The IL-2/IL-2R system: from basic science to therapeutic applications to enhance immune regulation

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Published online: 9 November 2013
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Abstract IL-2 plays a critical role in the normal function of the immune system. A trophic factor for lymphocytes, IL-2 is required for mounting and sustaining adaptive T cell responses; however, IL-2 is also critical for immune regulation via its effects on regulatory T cells (Treg cells). Over the years, we have contributed to the understanding of the biology of IL-2 and its signaling through the IL-2 receptor and helped define the key role played by IL-2 in Treg development and function. Our data show that Treg cells have a heightened sensitivity to IL-2, which may create a therapeutic window to promote immune regulation by selective stimulation of Treg cells. We are now developing new efforts to translate this knowledge to the clinical arena, through our focused interest in Type 1 diabetes as a prototypic autoimmune disease. Specifically, we aim at developing IL-2-based therapeutic regimens and incorporate means to enhance antigen-specific Treg responses, for improved and more selective regulation of islet autoimmunity. In parallel, we are pursuing studies in preclinical models of autoimmunity and transplantation to define critical factors for successful adoptive Treg therapy and develop clinically applicable therapeutic protocols.

Keywords Treg cells · IL-2 · Autoimmunity · Tolerance · Type 1 diabetes · Insulin

Abbreviations

AIRE	Autoimmune regulator transcription factor
DC	Dendritic cell
IGRP	Glucose-6-phosphatase catalytic subunit-related protein
GAD	Glutamic acid decarboxylase
GvHD	Graft-versus-host disease
mTECs	Medullary thymic epithelial cells
NOD	Nonobese diabetic

NK	Natural killer
SPK	Simultaneous pancreas-kidney transplants
T1D	Type 1 diabetes
Treg	Regulatory T cell
TSAs	Tissue-specific antigens
WT	Wild type

Introduction

A major focus of our laboratory has been to determine the underlying molecular and cellular mechanisms by which IL-2 regulates the immune system. IL-2 was discovered based on its potent T cell growth factor activity; hence, early mechanistic studies and clinical trials were focused on its immune stimulatory activities. IL-2 is a cytokine that is primarily produced by activated T lymphocytes. IL-2 exerts its biological effects under most physiological conditions through binding to the high affinity (K_d 10^{-11} M) IL-2R, consisting of IL-2R α (CD25), IL-2R β (CD122), and

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γ_c (CD132), which is primarily found on activated T conventional and Treg cells [1]. In situations of very high IL-2 levels, IL-2 can also induce signals through the intermediate affinity IL-2R (K_d 10^{-9} M), consisting of IL-2R β and γ_c , found on most natural killer (NK) and CD8⁺ T memory cells. The main role of IL-2R α is to capture IL-2 and to facilitate binding to IL-2R β and γ_c . These latter two subunits have significant cytoplasmic tails and initiate signaling through the Jak1/Jak3/STAT5, PI3K, and MAPK pathways [2]. These pathways impact gene expression to regulate cellular growth, death, and immune function in IL-2R-bearing cells.

Based on the initial concept that IL-2 was an essential growth factor for immune responses, the phenotype of IL-2- and IL-2R-deficient mice was much unexpected. Instead of impaired T cell proliferation and immunity, these mice show uncontrolled T cell proliferation and rapidly developed systemic lethal autoimmunity, where most mice die between 4 and 12 weeks of age [3–5]. Our laboratory provided the first definitive data to account for this paradox by showing that failed production of Treg cells caused this lymphoproliferative autoimmune syndrome [6]. Based on this understanding, clinical application of IL-2 must consider not only the immune stimulatory activity of IL-2 but also its capacity to boost immune regulation through its action on Treg cells. IL-2-dependent activation of natural killer (NK) and T effector cells depends on the application of high levels of IL-2 which does not discriminate between the effector and regulatory compartments. However, infusion of relatively low doses of IL-2 appears to selectively support Treg cells and offers new opportunities for IL-2-based immunotherapy by boosting immune suppressive mechanisms to inhibit unwanted immune responses.

In this article, we will summarize our contributions that help shape the current understanding by which IL-2 regulates tolerance, including why low IL-2R signaling selectively supports Treg cells, and discuss how we are translating this knowledge in preclinical and clinical studies. Many of our experiments on Treg cells depended on the capacity of adoptively transferred Treg cells to prevent autoimmunity associated with IL-2R β -deficient mice. These studies revealed the importance of an empty Treg niche for long-term persistence of the donor Treg cells. This has led to efforts to manipulate the Treg niche (“space”) in wild-type (WT) mice with the goal of improving adoptive Treg immunotherapy. Promoting immune regulation through enhancing Treg cells offers the hope to suppress many unwanted immune responses in a plethora of autoimmune diseases, in graft-versus-host disease (GvHD), and in transplantation to prevent graft rejection. Finally, we describe some of our contributions to the study of human Type 1 diabetes (T1D), which provide further rationale and context for developing novel

therapeutic approaches based on in-depth knowledge of the human disease. Our focus is to apply our understanding of the IL-2 system to promote immunoregulation in T1D patients.

IL-2R signaling in the regulation of Treg development and homeostasis

IL-2R signaling in the thymic development of Treg cells

We performed two critical experiments to establish that the main non-redundant function of IL-2 was related to the production of Tregs cells. First, we developed a transgenic model in which WT IL-2R β was expressed as a transgene in IL-2R $\beta^{-/-}$ mice. By using the proximal *lck* promoter to drive transgenic IL-2R β expression, readily detectable levels of IL-2R β were observed only for thymocytes. Importantly, these transgenic mice were fully protected from the lethal autoimmunity associated with the IL-2R $\beta^{-/-}$ phenotype, and functional studies showed that the peripheral T cell compartment was non-responsive to IL-2 [7]. This experiment made two key points. First, IL-2R β provides an essential signal during thymic development through selective expression of the transgene to promote immune tolerance. Second, the lack of IL-2 responsiveness by peripheral T cells in this transgenic model provides strong evidence that a failure in peripheral IL-2-dependent apoptosis in autoreactive T cells was unlikely to account for autoimmunity associated with IL-2 and IL-2R deficiency, which was the prevailing notion at the time. Subsequent experiments showed that CD4⁺ CD25⁺ T cells, which mark Treg cells, were absent in the thymus and spleen but readily detected at normal levels in these tissue from this transgenic model [8]. This finding associated expression of the transgene in the thymus to the production of Treg cells.

The second key experiment was when we showed that adoptive transfer of a low number of Tregs into neonatal IL-2R $\beta^{-/-}$ mice also fully protected them from lethal autoimmunity [8]. Collectively, these data showed that the lack of Treg cells in IL-2R β deficient mice was due to a failure in their thymic production and peripheral persistence. Although the recipients received a single infusion ($1\text{--}3 \times 10^5$ cells) of WT Treg cells, remarkably, donor-derived Treg cells were detected at normal levels in these mice, which live to a normal old age. This adoptive transfer approach revealed that Treg cells are highly proliferative and undergo enormous self-renewal without exhaustion. Indeed, when assayed any time after transfer, 30–40 % of donor Tregs are proliferating as assessed by Ki67 expression.

Fig. 1 Main sites of action of IL-2 on thymic and peripheral Treg cells

Much subsequent work by us and others has provided more information concerning the role of IL-2 during thymic Treg development (Fig. 1). After *Foxp3* was discovered as a key marker for Treg cells [9], IL-2- and IL-2R-deficient mice were found to contain $Foxp3^+$ $CD25^-$ T cells, suggesting that Treg development was not IL-2-dependent [10, 11]. These cells, however, express lower levels of *Foxp3* and represent immature, non-functional Treg cells [12]. Immature Treg cells are also found in the thymus of normal mice and are further characterized by lower levels of molecules important for Treg function, e.g., CTLA4, CD73, and CD39 [13]. During Treg development, IL-2 functions as a key maturation factor by upregulation of *Foxp3*, *CD25*, and Treg suppressive molecules through activation of STAT5 [14–16]. The link between IL-2 and *Foxp3* provides a mechanism to enforce expression of the Treg functional program, and IL-2 likely reinforces this program in the periphery [17]. This maturation step requires IL-2 because essentially all Treg cells in IL-2Rβ^{-/-} mice are immature. However, few $Foxp3^{hi}$ cells are found in IL-2Rβ^{-/-} mice, but the expression of CTLA4, CD73, and CD39 remains low [13]. This finding suggests that along with *Foxp3* there remain other unidentified IL-2-dependent activities for maturation of Treg cells. IL-2 also provides growth and survival signals that help increase mature Treg numbers in the thymus.

We and others have examined other knockout and transgenic mouse models to study the cytokine dependency of Treg cells. Although the IL-15R also shares IL-2Rβ,

Treg development and function are normal in IL-15- and IL-15Rα-deficient mice [18, 19], indicating that IL-15 is not a major cytokine for Treg cells in vivo. IL-7 is another cytokine that activates STAT5 and participates in Treg thymic development. Immature $Foxp3^{lo}$ Treg cells associated with IL-2R deficiency are not detected in mice doubly deficient for IL-7 and IL-2Rβ or IL-7 and IL-2 [18, 19]; this demonstrates a role for the IL-7R in Treg development. Although IL-7 has the potential to signal in a redundant manner with IL-2, this cytokine is distinctly localized in the thymus such that IL-7 cannot replace IL-2 [20]. It remains unclear how IL-7 shapes the Treg pool, but one possibility is that IL-7R signaling partially opens chromatin for *Foxp3*. This might provide a mechanism to facilitate Treg maturation as there are few IL-2 producing cells in the thymus and IL-2 levels are expected to be limiting. Along this line, thymic-derived Treg cells show enhanced IL-2-dependent STAT5 activation that would also promote Treg maturation in the presence of low IL-2 levels [13, 21]. Thus, there are likely several mechanisms in place to ensure that Treg cells effectively use the available IL-2 for their maturation.

IL-2R signaling in the regulation of the peripheral T cell compartment

The reduced proportions of Treg cells associated with IL-2- and IL-2R-deficient mice provided an initial hint that IL-2 is required for peripheral Treg homeostasis. One

complexity with this interpretation is that these Treg cells are actually immature Foxp3^{lo} cells that have seeded the periphery from the thymus. However, interfering with IL-2R signaling blocks the rapid expansion of Treg cells that occurs in WT neonatal mice [22] and anti-IL-2 blockade reduces Treg numbers in the periphery of WT mice [23]. In addition, mixed chimeras prepared with bone marrow from WT and transgenic IL-2Rβ^{-/-} mice that expressed high levels of transgenic IL-2Rβ only in the thymus resulted in recipient mice with similar representation of both types of Treg cells in the thymus, whereas the periphery was dominated by WT Treg cells [13]. Collectively, these data make a strong case for IL-2 playing a critical role in peripheral Treg homeostasis (Fig. 1). Gene enrichment analysis of global gene expression profiling of Treg cells from WT and IL-2R signaling impaired mice showed enrichment of genes important for cell growth and death [11, 21]. Major mediators of Treg homeostasis are pro-apoptotic Bim and anti-apoptotic Mcl-1, the latter which is regulated by IL-2R signaling [24, 25]. IL-2R signaling also promotes peripheral Treg homeostasis by driving the development of Klrp1⁺ Treg cells [26]. This Treg subset is highly activated and expresses increased levels of tissue-seeking chemokine receptors and Treg suppressive mediators. This subset develops from “resting” Klrp1⁻ Treg cells where Klrp1 is expressed after 8–10 cell divisions. These Klrp1⁺ Treg cells are terminally differentiated and short-lived. The IL-2-dependent development of these Treg cells and their subsequent death represent another means by which highly proliferative Treg populations are maintained at a constant number in the periphery (Fig. 1).

IL-2 is much more than a homeostatic regulator of Treg cells. Gene expression profiling also revealed enrichment in some genes important for Treg tissue localization and function [11, 21]. Signaling from the IL-2R promotes Treg survival and Foxp3 expression, which in turn helps enforce and maintain the Treg suppressive program [17]. In this regard, it is noteworthy that IL-2^{-/-} Bim^{-/-} mice contain normal numbers of Foxp3⁺ cells in the periphery, but these cells were not functional and these mice developed lethal autoimmunity [24]. These findings clearly separate IL-2-dependent regulation of Treg growth/survival activities versus regulation of function and strongly support a role for IL-2R signaling in direct regulation of the Treg suppressive program. IL-2 also supports the conversion of conventional peripheral CD4⁺ T cells into induced Treg cells [27, 28].

The Treg TCR repertoire and tolerance

The empty mature Treg niche associated with IL-2Rβ-deficient mice also provides a model to study properties of Treg cells independent of IL-2. One area where we

have exploited this model is related to the TCR repertoire of donor Treg cells as they suppress autoimmunity upon transfer into IL-2Rβ^{-/-} mice. We showed that the TCR repertoire of Treg cells is sufficiently cross-reactive to be selected in fully MHC mismatched allogeneic recipients such that these cells persist and suppress autoimmunity [29]. The allogeneic donor Treg cells were not a target of a host-versus-graft response. Remarkably, besides suppressing autoimmunity, the donor Treg cells supported potent transplantation tolerance to allogeneic skin grafts, provided the skin expressed the MHC allogeneic antigens of the Treg cells. Tolerance to allogeneic skin was in part due to Treg-mediated suppression of allogeneic rejection and in part to a substantial pool of alloantigens associated with the highly replicating donor Treg cells that are available and promote thymic negative selection of the host pool of alloreactive T cells [30]. These findings raise the possibility that the transfer of Treg cells from the same donor used for an organ or tissue graft could lead to potent immune tolerance, provided conditions are found to promote stable engraftment of the donor Treg cells in the recipients. This represents an ongoing effort, as discussed below.

Tregs have a highly diverse TCR repertoire. Nevertheless, we noted that polyclonal autoreactive T cells in IL-2Rβ^{-/-} mice were suppressed by a fixed and limited number of donor Treg cells, indicating that full Treg TCR diversity is not required to maintain self-tolerance. Indeed, direct assessment of the TCR repertoire of donor syngeneic and allogeneic Treg cells, obtained 2–4 months after “curing” IL-2Rβ^{-/-} mice, showed narrowing of the TCR repertoire with a more restricted Treg repertoire associated with allogeneic Treg cells [31]. Extending this type of analysis using syngeneic Treg cells that expressed a single TCR β-chain further supports the notion that a fraction of the Treg TCR repertoire is sufficient to control autoreactive T cells that express a more diverse repertoire. TCR sequencing of the TCR α-chains from these donor Treg cells revealed considerable repertoire reshaping that was distinct between individual recipients [31]. This finding suggests that a mechanism is in place to select proper Treg TCR specificities to suppress autoreactive T cells. As noted, while a fully diverse Treg repertoire is not required to suppress self-reactive T cells, too severe constraints on the Treg TCR repertoire eventually lead to uncontrolled autoimmunity. We are currently quantifying more precisely the extent of Treg TCR diversity that is necessary to effectively suppress autoreactive T cells. The inter-relationship between phenotypic distinct Treg subsets is also being evaluated in part through assessing the extent to which their TCR repertoire is shared.

Type 1 diabetes: an autoimmune disease with chronic immune dysregulation

Overview

Type 1 diabetes (T1D) is a chronic autoimmune disease that eventually leads to the virtually complete destruction of pancreatic beta cells [32]. As a consequence, patients become dependent on insulin injections for their lifetime. However, this remains an imperfect therapy that does not prevent acute and chronic disease complications, which can be severely debilitating and even life-threatening. T1D is commonly diagnosed in children and adolescents, but also occurs in adults. More than 40 susceptibility loci have been linked to diabetes risk; consistently with an autoimmune pathogenesis, many are involved in key immunological functions and were also linked to other autoimmune diseases [33]. Prospective studies in genetically at-risk cohorts have shown that islet autoimmunity can be triggered in early childhood [34]. The disease process involves both humoral and cellular autoimmune responses against several islet cell autoantigens, with immune infiltrates (insulitis) developing in the pancreatic islets, which contain autoantigen-specific T cells [35, 36]; the best studied autoantigens include insulin/proinsulin, glutamic acid decarboxylase 65 (GAD65), the tyrosine phosphatase-like protein IA-2, the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and the cation efflux transporter ZnT8. Standardized radioimmunoassays measure autoantibodies to insulin, GAD65, IA-2, and ZnT8, which are diagnostic and predictive markers employed as screening tools in clinical trials [37]. Autoantibodies and autoreactive T cells are typically detected prior to and around the time of clinical diagnosis [38], but in some patients can persist for many years after diagnosis, further supporting the chronicity of islet autoimmunity.

Both central and peripheral tolerance mechanisms play a role in T1D pathogenesis

The expression of tissue-specific antigens (TSAs) in the thymus has been shown to play a key role for immunological self-tolerance. However, genetic mechanisms (allelic variation, alternative splicing, epigenetic regulation) influence such expression sometimes leading to insufficient levels or differential expression of isoforms compared to the target organ, which ultimately can lead to imperfect central tolerance for selected autoantigens [39]. We and others have shown that this is the case for insulin. In man, insulin expression in the thymus is influenced by allelic variation and epigenetic regulation of the insulin gene [40, 41], noting that this has been identified as a susceptibility locus for T1D and insulin is a key autoantigen [42, 43]. We also

showed that other T1D autoantigens, such as IA-2 and IGRP, exist in several spliced variants with different expression patterns in human pancreas and thymus, suggesting that self-tolerance may not be achievable to epitopes expressed only by the pancreas [44–47]. There is evidence that the epitopes and levels of expression of these autoantigens in the thymus affect negative selection of autoreactive T cells as well as the development of Treg cells [48].

Thymic expression of self-antigens is largely mediated by a specialized subset of medullary thymic epithelial cells (mTECs) [39], under the control of the autoimmune regulator transcription factor (AIRE) [49]. However, we and others have shown insulin gene transcripts and insulin protein in CD11c⁺ dendritic cells (DCs), both in man [50] and rodents [51, 52], which are present in the thymus and in peripheral lymphoid tissues [50, 53]. Insulin expression by AIRE-expressing DCs is critical for peripheral tolerance to this antigen [54]. Additional TSA-expressing cells have been reported in the periphery and include stromal cells [55, 56] as well as lymph node-resident lymphatic endothelial cells and fibroblastic reticular cells [57, 58]. Studies in the nonobese diabetic (NOD) mouse model [59], which closely resembles human T1D, show that insulin gene expression is progressively lost in the pancreatic lymph node in association with proinflammatory changes that occur as mice progress toward diabetes; these observations link reduced TSA expression with impaired peripheral tolerance and islet autoimmunity [60]. Thus, multiple mechanisms influence both thymic and peripheral tolerance to insulin (and other islet autoantigens) and play a role in T1D pathogenesis, which are thought to negatively impact Treg function.

Clinical trials

Over the last three decades, clinical trials have been conducted in an attempt to halt islet autoimmunity [61]. Most of these trials have enrolled recent onset T1D patients, hoping that effective suppression of autoimmunity would spare residual beta cells; the ultimate goal of these trials is to preserve insulin secretion to allow patients to maintain normal glucose metabolism without the need for insulin injections, hence modifying the severity of clinical manifestations and the likelihood of long-term complications. In addition, some trials have been conducted in high-risk relatives of patients in the attempt to prevent progression of islet autoimmunity to clinical disease. Most trials have relied on immunosuppressive drugs, administered chronically to suppress autoimmunity or in short course regimens in the hope that these would reset the immune system and restore self-tolerance. Results have shown limited efficacy with either approaches, both in terms of disease remission

(i.e., patients becoming insulin-independent) and preservation of stimulated C-peptide responses (a measure of insulin secretion) as primary outcomes. For example, earlier studies with Cyclosporine showed increased remission rates in treated children, but these were not sustained upon discontinuation of treatment. Short courses with anti-CD3 or Rituximab, which target T or B cells, respectively, resulted in better preservation of C-peptide responses in the treated patients but the decline in insulin secretion was delayed but not halted [62, 63]. In new onset patients treated with Abatacept to block co-stimulatory signals, there was some C-peptide preservation but the protective effect declined during treatment [64]. While responder patients can be identified in these trials that had a more significant therapeutic effect, the overall disease-modifying impact of these therapies has been limited.

Lessons from diabetes recurrence in transplant recipients

Another important lesson derives from our own studies of patients with T1D who later become recipients of simultaneous pancreas–kidney transplants (SPK). Over the past few years, we have shown that about 5–6 % of our SPK recipients will, on long-term follow-up, develop recurrence of T1D in the transplanted pancreas [65, 66]. Our studies show that T1D recurrence is possible despite immunosuppression that prevents rejection, by extensive documentation of the cardinal features of T1D in SPK recipients [66]:

1. The return of hyperglycemia, with no evidence of rejection and stable exocrine pancreas (urine amylase) and kidney (serum creatinine) graft function, with selective loss of C-peptide secretion;
2. The presence of insulinitis and/or beta cell loss, on biopsy of the transplanted pancreas in SPK recipients with T1D recurrence, but not in those with rejection or normal grafts;
3. The persistence or reappearance of autoantibodies, typically years prior to diabetes recurrence;
4. The presence of circulating autoreactive T cells around the time of diabetes recurrence and on follow-up; importantly, autoreactive CD4 T cells responding to several autoantigens were demonstrated both in the circulation and in the pancreas graft and/or draining lymph nodes in association with T1D recurrence. The TCR sequences (V-beta and CDR3) in samples collected over time suggested a memory phenotype [66, 67], and more recent phenotypic studies (unpublished) show that these autoreactive T cells are largely memory cells, including those in the insulinitis lesion;
5. The presence of autoreactive T cells in the circulation that correlated with disease activity and progression; in patients that received additional immunosuppression in the attempt to salvage the residual beta cell mass demonstrated at biopsy, autoreactive T cells were no longer detected after treatment. However, these reappeared on later follow-up and their return was followed by further and complete loss of C-peptide secretion;
6. The demonstration that antigen-specific autoreactive T cells isolated from these patients could mediate beta cell destruction when transplanted with human islets in immuno-deficient mice.

Considering our results in transplant recipients and the results from the clinical trials in recent onset T1D, we have to question whether chronic immunosuppression can be effective in the long run in treating islet autoimmunity. Another concern is that chronic immunosuppression, or even a short course with depleting agents, for example, thymoglobulin, as used in our transplant recipients at the time of diabetes recurrence, may favor the emergence of memory autoreactive T cells through mechanisms of homeostatic proliferation [68]. Moreover, we must consider that chronic immunosuppression is associated with serious side effects, which would limit its implementation especially when children are concerned.

Rationale for targeting the IL-2 pathway and Treg cells in T1D to promote immune regulation

An alternative approach to immunotherapy in T1D would entail boosting tolerogenic mechanisms by promoting regulation of adaptive T cell responses. Manipulation of the IL-2 pathway, given its importance for Treg development, homeostasis and function, appears as a prime candidate strategy. Many studies show that a reduction or functional alteration of Treg cells leads to spontaneous development of autoimmunity against multiple organs, resulting in various autoimmune diseases including gastritis, thyroiditis, and T1D [41–43]. For example, mutations or absence of Foxp3 leads to an immune compartment devoid of CD4⁺CD25⁺ Treg cells and mice die within one month from birth. Furthermore, acute ablation of the entire Foxp3⁺ Treg compartment in either neonatal or adult mice resulted in severe autoimmune disease that is similar to what is observed in Foxp3-deficient mice [44]. Importantly, adoptive transfer of WT Treg cells into Foxp3-deficient mice prevents disease development in these models [45] and our studies in the IL-2Rβ^{-/-} mice showed that transfer of Treg cells leads to a normal life span and prevention of autoimmunity with life-long donor Treg cell engraftment and suppressor function.

Moreover, there are several lines of evidence that support targeting the IL-2 pathway and Treg cells specifically in T1D. In patients, the IL-2RA gene is among several T1D susceptibility genes identified [33]. IL2RA polymorphisms are associated with reduced levels of soluble IL-2 receptor [69], lower STAT5a IL-2 responses in antigen-experienced CD4 T cells, reduced Foxp3 levels in Treg cells, and impaired Treg suppressive function [70]. Moreover, several functional defects of Treg cells have been reported in T1D patients, as well as resistance of effector T cells to Treg suppression [71]. In the NOD mouse, the IL-2 gene is a risk allele and abnormalities in the IL-2 pathway influence diabetes development and Treg function [72]. Intra-islet Treg cells in NOD mice express lower IL2RA levels and undergo increased apoptosis [73]. These defects can be ameliorated by treating NOD mice with low-dose IL-2, which also reverses new onset diabetes [74]. Moreover, adoptive Treg therapy has been shown effective in NOD mice, especially with the infusion of antigen-specific Treg cells [75–77].

Our group is exploring two approaches to promote immune regulation through the targeting of the IL-2 pathway and Treg cells, one based on the administration of low doses of IL-2 to selectively stimulate Treg cells, the other based on novel approaches to promote efficacy and engraftment of adoptive Treg therapy.

Selectivity of low-dose IL-2 for Treg cells

The use of IL-2 in immunotherapy is potentially counter-intuitive because IL-2 may act on the T effector cell compartment and in fact IL-2 has been largely used with the purpose of stimulating the immune response. However, the discovery that IL-2 is also critical for Treg cells explains the largely disappointing results when IL-2 was used to boost immunity in cancer and HIV/AIDS patients [78–80]. The realization that IL-2 promotes Treg growth and function, however, raises the possibility that IL-2 may offer a means to boost Treg cells and immune tolerance. The key lies in the administration of a dose that is selective for Treg cells and does not stimulate effector cells. In pre-clinical studies, low levels of IL-2 or agonists IL-2/anti-IL-2 complexes controlled T1D in NOD mice and also suppressed experimental autoimmune encephalitis in a mouse model of multiple sclerosis [73, 74, 81]. However, in NOD mice, diabetes was exacerbated and the T effector compartment was activated upon administering higher doses of IL-2 [73]. Overall, these studies indicate that there is a therapeutic window where IL-2 selectively promotes Treg cells and immune regulation.

One important question is what accounts for the apparent selectivity of low-dose IL-2 in Treg cells. Work in our

laboratory provides some initial clues. We developed mouse models where T cells are engineered to express IL-2R β -chains with mutations in their cytoplasmic tail, where tyrosine residues were mutated to phenylalanine to interfere with IL-2-dependent STAT5 and Shc-dependent PI3K activation. Based on the known importance of IL-2R signaling for Treg development and homeostasis, we expected that these mice would lack mature, functional Treg cells and exhibit severe autoimmunity. To our surprise, these mice were outwardly healthy and contained normal numbers and proportions of mature Treg cells in the thymus and peripheral immune tissues [21]. In contrast, these mutations substantially blocked T effector responses in vitro and production of T memory responses in vivo. When considering the mice with the most severe IL-2R β mutations, IL-2-dependent STAT5 activation was markedly impaired in Treg cells. Collectively, these data indicate that minimal IL-2R signaling readily supports the key activities of Treg cells leading to normal Treg development, peripheral homeostasis, and immune tolerance, while this level of signaling did not support IL-2-dependent immunity. It is important to note that Treg cells with this type of lower IL-2R signaling still showed a gene program that is not fully functional. Thus, the most critical, but not all, IL-2-dependent activities function effectively with lower IL-2R signaling. We have ongoing studies, both in mouse and humans, which further support the concept that low-dose IL-2 selectively stimulates Treg cells.

Clinical trials with low-dose IL-2

The concept that low-dose IL-2 may have selective effects on Treg cells without stimulating effector cells underpins the application of this therapy in clinical trials. Recent studies have shown that low-dose IL-2 confers therapeutic benefit in two immune-mediated conditions, GvHD and hepatitis C virus-induced vasculitis [82–84]. In these studies, Treg numbers and function were also increased, in the absence of stimulation of effector and memory T cell responses. In a study of T1D patients, treatment with IL-2 and rapamycin resulted in worsened C-peptide secretion, likely because the IL-2 dose used was not low enough to only stimulate Treg cells; indeed, effector cell types were stimulated [85]. Moreover, there is evidence that rapamycin impairs insulin secretion [86]. Thus, if a proper low dose can be identified, low-dose IL-2 therapy might promote peripheral immune regulation through improved Treg function. In this regard, an IL-2 dose finding study has been recently completed in a small cohort of T1D patients [87]. This was a randomized placebo-controlled phase I/II trial to define the lowest IL-2 dose that could induce Treg cells in 24 adult T1D patients, randomized in groups of 6

patients to placebo or IL-2 at doses of 0.33, 1 or 3 million international units per day for a 5-day course. In this study, IL-2 therapy was safe and led to dose-dependent increased numbers of Treg cells with no or minimal increase in effector T cells and NK cells, in the absence of deleterious effects on insulin secretion.

All of the above provide a strong rationale for more extensive evaluation of low-dose IL-2 therapy in T1D patients. Stimulation of peripheral immune regulation with low-dose IL-2, if at doses that are low enough to prevent side effects or that compare favorably to those of immunosuppressive agents, could represent an acceptable chronic treatment to control islet autoimmunity and preserve beta cell function. Perhaps, it could even be safer than insulin therapy, as it would not carry the risk of hypoglycemia. If sufficient beta cell mass and function could be maintained with low-dose IL-2, there should be a lower risk of chronic diabetes complications.

Finally, low-dose IL-2 therapy could be used in combinatorial regimens in which the other therapeutic agent is one or more self-antigens. Antigen-based therapies have been tried in T1D, with variable success depending on antigen, route of administration and doses, with the auto-antigens GAD and insulin [88–94]. If low-dose IL-2 could be given concurrently with antigen, this could lead to preferential expansion of antigen-specific Treg cells. This hypothesis could be tested in patients with T1D, who immunize themselves multiple times daily with insulin injections, which in most induce insulin antibodies. Tetramer reagents could be used to determine whether insulin-specific Treg cells are induced in patients treated with low-dose IL-2. This is of particular significance because of the key role in the disease pathogenesis for insulin autoimmunity, its well-documented genetic basis, and because insulin, given orally to prediabetic subjects, has been shown to delay progression to diabetes [93]. Moreover, a plasmid encoding proinsulin has been shown to lead to deletion of proinsulin-specific CD8⁺ T cells and preserve C-peptide in new onset patients [92]. Future studies will determine whether low-dose IL-2 and antigen can promote antigen-specific regulation of autoimmune responses in T1D, a concept that can be applied to other autoimmune diseases.

There is much effort ongoing toward the development of clinical strategies for adoptive Treg therapy in T1D [95–97]. Such trials present several challenges: (1) achieving sufficient number of Treg cells for adoptive therapy requires *in vitro* expansion [54–59], which may also alter the phenotype of the Treg cells and may include contamination with proinflammatory cells, some of which may be autoreactive; (2) expanded cells are unselected, polyclonal Treg cells, which in mouse models have lower potency than antigen-specific ones; (3) upon infusion, expanded Treg cells may

lead to increased proportions of Treg cells in the circulation, but this effect is not sustained over time; (4) there is concern that expanded Treg cells may require IL-2 to survive upon infusion in the patient.

Key requirements for successful adoptive Treg cell immunotherapy

Another strategy would involve adoptive transfer of Treg cells using protocols that favor their long-term engraftment through *in vivo* antigen-driven selection and expansion for improved therapeutic efficacy. We have modeled such an approach in our earlier studies in the IL-2R $\beta^{-/-}$ model, showing that under the right biological conditions adoptive transfer of even a small, unmanipulated number of Treg cells is very effective and results in long-term engraftment and long-term persistence of donor Treg cells. Furthermore, we found that upon adoptive transfer, donor Treg cells were subjected to biological selection which allowed a population of therapeutic Treg cells to engraft and regulate immune responses that prevent lethal autoimmunity and confer tolerance to skin grafts. Collectively, our data, discussed below, indicate that four requirements are critical for adoptive Treg therapy that does not require *in vitro* expansion: (1) creating space in the Treg niche; (2) overcoming competition from host Treg cells; (3) sufficient availability of IL-2; and (4) availability of relevant antigens (Fig. 2).

The importance of space, overcoming host Treg competition, and IL-2

Key features of the IL-2R $\beta^{-/-}$ model is the lack of continuous competition from endogenous Treg cells and adoptively transferring donor Treg cells very early following birth, a factor that could contribute to successful donor engraftment, given the limited competition from host immune cells. Similarly, T1D prevention was achieved with transfer of antigen-specific NOD Treg cells in NOD mice that were devoid of either T or Treg cells [55, 63–65], resulting in space in the natural Treg niche and lack of competition from endogenous Treg cells, resembling our IL-2R $\beta^{-/-}$ model. In transplantation models, success of adoptive Treg therapy was achieved in likewise immunocompromised recipients, including irradiated, athymic, T cell depleted, or Rag $^{-/-}$ hosts [66–70]. While long-term donor Treg engraftment was not examined in these studies, it is likely that the recipient lymphopenic environment fostered engraftment due to the reduced competition from host immune cells. We have also shown that there is a residual host Treg population that survives ablation and is

Fig. 2 In vivo environment to support adoptive Treg immunotherapy

highly proliferative and functional [71]; IL-2 is the dominant cytokine driving their proliferation and survival in this lymphopenic environment [72]. Although there is low-level IL-2 following ablation compared to other STAT5-dependent cytokines, such as IL-7 and IL-15, expression of the high-affinity IL-2R and lower threshold of IL-2R signaling required for Treg cell homeostasis gives these cells a competitive advantage in environments containing minimal IL-2.

The importance of antigen for adoptive Treg therapy

Many groups have shown that antigen is critical for Treg cell homeostasis and that Treg cells could be found within tolerant grafts, indicating that alloreactive Treg cells could potentially be recruited to or even retained at sites of alloantigen expression and actively participate in tolerance induction and/or maintenance [74–77]. Moreover, the ability of Tregs to prevent thyroiditis was lost when the thyroid had been ablated, but rats retained the ability to prevent autoimmune diabetes [78], suggesting that the presence of antigen maintains a population of antigen-specific Treg cells. Another study showed constant presence of alloantigen was required to inhibit allogeneic responses and induce tolerance to allogeneic bone marrow cells [66, 67]. Antigen-specific Treg cells are much more effective than polyclonal Treg cells in controlling disease progression in NOD mice and controlling alloresponses [55, 63, 64, 67, 79]. In addition, several reports using engineered antigen-specific Treg cells have promising results for diabetes therapy [80, 81].

Allogeneic Treg cells are likely the initial population of donor Treg cells that could be examined for tolerance induction in the transplant setting. Allogeneic Treg cells could engraft, persist, and induce tolerance in our IL-2R $\beta^{-/-}$ model and there was a specific donor Treg enrichment to host alloantigen following adoptive transfer of allogeneic Treg cells into IL-2R $\beta^{-/-}$ mice, indicating Treg infusion allowed biological selection of donor inoculums resulting in therapeutic Treg cells capable of regulating autoimmunity and tolerance [38]. This dominant transplantation tolerance was toward the MHC antigens

that the Treg cells were initially selected for. A major benefit from utilizing antigen-specific donor Treg cells is that these Treg cells will act only where antigen is present, thereby providing local immunotherapy. Importantly, infusion of antigen-specific Treg cells following immunomodulation may allow for the transfer of far fewer cells for therapy. Unpublished findings from our laboratory also demonstrate that antigen-specific Treg cells require the presence of their cognate antigen for stable engraftment following irradiation. These data have important implications in designing Treg immunotherapy in autoimmune setting, such as T1D, in that insulin-specific or other islet-specific Treg cells may be better induced by contemporary administration of relevant autoantigens; it is also possible that residual beta cells or islet cell transplants may provide an adequate source of antigen. Alternatively, infusion of donor allogeneic Treg cells could potentially replenish this population, but pose more of challenge than syngeneic antigen-specific Treg cells because of their immunogenicity. Successful engraftment of allogeneic donor Treg cells in IL-2R $\beta^{-/-}$ mice was likely partial due to the fact that these mice contain less robust T effectors compared to WT mice [7, 82]. However, controlling allogeneic responses with rapamycin may overcome this immunogenicity, while promoting Treg expansion and function [51, 83, 84].

Novel approaches for adoptive Treg therapy

Designing strategies that allow adoptive Treg therapy without in vitro expansion and that address the critical requirements described above is actually difficult to achieve in most clinical situations, or models that resemble them, because major IL-2 signaling abnormalities or immune deficiencies that would create a Treg niche and prevent host competition are not present. Of note, the first clinical trials using in vitro expanded human, polyclonal Treg cells have been in the prevention of GvHD, which have shown promising results and excellent safety profiles [59–61] and likely have been favored by the presence of lymphopenia and reduced host competition. Based on the information gleaned from our studies in the IL-2R $\beta^{-/-}$ model, we are now pursuing studies in NOD mice and derivatives to identify

strategies that combine adoptive Treg therapy with immune manipulation to create a niche for the infused cells, control host competition, provide IL-2 to support survival, and overall provide a supportive environment for long-term engraftment (Fig. 2). Our ongoing studies began with proof of principle experiments that involved total body irradiation; we are now exploring combined, short course regimens with therapeutic agents that have been used in clinical trials in T1D patients, and therefore have strong translational potential. These include regimens with cyclophosphamide and anti-CD3 to create space and control competition and low-dose IL-2 to promote Treg homeostasis without stimulating effector T cells [98].

Closing remarks

There is an unmet need to identify therapies that can regulate the immune system to control autoimmune disease, such as T1D, but do not involve conventional immunosuppression. Besides its lack of specificity and many side effects, we have shown that chronic immunosuppression that prevents rejection does not afford protection from T1D recurrence in pancreas transplant recipients. In studying the IL-2 system, we have contributed to uncovering its critical role in Treg development, homeostasis, and function; we have defined the existence of differential sensitivity of effector and Treg cells, suggesting that at the proper doses IL-2 may operate within a therapeutic window to selectively regulate the immune system without stimulating immunity. Moreover, our genetically manipulated models have allowed us to define critical requirements for adoptive Treg therapy that would not require in vitro expansion of Treg cells. Given the defects involving the IL-2 pathway and Treg cells in human T1D, there is strong rationale for pursuing low-dose IL-2 and adoptive Treg therapy, with emphasis on the exploitation of T1D relevant antigen-specific regulation, informed by the lessons learned throughout our collective studies.

Acknowledgments Our work is supported by NIH R01AI055815 (TRM), R01DK093866 (TRM), JDRF 17-2011-594 (AP), JDRF 17-2012-3 (AP), NIH 1 U01 DK085499-01 (AP), ADA 7-09-JF-06 (AB), and NIH 1R56AI101278-01A1 (AB) and the Diabetes Research Institute Foundation, Hollywood, FL.

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