg cell stability, as it has been shown that Foxp3 expression is modulated by DNA methylation via CpG islands in its promoter [93]. Also, as suggested by Blazar *et al.*, it could be possible to use clinical-grade lentiviral vectors in order to redirect polyclonal Treg cells to the specific targets, as well as to prevent Treg cell conversion to the Teff cells [94]. Thus, Treg cells could be engineered to constantly express Foxp3, so that the infused Treg cells keep Foxp3 expression.

Antigen-specific immunotherapy

The efficiency of Treg-mediated immunotherapy could be greatly enhanced by focusing on auto-antigen specific Treg cells. While Treg cells can suppress antigen non-specifically *in vitro*, these cells need to home to and suppress antigen-specific responses in the target organ in order to mediate disease protection [69]. This would also reduce potential adverse effects of systemic immunosuppression in treated individuals. However, the identification and isolation of antigen-specific Treg cells, existing at very low frequencies in blood, poses significant hurdles for their use in cellular infusion protocols. A potentially

promising avenue might therefore be to increase the endogenous antigen-specific Treg population. Expansion and/or *de novo* induction of Treg cells of a given specificity can theoretically be achieved by an antigen vaccination strategy. This has proven efficient in the NOD mouse model, as well as in other murine models of T1D [95-100]. The feasibility of translating these therapies to humans remains to be assessed. One potential limitation of the process is the identification of those antigens that are the most relevant as targets, as the human auto-antigen-specific T cell repertoire is diverse and the optimal antigen target could vary between patients [95]. Moreover, the possibility of conversion of antigen-specific Treg cells into Teff cells would pose an even greater danger in the context of antigen-specific Treg cell therapies. A deeper understanding of the factors that modulate this phenotypic and functional plasticity in Foxp3$^+$ Treg cells will be needed in order to implement Treg-cell based therapies in autoimmune disease.

Conclusion

In conclusion, T1 D progression is associated with a temporal loss of CD4$^+$Foxp3$^+$ Treg cells in β-islets, which perturbs the Treg/Teff cell balance and unleashes the anti-islet immune responses. Moreover, IL-2 deficiency is an important trigger to intra-islet Treg cell dysfunction and progressive loss of self-tolerance in the islets. Currently there is great interest in the use of various immunotherapeutic agents including IL-2 modulatory strategies, to prevent T1 D in genetically susceptible individuals and/or cure the overt disease. The induction and maintenance of long lasting tolerance to islet autoantigens remains a major goal of T1 D research. CD4$^+$ Treg cells represent major players in the control of T1 D and offer much hope for effective antigen-specific immunoregulation in the immediate future. However, several critical issues arise when considering the treatment of autoimmune disorders like T1D:

- Genetic-based identification of immune defects and biomarkers of disease progression

Studies documenting quantitative or qualitative defects in CD4$^+$Foxp3$^+$ Treg cells as a contributor to human T1 D are inconclusive at best. The inability to detect immune dysregulation in human T1 D as unequivocally as in the murine models could be attributed to the lack of specific and stable markers of human FOXP3$^+$ Treg cells. Indeed, the accurate immune monitoring of human Treg cell frequency and function in various clinical settings is primordial to our understanding of the fundamental role of these cells in the pathophysiology of many human diseases. Moreover, we have no reason to assume that the primary immune dysfunction is identical among individuals. Indeed, the existence of the two rodent models of the NOD mouse and the BB rat, which display distinct immune dysfunction genotypes/phenotypes, clearly demonstrates the existence of at least two distinct mechanisms that can lead to loss of γ-cell tolerance. Based on the genetic diversity of the human population, the primary dysfunction can thus be assumed to differ between individual T1 D subjects. Additionally, assuming that a primary Treg defect is important in human T1 D, it can be expected that many healthy controls will have the same defect but not get T1 D because of other genetic or environmental contributors. Conversely, this defect may not be an absolute requirement and may be absent from many of the cases. A more refined approach, based on genetic-based selection of clinically stratified T1 D subjects, may now be feasible, given the recent breakthroughs in the genetics of T1D [101]. Knowledge of how known and novel T1 D loci affect Treg cell development and function can be expected to lead to assessments of immune function that provide meaningful information for the individual being tested.

The detection of T1D-specific antibodies is currently used for meaningful and reliable prediction of T1 D, years before clinical onset, but likely reflects ongoing autoimmune responses towards β-islets. Although still under development, assays of immune responses, and in particular antigen-specific T cell responses could become an alternative screening tool. However assays are urgently required to measure not only the number/function in pro-inflammatory, diabetogenic cells, but also the induction, expansion and function of islet-specific Treg cells. Reliable assays to detect a primary (i.e. genetically determined and preceding the autoimmune process) immune dysfunction exist in the rodent models but not in humans. Hence the critical question remains of whether biomarkers can be developed to detect the primary, genetically-determined, immune dysfunction that leads to T1 D rather than the consequences of autoimmunity induced on a given genetic background by environmental triggers.

- When could a treatment be initiated/applied universally to all T1D-susceptible subjects?

T1 D develops progressively, over several years, and is only diagnosed once most of the damage to the pancreas has already been done. Insights into human pathogenesis are scarce, but the NOD model displays a step-wise pathogenesis, whereby insulitis occurs long before islet-destruction. This suggests the existence of several so-called checkpoints, when distinct immunological events are at play. As such, therapeutic intervention can be expected to have a different impact, depending on what stage the disease development is at. These pathogenesis phases, however, are still ill-defined in humans. The genetic and physiological hallmarks of disease risk and progression have previously been thoroughly reviewed [101].

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Authors' contributions
All authors contributed to the writing of this manuscript. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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