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ICOS-Dependent Homeostasis and Function of Foxp3+ Regulatory T Cells in Islets of Nonobese Diabetic Mice

Mara Kornete,*1 Evridiki Sgouroudis,*1 and Ciriaco A. Piccirillo*†

A progressive waning in Foxp3+ regulatory T cell (Treg) functions is thought to provoke autoimmunity in the NOD model of type 1 diabetes (T1D). A deficiency in IL-2 is one of the main triggers for the defective function of Tregs in islets. Notably, abrogation of the ICOS pathway in NOD neonates or BDC2.5-NOD (BDC2.5) mice exacerbates T1D, suggesting an important role for this costimulatory pathway in tolerance to islet Ags. Thus, we hypothesize that ICOS selectively promotes Foxp3+ Treg functions in BDC2.5 mice. We show that ICOS expression discriminates effector Foxp3+ T cells from Foxp3- Tregs and specifically designates a dominant subset of intra-islet Tregs, endowed with an increased potential to expand, secrete IL-10, and mediate suppressive activity in vitro and in vivo. Consistently, Ab-mediated blockade or genetic deficiency of ICOS selectively abrogates Treg-mediated functions and TID protection and exacerbates disease in BDC2.5 mice. Moreover, T1D progression in BDC2.5 mice is associated with a decline in ICOS expression and expansion and suppression by intra-islet Foxp3+ Tregs. We further show that the ICOS+ Tregs, in contrast to their ICOS- counterparts, are more sensitive to IL-2, a critical signal for their survival and functional stability. Lastly, the temporal loss in ICOS+ Tregs is readily corrected by IL-2 therapy or protective I22 gene variation. Overall, ICOS is critical for the homeostasis and functional stability of Foxp3+ Tregs in prediabetic islets and maintenance of TID protection. The Journal of Immunology, 2012, 188: 1064–1074.

Type 1 diabetes (T1D) is a chronic autoimmune disease resulting from a T cell-dependent destruction of the insulin-producing β-islets of Langerhans (1, 2). The NOD mouse model develops spontaneous T1D and shares many features with human T1D, such as development of autoantibodies and hyperglycemia (1). NOD mice exhibit profound dysregulated immune responses, and a progressive loss in immunoregulatory mechanisms is thought to underlie the pathogenesis of T1D (3). Regulatory T cells (Tregs), which constitutionally express CD4, Foxp3, and CD25 (4–6), are a major mechanism of peripheral tolerance and have been implicated as a critical point in T1D progression, as depletion of CD25-expressing cells or genetic ablation of Foxp3 results in accelerated T1D (7, 8).

Studies point to a progressive waning in Treg functions as a trigger of T1D onset and progression (9–12). Recently, we showed that T1D progression is associated with a temporal loss in the specific capacity of Foxp3+ Tregs to expand/survive in β-islets, which in turn perturbs the Treg/effector T cell (Teff) balance and unleashes anti-islet immune responses (11, 13). Moreover, a deficiency in IL-2, a cytokine essential for the function and fitness of Tregs within islets, was shown to trigger this defective function of Tregs, in turn provoking a Treg/Teff imbalance in islets (13, 14). These results are also reminiscent of earlier studies showing that T cells from prediabetic NOD mice become hypoproliferative and poor IL-2 producers at the onset of insulitis, a time point coinciding with the waning of Treg functions (15). Consistently, prophylactic treatment with low doses of IL-2 in NOD mice can prevent and even reverse established disease in NOD mice by restoring the survival of intra-islet Tregs (14).

Foxp3+ Treg function in NOD mice is costimulation dependent as disruption of CD28–B7 pathway abrogates Treg development and homeostasis and leads to acceleration of T1D in NOD mice (16, 17). The contribution of other costimulatory pathways is currently not well understood. Notably, ICOS is a CD28 superfamily-related molecule, which plays an important role in T cell activation/survival (18). ICOS enhances IL-4 and IL-10 secretion in Th2 cell responses (18, 19), and blockade of the ICOS–ICOS-ligand pathway during myelitis exacerbated disease by enhancing IFN-γ production (20). Notably, ICOS mRNA is abundantly expressed in pancreatic lymph nodes (pLNs) of T1D-protected BDC2.5-NOD (BDC2.5) TCR transgenic mice (21). In contrast to T1D-resistant ICOS−/− NOD mice, ICOS blockade in BDC2.5 mice and NOD neonates or ICOS deficiency in BDC2.5 mice exacerbated T1D. However, in the reported studies, the expression or function of ICOS was solely examined on CD25-expressing (CD25+CD69+ T cells), not purified Foxp3+. Tregs enriched from inflamed pancreatic sites (21–23).

Notably, IL-2 therapy augments Treg numbers in the pancreas and induces the expression of various Treg-associated proteins such as Foxp3, CD25, Bcl-2, and ICOS (24). IL-2 is known to enhance ICOS expression on activated T cells, suggesting that a positive feedback loop exists between IL-2 and ICOS signaling pathways (25). Collectively, these lines of evidence suggest that ICOS may selectively control immunoregulatory mechanisms of T1D, including Foxp3+ Treg functions.

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Abbreviations used in this article: BDC2.5, BDC2.5-NOD; BMDC, bone marrow-derived dendritic cell; LN, lymph node; MFI, mean fluorescence intensity; PI, propidium iodide; pLN, pancreatic lymph node; T1D, type 1 diabetes; Teff, effector T cell; Treg, regulatory T cell; WT, wild-type.

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In this study, we hypothesized that ICOS represents a critical factor in the stabilization of Foxp3 Treg functions in the NOD mouse model of T1D. We identify ICOS to be preferentially expressed by a subset of Foxp3 Tregs, in contrast to Foxp3 Teffs, in islets of prediabetic BDC2.5 mice. We also demonstrate that T1D is associated with a progressive loss in ICOS expression and expansion of Foxp3 Tregs in islets of prediabetic mice. This ICOS Treg subset, in contrast to its ICOS counterpart, is endowed with prominent proliferative and suppressive capacities in situ and readily secretes IL-10 upon islet-Ag stimulation. Moreover, icos deficiency or Ab blockade in BDC2.5 mice dampens the competitive fitness of Tregs in islets and cripples their capacity to protect against T1D. We also show that IL-2 therapy or protective IL2 gene variation readily restores the frequency of ICOS Tregs in islets. Strikingly, IL-2 preferentially activates the STAT5 pathway in the ICOS Treg subset, in contrast to its ICOS counterpart, in turn favoring ICOS Treg cell survival and function. Overall, ICOS costimulation sustains IL-2–driven stability of Foxp3 Treg functions in prediabetic islets and maintains protection from T1D.

Materials and Methods

Mice

NOD and NOD ICOS−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). NOD.B6Idd3 congenic mice were obtained from Taconic Farms. NOD.TCR−/− and BDC2.5 and Thy1.1 BDC2.5 CD4+ transgenic mice were a generous gift from C. Benoist (Harvard University). NOD.Foxp3GFP mice were generated by back-crossing them to B6.Foxp3GFP reporter mice for 12 generations (26). BDC.Idd3, BDC2.5 ICOS−/−, and Thy1.1 BDC2.5.Foxp3GFP mice were generated in-house. All mice strains were maintained in specific pathogen-free conditions at McGill University and McGill University Health Centre.

Abs and flow cytometry

Stainings for surface phenotyping were done with the following fluorochrome-conjugated or biotinylated mAbs: anti-CD3 (145-2C11), anti-CD4 (RM5), anti-CD25 (PC6), anti-Vβ4 (CTV4), anti-ICOS (7E.17G9), and anti-Thy-1.1 (HIS51). The expression of Foxp3 (FJK-16s), annexin V, propidium iodide (PI) (eBioscience, San Diego, CA), Ki-67 (B56), p-STAT5 (pY694), and Bcl2 (3F11) (BD Bioscience, Mississauga, ON, Canada) was determined by intracellular staining performed according to the manufacturer’s protocol. To determine the cytokine production, T cells were restimulated for 4 h at 37°C with PMA (20 ng/ml), ionomycin (1 nM) (Sigma-Aldrich, Oakville, ON, Canada), and BD GolgiStop (1:1000 dilution), and then stained intracellularly with anti-IL-10 (JES5-16E3), anti-IFN-γ (XMG1.2), and anti-IL-2 (JES6-5H4). In some instances, T cells were stimulated in an Ag-specific manner with NOD bone marrow-derived dendritic cells (BMDCs) (4:1 cell ratio) and BDC mimetope (2 ng/ml) for 72 h. Teff proliferation was assessed by CFSE dilution or cell cultures were pulsed with 1 μCi tritiated thymidine for the last 12–16 h of culture. To perform apoptosis and STAT5 phosphorylation assays, FACS-sorted Tregs or Teffs (1 × 106) were cultivated with APCs (4 × 105) and BDC2.5 mimetope (10 ng/ml) in the presence or absence of IL-2 (25–100 U/ml) for 36 and/or 72 h of culture.

In vivo IL-2 treatment

Mice were treated daily with i.p. injections of 25,000 IU recombinant human IL-2 (gift from the Surgery Branch, National Cancer Institute) for 5 consecutive days as described in Ref. 14. Control mice were treated with PBS. The experiment was terminated 2 d after the last injection.

Anti-ICOS mAb administration

Anti-ICOS mAb (7E17G9.G; Bio X Cell, West Lebanon, NH) was administered i.p. either at low (50 μg) or high (100 μg) dose on days 0, 3, and 5 after T cell transfer in NOD.TCRα−/− recipients.

Diagnosis of diabetes

Blood glycemia levels were determined every 2–3 d with Hemoglukotest kits (Roche Diagnostics, Montreal, QC, Canada), and T1D was diagnosed at values >300 mg/dl.

Statistical analysis

Results are expressed as means ± SD. Analyses were performed with a Student’s t test, except for diabetes incidence, where the Kaplan–Meier survival test was used. Values of p < 0.05 were considered significant.

Results

ICOS expression designates a dominant Foxp3 Treg subset in prediabetic islets

ICOS blockade in NOD mice results in T1D exacerbation, suggesting that ICOS may play a role in Treg-mediated self-tolerance (21). We first sought to characterize ICOS expression within the Foxp3 and Foxp3 Teff subsets in nondraining LN (data not shown), draining pLN, and pancreas at the early stage of insulitis in 3- to 4-wk-old BDC2.5 mice, whose TCR is specific for a β-islet–specific autoantigen. We observed a substantial increase in ICOS expression specifically on intrapancreatic Foxp3 Tregs (Fig. 1A, top row). The frequency of Foxp3 ICOS Tregs among CD4 T cells was significantly higher in the pancreas compared with that in draining pLN (8.8 ± 1.8% versus 1.5 ± 0.7%, p ≤ 0.01) (Fig. 1A, bottom row, left panel) and represents a dominant fraction of total Foxp3 Treg in the pancreas compared with that in draining pLN (57 ± 10.2% and 18.9 ± 4.5%, p ≤ 0.002) (Fig. 1A, bottom row, middle panel). Similarly, the mean fluorescence intensity (MFI) of ICOS was also significantly greater on Tregs in the pancreas relative to the pLN (144.3 ± 11.9 versus 38.5 ± 15.9, p ≤ 0.001) (Fig. 1A, bottom row, right panel). Only a small proportion of ICOS Teffs in pLN and pancreas was detected among the CD4 T cell pool (0.96 ± 0.4% versus 5.3 ± 1.5%) (Fig. 1A, bottom row, left panel) and particularly among Foxp3 Tregs (1 ± 0.4% versus 6 ± 1.6%) (Fig. 1A, bottom row, middle panel), suggesting that ICOS costimulation may not be required for the optimal function of autoreactive CD4 T cells.

The striking difference in ICOS expression on pancreatic Foxp3 Tregs relative to their counterparts in pLN suggested that Tregs acquire distinctive functional and phenotypic properties in situ. In pLN, approximately half of the Foxp3 Treg pool expressed CD25 (49.7 ± 4.3%), and only a small proportion of Foxp3 Tregs coexpressed both CD25 and ICOS (7.5 ± 0.8%) (Fig. 1B). In stark contrast, a significant frequency of Foxp3 Tregs coexpressed CD25 and ICOS in the pancreas of BDC2.5 mice (53.8 ± 5.2% versus 7.5 ± 0.8%, p ≤ 0.0007) (Fig. 1B). We also examined how ICOS expression is related to the proliferation of the Foxp3 Treg pool within the pancreatic lesion. A significant proportion of cycling Tregs (based on Ki-67 expression) expressed ICOS in the pancreas relative to pLN (24.4 ± 4.5% versus 9.2 ± 3.3%, p ≤
Notably, the ICOS⁺ subset exhibited a much greater proliferative potential compared with the ICOS⁻ subset of Foxp3⁺ Tregs (53.1 ± 5.7% versus 16.9 ± 4.2%, p < 0.001) (Fig. 1C, right panel). Remarkably, pancreatic ICOS⁺ and ICOS⁻ Foxp3⁺ Treg subsets did not differ in their activation status as both fractions were found within the CD62L lowCD44high Teff memory pool, irrespective of their proliferative potential (data not shown), suggesting that ICOS⁺Foxp3⁺ Tregs represent tissue-resident memory Tregs in islets. Thus, ICOS expression discriminates effector Foxp3⁻ Teffs from Foxp3⁺ Tregs and specifically designates a dominant subset of intra-islet Tregs, endowed with an increased potential to expand in islets of prediabetic BDC2.5 mice.

ICOS costimulation promotes the competitive fitness of Tregs in vivo

To determine whether ICOS expression conferred a competitive advantage to Ag-specific Foxp3⁺ Tregs in vivo, we examined Foxp3⁺ Treg responses in ICOS-deficient (ICOS⁻⁻) BDC2.5 mice compared to BDC2.5 (WT) mice. To this end, T cell-deficient NOD.TCRα⁻⁻ mice were adoptively transferred with CD4⁺ T cells from congenic (Thy1.1⁺) WT or (Thy1.2⁺) ICOS⁻⁻ BDC2.5 mice, and the relative occupancy of transferred T cells in pancreatic sites was evaluated 8 d after adoptive transfer. Our results show an increase in the proportion of WT (Thy1.1⁺) Foxp3⁺ Tregs compared with ICOS⁻⁻ (Thy1.2⁺) Tregs in draining LN (14.0 ± 0.8 versus 7.4 ± 0.7, p = 0.007) and the pancreas (25.9 ± 2.3 versus 10.7 ± 2.2, p < 0.0032) (Fig. 2A, left panel). The proportion of Foxp3⁻ T cells was slightly increased from ICOS⁻⁻ (Thy1.2⁺) donors (data not shown). We then examined whether the decreased frequency of ICOS⁻⁻ Foxp3⁺ Tregs resulted from a reduced cycling in situ. Our analysis of Ki-67 expression showed that ICOS⁻⁻ (Thy1.2⁺) Tregs expanded less efficiently compared with WT (Thy1.1⁺) Foxp3⁺ Tregs in draining LN (65.7 ± 2.8 versus 78.0 ± 1.2, p = 0.007) (Fig. 2A, right panel) and particularly in the pancreas (77.7 ± 4.2 versus 95.9 ± 1.2, p = 0.0061) (Fig. 2A, right panel). This decrease in ICOS⁻⁻ (Thy1.2⁺) Treg expansion also coincided with a reduction in CD25 expression in ICOS⁻⁻ Tregs compared with WT Tregs in pancreas (73.9 ± 2.4 versus 49.2 ± 4.0, p < 0.0001), suggesting a possible IL-2–ICOS cross-talk in Treg homeostasis in islets (data not shown).

We then examined the competitive fitness of Foxp3⁺ Tregs from WT or ICOS⁻⁻ BDC2.5 mice in non-lymphopenic hosts. To this end, we performed a crisscross adoptive transfer whereby WT (Thy1.1⁺) or ICOS⁻⁻ (Thy1.2⁺) CD4⁺ T cells were transferred in either WT (Thy1.1⁺) or ICOS⁻⁻ (Thy1.2⁺) recipient mice, and
As an alternate approach to assess the proliferation of WT and ICOS<sup>−/−</sup> T cell subsets, CFSE-labeled CD4<sup>+</sup> T cells from WT or ICOS<sup>−/−</sup> BDC2.5 mice were adoptively transferred into NOD recipients, and the extent of proliferation was determined in draining LN. The proportion of ICOS<sup>+</sup>Foxp3<sup>+</sup>Treg expansion was significantly lower relative to WT Tregs in pLN (65.1 ± 3.6 versus 81.4 ± 0.35, p ≤ 0.0026) (Fig. 2C, upper panel). No significant difference was observed between Teffs from either genotype, indicating that Teff priming and expansion is not impaired in the absence of ICOS in vivo (Fig. 2C, lower panel). Overall, our results show that ICOS<sup>+</sup>Tregs have a greater proliferative capacity than Teffs and that a decreased expansion potential underlies the defective competitiveness of Ag-specific ICOS<sup>−/−</sup>Tregs in inflamed sites.

**ICOS-deficient Foxp3<sup>+</sup>Tregs are less suppressive in vitro than WT Tregs**

We then asked whether ICOS affected the functional potency of Tregs. To address this question, we first characterized the function of Tregs with differential ICOS expression levels by suppression assays in vitro. CD4<sup>+</sup>CD25<sup>+</sup>ICOS<sup>+</sup>Tregs were more potent than CD4<sup>+</sup>CD25<sup>+</sup>ICOS<sup>−/−</sup>Tregs at suppressing CD4<sup>+</sup>Teffs at all ratios examined (Fig. 3A, right and left panels). To ensure that CD25<sup>+</sup>ICOS<sup>+</sup>Tregs were not contaminated with activated Teffs expressing these markers, ICOS<sup>+</sup>Foxp3<sup>+</sup>Tregs from BDC2.5. Foxp3<sup>GFP</sup> reporter mice were assessed for their ability to suppress the proliferation of Foxp3<sup>+</sup>(GFP<sup>+</sup>) Teffs in vitro. ICOS-expressing Foxp3<sup>+</sup>(GFP<sup>+</sup>) Tregs exhibited a greater suppressive capacity relative to their ICOS<sup>−/−</sup> counterparts (Fig. 3B), consistent with the increased ability of WT BDC2.5 Tregs, compared with ICOS<sup>−/−</sup>Tregs, to suppress the proliferation of WT or ICOS<sup>−/−</sup>Teffs in vitro (Fig. 3C). Blocking anti-ICOS Abs did not abolish suppression, suggesting that ICOS expression is not involved in the effector mechanism of suppression in vivo (data not shown). Moreover, whereas ICOS<sup>−/−</sup>Tregs upregulate ICOS upon TCR stimulation in vitro, they do not suppress Teffs as efficiently as ex vivo ICOS<sup>+</sup>Tregs (data not shown). This suggests that ICOS may have an important role in imprinting this potential in Tregs during their development, and possibly designates a unique Foxp3<sup>+</sup>Treg subset operating in the periphery, similar to an observation made by Ito et al. (28), in human studies. Thus, ICOS-expressing Foxp3<sup>+</sup>Tregs are endowed with a more suppressive phenotype compared with their ICOS<sup>−/−</sup>subset and indicate a role for ICOS in potentiating Treg suppressive functions.

**ICOS-deficient Foxp3<sup>+</sup>Tregs are unable to inhibit T1D induction in vivo**

We then assessed how WT and ICOS<sup>−/−</sup>Foxp3<sup>+</sup>Tregs compared in their capacity to inhibit pathogenic T cells during the pathogenesis of T1D. WT or ICOS<sup>−/−</sup>BDC2.5 CD4<sup>+</sup>Teffs were transferred into NOD.TCRα<sup>−/−</sup>hosts either alone or with WT or ICOS<sup>−/−</sup>BDC2.5 Tregs (1:8 Treg/Teff ratio), and the onset of T1D was monitored. Recipient mice transferred with WT or ICOS<sup>−/−</sup>Teffs alone developed T1D with a similar onset (11–12 d posttransfer) and similar incidence (80%) indicating that absence of ICOS co-stimulation is not essential for the pathogenic functions of Teffs (Fig. 4A). Expectedly, WT CD4<sup>+</sup>Teff efficiently suppressed the pathogenic potential of WT Teffs, as NOD.TCRα<sup>−/−</sup>recipient mice remained 90% T1D-free for up to 23 d posttransfer (Fig. 4A). Strikingly, recipient mice receiving WT Teffs and ICOS<sup>−/−</sup>Tregs rapidly developed T1D by day 13–15 posttransfer, demonstrating that ICOS<sup>−/−</sup>Tregs are unable to maintain self-tolerance compared with WT Tregs (Fig. 4A). These in vivo data are consistent with our observations that ICOS<sup>−/−</sup>or
Foxp3+ Tregs are unable to suppress Teffs in vitro (Fig. 3). These findings confirm that ICOS deficiency in Foxp3+ Tregs cripples their capacity to control autoreactive Teffs and mediate T1D protection in contrast to ICOS+ Tregs, which fully preserve their functions in vivo. Given the inability of ICOS−/− Tregs to suppress induction of T1D by Teffs, we then asked whether this was due to decreased expansion and accumulation of Foxp3+ Tregs in the pLN and pancreas. To examine this possibility, BDC2.5 CD4+ Teffs were transferred into NOD.TCRa2/2 mice either alone or with WT and ICOS−/− BDC2.5 Tregs, and the cellular frequency of cycling Foxp3+ cells (Ki-67 expression) was analyzed on days 15 and 23 posttransfer. The frequency of ICOS−/− Foxp3+ Tregs in the pLN and pancreas was significantly lower compared with recipients of WT Tregs (22.8 ± 4.2 versus 11.13 ± 3.02, p < 0.004) (Fig. 4A), and particularly after the onset of T1D. (22.8 ± 4.2 versus 5.6 ± 0.6).
ICOS and IL-10 led to the hypothesis that ICOS$^{+}$Foxp3$^{+}$ Treg-mediated T1D protection is related to this production of IL-10. To this end, ICOS$^{+}$ and ICOS$^{-}$ Treg subsets were FACs purified from BDC2.5.Foxp3GFP mice, activated with NOD BMDCs and mimotope in vitro, and the capacity to produce IL-10 was assessed by flow cytometry. Our results show that only ICOS$^{+}$ Tregs were able to secrete IL-10 after Ag-specific stimulation compared with ICOS$^{-}$ Treg (19 ± 7.1% versus 1.91 ± 4.1%, p ≤ 0.0001) (Fig. 5A). Similarly, ICOS expression correlated with IL-10 production in recently in vitro-activated CD4$^{+}$ T cells from pLN and pancreas of prediabetic NOD.Foxp3GFP mice. More specifically, a substantial proportion of ICOS-expressing CD4$^{+}$ T cells produced IL-10 (4.8 ± 1.8%) (Fig. 5B, left panel), and a striking proportion of Foxp3GFP$^{+}$ Tregs secreted IL-10 upon in vitro polyclonal stimulation (47.2 ± 6.4%) (Fig. 5B, right panel). The frequency of IL-10–secreting Foxp3$^{+}$ Tregs is significantly greater in the pancreas than in pLN (47.2 ± 6.35 versus 3.6 ± 0.5, p ≤ 0.0001), where little IL-10 production is detected (Fig. 5B, right panel). Surprisingly, the in vitro suppressive activity of ICOS$^{+}$ or ICOS$^{-}$ Treg subsets was not affected by IL-10 neutralization suggesting that redundant effector mechanisms of Treg suppression operate in vitro (data not shown).

To assess directly the requirement for ICOS expression in the production of IL-10 by Foxp3$^{+}$ Tregs, we examined the potential for IL-10 secretion in BDC2.5 ICOS$^{+/−}$ mice. To this end, WT or ICOS$^{+/−}$ BDC2.5 CD4$^{+}$ T cells were transferred into NOD.TCRα$^{−/−}$ recipients and the frequency of IL-10–producing Foxp3$^{+}$ Treg assessed in pLN and pancreas 8 d posttransfer. We observed that the most significant IL-10 production by Tregs occurs directly in the pancreas compared with that in the draining LN (Fig. 5C). Furthermore, we observe that the frequency of IL-10–producing Foxp3$^{+}$ Tregs is dramatically reduced in the pLN, and particularly in the pancreas, of ICOS$^{+/−}$ mice compared with that in WT mice (16.7 ± 7.7 versus 3.0 ± 0.7, p ≤ 0.002) (Fig. 5C). Overall, our results show that ICOS expression is required for IL-10 production by Tregs and likely drives this effector differentiation in the pancreas.

**Loss in ICOS expression and IL-10 production in Foxp3$^{+}$ Tregs coincides with T1D progression**

We and others have previously shown that Foxp3$^{+}$ Treg function wanes with TID progression, in turn enabling the diabetogenic T cells to escape regulation and promote destructive infiltration of islets (8, 9, 11–13, 35, 36). We then explored the possibility that breakdown in self-tolerance may be attributed to an age-related decline in ICOS expression on Tregs, at checkpoints known to demarcate the early (≤4 wk) from late (≥12 wk) stages of insulitis in the BDC2.5 model. We observed a remarkable decline in the cellular frequency of ICOS-expressing Foxp3$^{+}$ Tregs within the pancreas (68.6 ± 1.4 versus 47.4 ± 1.8, p ≤ 0.0008) (Fig. 6A, right panel), in stark contrast to the pLN. Similarly, ICOS expression on intrapancreatic Foxp3$^{+}$ Tregs is significantly reduced with age (144.3 ± 11.9 versus 50.7 ± 17.6, p ≤ 0.003) (Fig. 6A, left panel), suggesting that the suppressive potential of Tregs correlates with ICOS-mediated costimulation in Tregs. Moreover, this reduction in the cellular frequency of ICOS$^{+}$Foxp3$^{+}$ Tregs was directly related to the loss in expansion (Ki-67 expression) of ICOS-expressing Foxp3$^{+}$ Tregs directly within the pancreas of prediabetic mice (50.2 ± 3.8 versus 25.0 ± 2.6, p ≤ 0.003) (Fig. 6B) with time, a trend not observed in pLN (10.4 ± 0.3 versus 7.9 ± 0.7, NS) (Fig. 6B).

We and others have previously shown that TID progression in NOD mice correlates with a drastic reduction of intrapancreatic Treg numbers (11, 13). We then wondered whether TID onset...
could be attributed to a loss of ICOS expression on Foxp3+ Tregs. To this end, CD4+ T cells from BDC2.5.Foxp3GFP mice were transferred into NOD.TCRα2/2 recipients, and the cellular frequency of ICOS+Foxp3+ Tregs in the pLN and pancreas was analyzed relative to T1D onset. Notably, the expression level of ICOS as well as the frequency of ICOS+Foxp3+ Tregs sustained a drastic reduction as recipient mice progressed from prediabetes to overt T1D (1060 ± 92.8 versus 362.3 ± 68.6, p < 0.0001) (Fig. 6C). Thus, we show that T1D progression correlates with a loss of ICOS expression on Foxp3+ Tregs, particularly in the pancreas, indicating a loss of Treg suppression throughout T1D progression.

Herman et al. (21) showed that IL-10 and ICOS mRNA were highly expressed by pancreatic CD25+CD69+ Tregs relative to their draining LN counterparts. As pancreatic ICOS+ Foxp3+ Tregs possess the capacity to secrete high amounts of IL-10, we then assessed whether the loss in ICOS expression with T1D progression dampened the capacity of Tregs to produce IL-10 in our BDC2.5 transfer model (34). To this end, CD4+ T cells from pLN and pancreas of prediabetic and diabetic mice were reactivated ex vivo, and the secretion of IL-10 was assessed by

![FIGURE 5. Intra-islet ICOS+ Foxp3+ Tregs preferentially secrete IL-10. A, FAC-sorted pure ICOS+ and ICOS+ Foxp3+GFP T cells were isolated from pooled LN and spleen of 3- to 4-wk-old BDC2.5GFP reporter mice and then activated in the presence of NOD BMDCs at a 4:1 ratio and BDC2.5 mimotope (100 ng/ml) for 72 h. The frequency of IL-10-producing Foxp3+ Tregs, relative to ICOS expression, was assessed by flow cytometry (left panel). The proportion of IL-10–producing Foxp3+ Tregs among ICOS+ and ICOS− subsets are shown (right panel). B, Cell suspensions from pLN and pancreas of 4-wk-old NOD.Foxp3GFP reporter mice were reactivated in vitro for 4 h with PMA/ionomycin, and the frequency of IL-10–producing Foxp3+ Tregs, relative to ICOS expression, was determined by FACS (left panel). The proportion of IL-10–producing ICOS−Foxp3+ Tregs in pLN and pancreas are shown (right panel). C, NOD.TCRα2/2 mice were transferred with CD4+ T cells (1 × 106) isolated from pooled LN and spleen of 3- to 4-wk-old WT (Thy1.1+) or ICOS−/− (Thy1.2+) BDC2.5 mice. Eight days posttransfer, the frequency of IL-10–secreting Foxp3+ Tregs in each donor group was determined by FACS in pancreas and pLN, as in B. Data are representative of three separate experiments. Results represent the means ± SD.](http://www.jimmunol.org/)

![FIGURE 6. Temporal loss in ICOS expression and IL-10 production in Foxp3+ Tregs coincides with T1D onset and progression. A, Shown are the levels of ICOS expression on Foxp3+ Tregs (left panel) and proportions of ICOS+Foxp3+ Tregs (right panel) in pLN and pancreas of 3- to 4-wk-old and 12-wk-old BDC2.5 mice as analyzed by FACS. B, The extent (left panel) and proportion (right panel) of proliferating (based on Ki-67 expression) ICOS+Foxp3+ Tregs in pancreas of 3- to 4-wk-old and 12-wk-old BDC2.5 mice are shown. C, NOD.TCRα2/2 recipient mice were transferred i.v. with CD4+ T cells (1 × 106) isolated from pooled LN and spleen of 3- to 4-wk-old BDC2.5.Foxp3GFP reporter mice, and T1D onset was monitored as in Fig. 3. Fourteen to twenty-one days posttransfer, Foxp3+ and Foxp3− CD4+ T cells from NOD.TCRα−/− recipient mice were restimulated ex vivo with PMA/ionomycin, and the frequency of IL-10–producing Foxp3+ Tregs, relative to ICOS expression, was assessed in pLN and pancreas of prediabetic and diabetic mice (top panel). A representative dot plot of IL-10 production in ICOS+CD4+ T cells in the pancreas of prediabetic and diabetic mice is shown (top panel). ICOS expression (MFI, left y axis: A, pLN; △, pancreas) relative to the frequency of ICOS− Foxp3+ Tregs (right y axis: ■, pLN; □, pancreas) in prediabetic and diabetic mice (bottom panel). Results are representative of three independent experiments, (n = 7 mice/group). Results represent the means ± SD.](http://www.jimmunol.org/)
flow cytometry. Notably, the marked drop in the expression level of ICOS and frequency of ICOS-expressing Foxp3+ Tregs within the pancreas of diabetic mice correlated with a 3-fold decline in their capacity to produce IL-10 (21.5 ± 4.7 versus 5.8 ± 1.3, p ≤ 0.03) (Fig. 6C). Taken together, these data show that ICOS drives the differentiation of IL-10–producing Tregs particularly in the pancreas of prediabetic mice and that T1D progression correlates with a loss of Foxp3+ICOS+ Tregs within the pancreas.

**IL-2 preferentially supports the fitness and survival of ICOS-expressing Foxp3+ Tregs**

IL-2 is essential for the survival and function of Foxp3+ Tregs within islets, and a local deficiency in IL-2 was shown to render Tregs unfit and functionally defective, in turn provoking a Treg/Teff imbalance in situ (13). This is similar to earlier studies showing that T cells from prediabetic NOD mice become hypопroliferative and poor IL-2 producers at the onset of insulitis (3). Therefore, we examined whether the decline in the ICOS+ Treg pool, coincident with a lack of suppression of these cells and T1D progression, is associated with altered IL-2 levels. We first assessed IL-2Ra (CD25) expression in ICOS+ and ICOS- Treg subsets in response to IL-2 in vitro. To this end, ICOS+ and ICOS- Tregs from BDC2.5Foxp3+ BDC2.5 reporter mice were activated in vitro with APCs and mitomecto in the presence or absence of IL-2. We observe that ICOS+ Tregs are more responsive to IL-2 compared with ICOS- Tregs, as this resulted in a 28-fold increase in CD25 expression in ICOS+ Tregs and only a 10-fold increase in ICOS- Tregs in response to IL-2 compared with nontreated conditions (Fig. 7A, left panel). To confirm that ICOS+ Tregs preferentially respond to IL-2, STAT5 phosphorylation (p-STAT5) was assayed as an indicator of activation of the IL-2 signaling pathway. Our results show that administration of IL-2 leads to a significantly higher proportion (83.3 ± 2.6% versus 44.0 ± 1.8%, p ≤ 0.002) and MFI (1443 ± 19.1 versus 779 ± 4.2, p ≤ 0.008) of p-STAT5-positive ICOS+ Tregs compared with ICOS- Tregs (Fig. 7A, middle and right panels).

IL-2–mediated STAT5 phosphorylation is essential for survival of Foxp3+ Tregs (37–39). As ICOS+ Tregs are more sensitive to differential IL-2 levels, we then examined whether they also differed in their survival potential. We observe that ICOS+ Tregs, compared with ICOS- Tregs, are more susceptible to death by IL-2 withdrawal as indicated by the increased frequency of apoptotic cells in the absence of IL-2 compared with untreated cells (% annexin V+ PI+ cells; 90.3 ± 4.6 versus 62.3 ± 1.9, p ≤ 0.0003) (Fig. 7B, left panel). IL-2 treatment rescues ICOS+ Tregs from apoptosis, whereas ICOS- Tregs remain insensitive to IL-2 (45.4 ± 1.8 versus 39.0 ± 0.9, p ≤ 0.0002) (Fig. 7B, left panel). The IL-2–driven survival of ICOS+ Tregs was also confirmed by the upregulation of Bcl-2 expression in ICOS+ Tregs (14477 ± 7.5 versus 657 ± 4.6, p ≤ 0.008) in response to IL-2 in contrast to ICOS- Tregs whose Bcl-2 expression remained unchanged irrespective of IL-2 treatment (605 ± 13.2 versus 702 ± 6.8, NS) (Fig. 7B, middle and right panels). Overall, ICOS+ Tregs, in contrast to ICOS- Tregs, are more dependent on IL-2 signals, which bolster CD25 expression and prevent apoptosis in ICOS- Tregs. Notably, WT and ICOS-/- BDC2.5 Tregs do not differ in their intrinsic IL-2 response after activation as measured by p-STAT5 levels (data not shown); rather, the ICOS+ Tregs, compared with ICOS- Tregs, have different IL-2 requirements to maintain their functional fitness. Therefore, ICOS expression is not required for mediating IL-2 responses in ICOS+ Tregs, but rather delineates an IL-2–responsive Foxp3+ Treg subset in the pancreatic microenvironment.

II2 protective allelic variants or prophylactic IL-2 therapy restores ICOS expression in Tregs in BDC2.5 mice

A local IL-2 deficiency in islets is one of the underlying mechanisms in the age-related waning of Treg functions and loss of self-tolerance in NOD mice (13, 14, 24). Consistently, we have previously shown that Tregs within the pancreatic lesion of WT BDC2.5 mice exhibited reduced expansion, survival, and function, defects readily corrected by prophylactic IL-2 therapy or II2 allelic variation in NOD mice (13). Thus, we hypothesized that IL-2 promotes ICOS expression in vivo and sought to determine whether II2 allelic variants of T1D-protected congenic BDC2.5Idld3 mice could restore ICOS expression on Tregs and the enhanced frequency of ICOS Foxp3+ Tregs in situ. We observed an increase in the proportion of ICOS Foxp3+ Tregs (86.4 ± 1.7 versus 68.6 ± 1.4, p = 0.001) (Fig. 7C, right panel) and extent of ICOS expression (MFI) (282.3 ± 54.0 versus 144.3 ± 11.9, p ≤ 0.04) (Fig. 7C, left and middle panels) on Foxp3+ Tregs in the pancreas of 4-wk-old BDC2.5Idld3 mice relative to age-matched WT mice. Despite the substantial drop in the frequency of ICOS-expressing Foxp3+ Tregs by 12 wk of age in prediabetic BDC2.5Idld3 mice, the frequency was nonetheless significantly greater than that in WT mice (86.37 ± 1.7 versus 72.3 ± 0.4, p ≤ 0.005) (Fig. 7C, left panel). Moreover, the enhanced proportion of ICOS Foxp3+ Tregs coincided with a 2-fold enhancement in the fraction of actively proliferating ICOS-expressing Foxp3+ Tregs in 4-wk-old BDC2.5Idld3 mice relative to WT mice (39.1 ± 5.7% versus 24.4 ± 4.5%, p ≤ 0.01) (data not shown). This significant difference is maintained at later time points with disease progression (22.3 ± 2.8% versus 14.7 ± 3.4%, p ≤ 0.04) (data not shown). Overall, II2 allelic variation, known to increase IL-2 production in Tefs, also increases ICOS expression on Foxp3+ Tregs within the target organ, in turn, promoting their functions and T1D protection.

To confirm whether IL-2 deficiency is an underlying cause of the temporal loss of ICOS expression observed in Tregs within the pancreas (Fig. 5), a prophylactic IL-2 therapeutic regimen, known to promote T1D protection, was initiated in prediabetic BDC2.5 mice. In stark contrast to draining pLN, we observed a significant increase in the proportion of Foxp3+ Tregs within the pancreatic infiltrate of mice receiving IL-2 relative to PBS controls (13.3 ± 3.1% versus 9.4 ± 1.9%, p ≤ 0.03) (Fig. 7D, left panel). The proportion of ICOS Foxp3+ Tregs within the CD4+ T cell infiltrate of the pancreas was also enhanced in IL-2–treated BDC2.5 mice relative to PBS-treated controls (87.2 ± 4.3 versus 66.3 ± 3.8, p ≤ 0.0095), comparable with levels observed in BDC2.5Idld3 mice (87.2 ± 4.3 versus 84.5 ± 1.2) (Fig. 7D, right panel). IL-2 therapy also drives the expression of CD25 on Foxp3+ Tregs as the proportion of Foxp3+CD25ICOS+ was enhanced to levels observed in BDC2.5Idld3 mice in IL-2–treated BDC2.5 mice (87.2 ± 4.3 versus 84.5 ± 1.2) (Fig. 7D, right panel). In addition, our results show that the inability of ICOS+/- Tregs to suppress T1D in our T cell transfer model could not be reversed by prophylactic administration of IL-2, a condition that only partially bolsters Foxp3+ Tregs of ICOS+/- origin in contrast to WT Tregs (data not shown). Our data show that protective II2 allelic variants or low-dose IL-2 treatment restored ICOS and CD25 expression in intrapancreatic Foxp3+ Tregs, both conditions promoting T1D protection (13, 14). Overall, this suggests that ICOS expression by Foxp3+ Tregs plays an important role in IL-2–mediated rescue of Foxp3+ Tregs and T1D protection. Moreover, our results are reminiscent of those of Grinberg-Bleyer et al. (24), who demonstrated that IL-2 therapy can even reverse established T1D in NOD mice via a local effect on pancreatic
FIGURE 7. IL-2 is essential for ICOS expression in Foxp3+ Tregs and drives their fitness and survival. A. FACS-purified ICOS+ or ICOS− Foxp3+ Tregs (1 × 10^5) from pooled LN and spleen of 3- to 4-wk-old BDC2.5.Foxp3GFP reporter mice were stimulated with BDC2.5 mimetope (40 ng/ml) and irradiated spleen cells (4 × 10^5) in the presence or absence of IL-2 (5 μg/ml) for 36–72 h. The frequency of IL-2–responsive ICOS+ and ICOS− Foxp3+ Tregs was assessed by measuring CD25 expression in ICOS+ and ICOS− Treg subsets in unstimulated and IL-2–treated conditions demonstrated in histogram plots (left panel). The frequency of IL-2–responsive ICOS+ and ICOS− Foxp3+ Tregs was assessed by measuring p-STAT5 by flow cytometry. A representative histogram plot (middle panel) and MFI values (right panel) for p-STAT5 are shown. MFI values are expressed as ΔMFI of p-STAT5 in presence or absence of IL-2. B. The frequency of apoptotic cells (annexin V+/PI+) in ICOS+ and ICOS− Foxp3+ Tregs in the presence or absence of IL-2 was assessed by flow cytometry. Representative histogram plots of Bcl-2 expression in ICOS+ and ICOS− Tregs (middle panel) and MFI values (right panel) are shown. MFI values are expressed as ΔMFI of Bcl-2 expression in the presence or absence of IL-2. Each experiment was repeated three times with 10–15 mice per experiment. Results represent the mean ± SD. C. The level of ICOS expression (MFI) (left and middle panels) on Foxp3+ Tregs and the frequency (right panel) of ICOS+Foxp3+ Tregs in the presence of IL-2 (indicated by Bcl-2 expression) was assessed by flow cytometry. Representative histogram plots of ICOS+ and ICOS− Tregs in the presence or absence of IL-2 (indicated by Bcl-2 expression) were shown. MFI values are expressed as ΔMFI of ICOS expression in ICOS+ and ICOS− Tregs (middle panel) and MFI values (right panel) are shown. MFI values are expressed as ΔMFI of ICOS expression in the presence or absence of IL-2. Each experiment was repeated three times with 10–15 mice per experiment. Results represent the mean ± SD. D. Three- to four-week-old female BDC2.5 mice were injected daily with IL-2 (25,000 IU) for 5 consecutive days. On day 7 after the initial injection, the proportion of Foxp3+ Tregs within CD4+ T cells (left panel), the proportion of CD25-expressing Foxp3+ Tregs (middle panel), and the proportion of ICOS+ Foxp3+ Tregs (right panel) in pLN and pancreas was compared between PBS-treated and IL-2–treated BDC2.5 mice, as well as to BDC2.5Idd3 mice. Data are representative of at least three separate experiments (n = 5 mice/group). Results represent the mean ± SD.
Tregs by upregulating the expression of genes involved in Treg function and survival such as Bcl-2, CD25, Foxp3, and ICOS.

**Discussion**

A progressive loss in Foxp3+ Treg function in pancreatic sites unleashes the pathogenic potential of islet-reactive Teffs, consequently leading to overt T1D in NOD mice (8, 11, 33, 40–42). Foxp3+ Treg development and function are heavily dependent on costimulation, as shown by the exacerbated T1D onset in CD28−/− or B7−/− NOD mice (16, 17). Recently, ICOS has emerged as another critical costimulatory molecule in various autoimmune disorders (20, 43, 44). Genetic ablation or blockade of ICOS at various time points during disease progression yields divergent clinical outcomes, suggesting that ICOS may operate via cell type- or context-dependent mechanisms (20, 45). ICOS mRNA is highly expressed in pLN of T1D-protected BDC2.5 mice, and icos gene deficiency or ICOS blockade in neonatal BDC2.5 mice precipitates T1D (21, 22). Whereas ICOS−/− NOD mice are T1D resistant, ICOS blockade in NOD neonates resulted in T1D exacerbation (21, 23). However, it is unknown if ICOS differentially regulates Foxp3+ Teff and Foxp3+ Treg subsets or operates in different locations or phases of an anti-islet immune response (21, 23). These findings prompted us to evaluate the functional impact of differential ICOS activity in Teff and Treg subsets throughout T1D progression.

In this study, we make the formal demonstration that ICOS is a key costimulatory pathway that restrains islet autoimmunity by specifically affecting the homeostasis and function of Foxp3+ Tregs in prediabetic islets of NOD mice. We show that ICOS is a marker that discriminates effector Foxp3+ Teffs from Foxp3+ Tregs and specifically designates a subset of Tregs occupying islets of prediabetic mice. We also show that the intra-islet ICOS-expressing Foxp3+ Treg subset of neonatal BDC2.5 mice, in contrast to conventional Foxp3+ Tregs, is endowed with an increased potential to expand in situ, acquire an IL-10–secreting phenotype, and mediate greater suppressive activity in vitro and in vivo. We further show that the ICOS+ Treg subset, in contrast to its ICOS− counterparts, is more sensitive to IL-2–mediated survival, and the temporal loss in ICOS+ Tregs is readily corrected by IL-2 therapy or protective Il2 gene variation. ICOS sustains the stability of Foxp3+ Treg functions in prediabetic islets, and alterations in the ICOS pathway by ICOS deficiency or mAb-mediated blockade of ICOS in BDC2.5 mice specifically cripple Foxp3+ Treg fitness and function and abrogate T1D protection. Thus, ICOS maintains the homeostasis and functional stability of Foxp3+ Tregs in prediabetic islets.

A local IL-2 deficiency was shown to compromise Foxp3+ Treg survival and function within islets and exacerbate T1D progression, a defect that is readily restored by the protective Il2 allelic variants or IL-2 therapy in prediabetic or overtly diabetic NOD mice (11–14, 24). Several lines of evidence support a model whereby a positive feedback IL-2/ICOS loop stabilizes Treg survival and function in the inflamed pancreatic milieu (25). First, Il2 allelic variation in T1D-resistant BDC2.5Il2d3 mice produces more potent Tregs expressing higher ICOS levels than those of WT Tregs. Second, low-dose IL-2 therapy in BDC2.5 mice at the time of insulitis restored ICOS levels on intra-islet Foxp3+ Tregs comparable with those of BDC2.5Il2d3 mice. Third, we show that ICOS+ Tregs, in contrast to ICOS− Tregs, are more responsive to IL-2 in terms of STAT5 phosphorylation and activation, a signaling pathway essential for their survival, fitness, and functional stability. IL-2–mediated activation of the STAT5 pathway results in the upregulation of ICOS surface expression and increased foxp3 and cd25 gene expression (data not shown), which in turn stabilizes the suppressive Treg phenotype and prevents T1D. Moreover, when IL-2 is limiting, ICOS+ Tregs are highly susceptible to apoptosis, and IL-2 readily rescues them from apoptosis by upregulating Bcl-2, features not observed in the ICOS− Treg subset. These data show that ICOS+ Tregs are more dependent on IL-2 to maintain their functional fitness. Thus, a limited bioavailability of IL-2 in pancreatic environments may compromise ICOS-mediated stabilization Foxp3+ Treg function in islet autoimmunity.

The role of ICOS to instruct T cells to produce IL-10 has been documented (18). In a mouse model of asthma, the development of Ag-induced IL-10–producing Tregs and consequential suppression of allergen-induced airway hyperreactivity was dependent on an intact ICOS–ICOS-ligand pathway (46). Moreover, IL-10 has been shown to be immunoprotective in T1D in some contexts (47). However, the potential for IL-10 production by Tregs has not been as extensively examined as in other models where such production plays a key modulatory role in disease. In this study, we report that IL-10 production by Foxp3+ Tregs is restricted within the ICOS+ Foxp3+ Treg subset in contrast to ICOS− Foxp3+ Tregs or Foxp3− Teffs. The greater propensity for ICOS+Foxp3+ Tregs to cycle in inflamed pancreatic sites, in conjunction with their dependence on IL-2, may promote the differentiation of the IL-10–secreting phenotype in islet-reactive Foxp3+ Tregs from recently activated Foxp3+ Tregs in pancreatic sites. Moreover, the loss of ICOS expression and consequential IL-10 production by Ag-specific Foxp3+ Tregs with T1D progression indicates that ICOS might trigger IL-10 production by Foxp3+ Tregs. More importantly, the differentiation of IL-10–producing Foxp3+ Tregs in islets is impaired in ICOS−/− mice where Tregs fail to produce significant levels of IL-10 in the pancreatic sites compared with WT Tregs. Diminished expression of ICOS in Tregs of recently diagnosed human T1D patients supports the link between ICOS costimulation and the potency of the Foxp3+ Treg compartment (32).

Our data do not exclude the possibility that the defective Treg function observed in ICOS−/− mice may reflect a role for ICOS in imprinting such function during Treg development. Whether ICOS+ Tregs are generated in the thymus or from ICOS− Tregs in the periphery is unknown. We show that ICOS− Tregs are readily able to upregulate ICOS upon TCR activation in vitro, or in response to lymphopenia in vivo. Notably, mere induction of ICOS expression does not enable these cells to acquire the same functional features of ex vivo ICOS+ Tregs. This suggests that the thymus might not only play a critical role in the selection of Foxp3+ Tregs but also has the ability to imprint different Treg subsets with different functional potentials in the periphery. Alternatively, the important accumulation of ICOS+ Tregs in the pancreas may indicate that the inflammatory signals derived from the target organ can influence the expansion and recruitment of these cells. Consistently, Herman et al. (21) demonstrated that the gene expression profile of islet-infiltrating Tregs was drastically different from that of Tregs in pLN, suggesting that the inflammatory environment of the target tissue directs unique transcriptional programs in Tregs that may be related to their mechanism of action in situ.

Overall, our studies point to ICOS as an essential signal for the control of the pool size and instruction of the Treg suppressive phenotype within the islet microenvironment. Our findings extend our current understanding of the role of ICOS in T1D pathogenesis and suggest that IL-2 insufficiency may provoke a temporal loss in ICOS expression, which correlates with a functional waning of Tregs and breakdown in self-tolerance. As the icos gene may be functionally polymorphic in human T1D and contribute to T1D risk, it is tempting to speculate that such genetic variation may be affecting Treg-dominant mechanisms of islet tolerance (48). The elucidation of the mechanisms involved in maintaining...
self-tolerance will add to our current understanding and therapy of autoimmune disorders such as T1D.

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