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Impact of Protective IL-2 Allelic Variants on CD4⁺Foxp3⁺ Regulatory T Cell Function In Situ and Resistance to Autoimmune Diabetes in NOD Mice

Evrildiki Sgouroudis, Alexandre Albanese, and Ciriaco A. Piccirillo

Type I diabetes (T1D) is a T cell-dependent autoimmune disease resulting in the destruction of the insulin-producing β cells of Langerhans in the pancreas, leading to insulin deficiency (1, 2). Studies in NOD mice show that the lag time between the establishment of insulitis and overt clinical T1D onset may result from a progressive loss of immunoregulatory mechanisms, which include naturally occurring CD4⁺ regulatory T (nTreg) cells (3–6). CD4⁺ nTreg cells, constitutively expressing CD25 and the Foxp3 transcription factor (7–9), represent a major mechanism of peripheral self-tolerance, as their functional abrogation increases immunity to tumors, grafts, and pathogens and induces multispecific autoimmunity (10). Defects in CD4⁺ Foxp3⁺ nTreg cell development or function promote T1D susceptibility (11, 12) and have been implicated as a central control point in T1D progression.

T1D susceptibility is inherited through multiple insulin-dependent diabetes (Idd) genes. NOD.B6 Idd3 congenic mice, introgressed with an Idd3 allele from T1D-resistant C57BL/6 mice (Idd3B6), show a marked resistance to T1D compared with control NOD mice. The protective function of the Idd3 locus is confined to the I2 gene, whose expression is critical for naturally occurring CD4⁺ Foxp3⁺ regulatory T (nTreg) cell development and function. In this study, we asked whether Idd3B6 protective alleles in the NOD mouse model confer T1D resistance by promoting the cellular frequency, function, or homeostasis of nTreg cells in vivo. We show that resistance to T1D in NOD.B6 Idd3 congenic mice correlates with increased levels of IL-2 mRNA and protein production in Ag-activated diabetogenic CD4⁺ T cells. We also observe that protective I2 allele variants (Idd3B6 resistance allele) also favor the expansion and suppressive functions of CD4⁺Foxp3⁺ nTreg cells in vitro, as well as restrain the proliferation, IL-17 production, and pathogenicity of diabetogenic CD4⁺ T cells in vivo more efficiently than control do nTreg cells. Lastly, the resistance to T1D in Idd3 congenic mice does not correlate with an augmented systemic frequency of CD4⁺Foxp3⁺ nTreg cells but more so with the ability of protective I2 allele variants to promote the expansion of CD4⁺Foxp3⁺ nTreg cells directly in the target organ undergoing autoimmune attack. Thus, protective, I2 allele variants impinge the development of organ-specific autoimmunity by bolstering the IL-2 producing capacity of self-reactive CD4⁺ T cells and, in turn, favor the function and homeostasis of CD4⁺Foxp3⁺ nTreg cells in vivo. The Journal of Immunology, 2008, 181: 6283–6292.
mice have reduced T cell proliferative and IL-2 production capabilities, hallmark features, which coincide with a skewing toward pathogenic, β-cell-specific Th1 cell effector function (27). Recently, we have shown that Foxp3+ nTreg cell function declines with age in NOD mice, despite stable cellular frequencies of Foxp3+ nTreg cells, and that a possible loss in nTreg cell expansion in inflammatory sites may perturb the equilibrium between effector and nTreg cells within pancreatic sites, and amplifies local immune diabetogenic responses (12). Interestingly, Tang et al. clearly demonstrated that administration of low-dose IL-2 promoted Treg cell survival and protected NOD mice from developing T1D (28).

Presently, there is limited understanding in the regulation of T1D progression. Defining the mechanisms underlying the protective effects of T1D susceptibility gene variants, such as Idd3, is critical to understand how genetic variation may impinge natural checkpoints in T1D progression. Herein, we hypothesized that the protective IL2 allele variants (Idd3 locus) confer T1D protection by supporting CD4+ nTreg cell function and expansion in vivo. We show that Idd3 controls IL-2 secretion by islet-reactive CD4+ effector T (Teff) cells, favoring the cycling and function of CD4+ Foxp3+ nTreg cells locally in the pancreas, which in turn affords resistance to T1D. Thus, T1D genes may directly impinge the functional homeostasis of CD4+ Foxp3+ nTreg cells and, in turn, contribute to T1D susceptibility.

Materials and Methods

Mice

Mice strains were maintained in specific pathogen-free conditions at McGill University. NOD.TCRα−/− and BDC2.5 CD4+ transgenic mice were a gift from Christophe Benoist (Harvard University, Boston, MA). BDC2.5 TCR transgenic NOD mice contain a monoclonal, β-islet-specific CD4+ T cell repertoire (α/β), thus providing a rapid, synchronous system for the analysis of Ag-specific T cell responses in vivo. NOD.B6 Id3 congenic mice (line 1098) were obtained from Taconic Farms, and BDC.Id3 mice were generated by in-house breeding.

Cell purification

CD4+, CD4+CD25−, and CD4+CD25− T cell subsets were purified from lymph node (LN) or spleens using the autoMACS cell sorter (Miltenyi Biotec) or FACSAria flow cytometer (BD Biosciences), as described previously (29).

Flow cytometry

Stainings were done with the following fluorochrome-conjugated or biotinylated mAbs: anti-CD4 (clone RM5), anti-CD25 (clone PC61), and anti-Vβ4 (clone CTBV4) (eBioscience). Anti-Foxp3 (clone FJK-16s) and anti-Bcl-2 (clone 10C4) (eBioscience) intracellular staining was performed according to the manufacturer’s protocol. Stained cells were acquired on a FACSAria or FACSCalibur (BD Biosciences) and analyzed with FlowJo software.

Ki-67 proliferation analysis

Pancreata were digested with collagenase type IV (Invitrogen) at 37°C, extensively washed in HBSS, followed by a 10-min incubation at 37°C in nonenzymatic dissociation solution (Invitrogen). Cells were stained with anti-Ki-67 (clone B56) (BD Biosciences), anti-CD4, anti-Foxp3, and anti-CD25 (eBioscience).

Adoptive transfers

FACS-purified CD4+CD25− and CD4+CD25− T cells were transferred i.v., either alone or in combination, into NOD.TCRα−/−, NOD, or NOD.B6 Id3 recipient mice (1:10 nTreg:TCR ratio; 2–3×105/mouse), as previously described (12). In some cases, T cells were CFSE labeled (Invitrogen), and expansion of donor T cells was evaluated, as previously described (30).

In vitro proliferation assays

Proliferation assays were performed by culturing CD4+ T cells from NOD or BDC2.5 mice (5×105) in 96-well flat bottom microtiter plates with irradiated, spleen cells (1–2×105) and soluble anti-CD3 (1 μg/ml) or BDC2.5 minetope (RVRLWLVREME) (100 ng/ml) for 72 h at 37°C in 5% CO2. Cell cultures were pulsed with 1 μCi [3H]Tdr for the last 6–12 h and analyzed as previously shown (29). All experiments were repeated at least three times. Suppression assays were performed by culturing cell-sorted CD4+CD25− BDC.Id3 T cells with titrated numbers of highly purified BDC2.5 or BDC.Id3 CD4+CD25− Treg cells, irradiated APCs, and BDC2.5 minetope (10 ng/ml) for 72 h. Cell cultures were pulsed with 1 μCi [3H]Tdr for the last 6–12 h.

Intracellular cytokine production

Purified T cell subsets were stimulated 4–5 h with PMA and ionomycin. In some instances, T cells were isolated from the pancreatic or distal LN of recipient mice following adoptive transfer, and were activated ex vivo overnight with bone marrow-derived dendritic cells (BMDC) and BDC2.5 minetope, and treated with GolgiStop (BD Biosciences) for the last 2–3 h of culture. Intracellular cytokine staining (ICS) was performed using fluorochrome-conjugated anti-mouse mAb IL-2 (clone JES6-5H4), IFN-γ (clone XMG1.2), IL-17 (clone eBio1787) (eBioscience), or appropriate isotype controls (BD Biosciences), as previously shown (12).

Diagnosis of diabetes

Blood glycemia levels were determined every 2–3 days with Hemo-glykat est kits (Roche Diagnostics), and T1D was diagnosed at values >300 mg/dl.

RT-PCR

Analysis of IL-2 gene expression in resting and activated CD4+ T cell subsets was achieved by normalizing the IL-2 densitometric value with the intensity of the G3PDH amplicon for each sample, and reported as arbitrary IL-2/G3PDH ratios, as previously described (29).

Statistical analysis

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

Results

Resistance to the progression of T1D in NOD.B6 Id3 congenic mice correlates with increased production of IL-2 by autoreactive CD4+ T cells

We confirm that NOD.B6 Id3 mice have a delayed onset and incidence of T1D compared with wild-type (WT) NOD mice (15). Female NOD mice start to develop T1D by 14 wk of age and incidence reaches 85% by 28 wk. In contrast, only 10% of NOD.B6 Id3 female mice were diabetic by 28 wk of age, with the earliest onset occurring at 25 wk of age (Fig. 1A), a finding consistent with the T1D protection seen in BDC.Id3 mice (data not shown). We then assessed whether differences in the production of TNF-α and IFN-γ by CD4+ T1 cells, important inflammatory mediators in this model, correlated with T1D protection in BDC.Id3 mice. We show a significant, albeit modest, decrease in the percentage of CD4+Vβ4+IFN-γ+ T cells in peripheral LN (7.57 ± 0.30% vs 5.83 ± 0.26%; p = 0.001), pancreatic LN (pancLN, 8.52 ± 0.46% vs 7.14 ± 0.20%; p = 0.03), and in spleen (data not shown) of prediabetic BDC.Id3 mice compared with BDC2.5 control mice following Ag-specific stimulation (Fig. 1B). Moreover, we also observe a substantial decrease in the frequency of CD4+Vβ4+TNF-α+ T cells in peripheral LN (7.85 ± 0.97% vs 5.09 ± 0.17%; p = 0.008), panc LN (7.51 ± 0.43% vs 3.36 ± 0.6%; p = 0.002), and in spleen (data not shown) of prediabetic BDC.Id3 mice compared with BDC2.5 controls under similar stimulatory conditions (Fig. 1B). The T1D resistance in NOD.B6 Id3 mice also correlated with a significant reduction in CD4+ T cell infiltration in the pancreas compared with WT NOD mice (1.04 ± 0.3% vs 7.38 ± 1.9%; p = 0.004) (Fig. 1C), an observation also made in BDC.Id3 mice (data not shown). Thus, the T1D resistance in Id3 congenic mice correlates with a significant reduction in the accumulation of Teff cells, particularly Th1 cells in lymphoid tissues or pancreas.
As the II2 gene is the strongest and primary candidate for T1D protection in the Idd3B6 locus, and it has been shown that the II2 promoter from Idd3B6 alleles possesses some sequence variation compared with the susceptible NOD allele (31, 32), we wondered whether the Idd3B6D interval increased IL-2 gene transcription and protein production in activated CD4+ T cells of NOD.B6 Idd3 mice. To this end, CD4+ T eff cells from prediabetic NOD and NOD.B6 Idd3 mice were activated with plate-bound anti-CD3, and IL-2 expression was assessed by RT-PCR in resting or TCR-stimulated conditions (Fig. 1D). In contrast to unstimulated conditions, II2 expression levels in NOD.B6 Idd3 CD4+ T eff cells, albeit modestly greater than NOD T eff cells by 12 h of TCR engagement, were ~2–3-fold greater than their NOD CD4+ CD25+ T cell counterparts by 24–48 h post-TCR stimulation. This increased IL-2 expression in NOD.B6 Idd3 CD4+ T eff cells also correlated with their augmented proliferation compared with NOD controls at 72 h poststimulation (Fig. 1D and Fig. 2A). To determine whether increased IL-2 transcription correlated with increased IL-2 production in Idd3 congenic mice, CD4+ T eff cells from prediabetic NOD and NOD.B6 Idd3 or BDC2.5 and BDC.Idd3 mice were activated with PMA/ionomycin or BMDC in the presence of BDC2.5 mimotope, respectively, and IL-2 protein production was determined by FACS 24 h poststimulation. Our results show that activated T eff cells revealed an at least 2-fold increase in the fraction of cells producing IL-2 in Idd3 T eff cells compared with NOD control T eff cells without affecting the IL-2 mean fluorescence intensity in CD4+ T eff cells of either genotype, suggesting that Idd3B6 does not control the overall amount of IL-2 on a per cell basis (Fig. 1E). Thus, the protective Idd3B6 allele causes an increase in the frequency of activated CD4+ T eff cells producing IL-2, consistent with a recent study documenting similar increases in diabetogenic CD8+ T eff cells from NOD.B6 Idd3 mice (22).

Protective Idd3B6 alleles augment CD4+Foxp3+nTreg cell function in vitro

Given the critical role of IL-2 in CD4+Foxp3+nTreg cell functions, we then hypothesized that the increased production of IL-2 by activated NOD.B6 Idd3 T eff cells may potentiate nTreg cell suppressive function and restrain the proliferative capacity of responding CD4+ T eff cells. We first assessed the proliferation of CD4+ T eff cells from NOD and NOD.B6 Idd3 WT mice or BDC2.5 and BDC. Idd3 CD4+ TCR transgenic mice, whose monoclonal TCR repertoire is specific for an as of yet unknown pancreatic β-islet Ag.
activated CD4⁺ T cells from NOD and NOD.B6 Idd3 or BDC2.5 and BDC. Idd3 (Fig. 2B). However, activated CD4⁺ T cells from NOD.B6 Idd3 and BDC. Idd3 revealed a significantly increased proportion of CD4⁺Foxp3⁺ nTreg cells at 72 h postactivation when compared with NOD and BDC2.5 T cells, consistent with the reduced proliferation of CD4⁺ Teff cells (Fig. 2, A and B). Notably, the higher percentage of CD4⁺Foxp3⁺ nTreg cells within the activated CD4⁺ T cell pool from Idd3B6 congenic mice correlated with increased proliferation (11.4 ± 1.2 vs 7.7 ± 0.6; p ≤ 0.009) (Fig. 2C) and increased numbers (data not shown) of CD4⁺ Foxp3⁺ nTreg cells, suggesting that Idd3B6 congenic mice are intrinsically more potent in their function than are WT NOD controls.

We then performed in vitro suppression assays to directly assess whether enhanced CD4⁺CD25⁺ nTreg cell-mediated suppression is affected in BDC.Idd3 congenic mice. Our results show that CD4⁺CD25⁺ nTreg cells from BDC.Idd3 mice were more efficient than BDC2.5 controls in suppressing anti-CD3-induced T cell proliferation at all nTreg/responder T cell ratios examined, a finding consistent with CD4⁺CD25⁺ nTreg cells from BDC.B6 mice (Fig. 2D and data not shown). Overall, these findings demonstrate the ability of the protective Idd3 allele to favor the function of CD4⁺ Foxp3⁺ nTreg cells, which in turn dampen the proliferation of activated CD4⁺ Teff cells in vitro more efficiently than in their BDC2.5 counterparts.

Expansion of islet-reactive CD4⁺ T cells is dampened in NOD.B6 Idd3 mice

We sought to assess the impact of the protective Idd3B6 locus on diabetogenic CD4⁺ T effector cell activation and expansion in vivo. To this end, CD4⁺CD25⁻ T effector cells from BDC2.5 or BDC.Idd3 mice were CFSE labeled, adoptively transferred into NOD or NOD.B6 Idd3 recipients, and proliferation was monitored by CFSE dilution. BDC2.5 and BDC.Idd3 T effector cells proliferated and accumulated abundantly in the pancLN of NOD and NOD.B6 Idd3 recipient mice (Fig. 3, A and B), but not in other peripheral LN (data not shown), confirming that T cell priming was β-islet Ag specific. Our results also reveal a more significant reduction in the Ag-driven proliferation and total accumulation of both BDC2.5 and BDC.Idd3 T effector cells in the pancLN of NOD.B6 Idd3 mice compared with NOD controls (Fig. 3, A and B). Interestingly, BDC. Idd3 T effector cell Ag-induced proliferation was as efficient as that of BDC2.5 T effector cells in NOD recipient mice. This suggests that priming of diabetogenic T cells was not affected by the action of the Idd3B6 allele in donor T effector cells. Instead, suppression of islet-reactive CD4⁺ T effector cell priming is seemingly dependent on the presence of the protective Idd3B6 allele in recipient mice (Fig. 3, A and B). Thus, the protective Idd3B6 allele promotes a more suppressive environment, which impedes the activation and accumulation of Ag-specific, diabetogenic CD4⁺ T effector cells in vivo.

To exclude the possibility that DC from Idd3 congenic mice might be more tolerogenic in nature and may be, on their own, responsible for the suppressive environment in these mice, we activated BDC2.5 CD4⁺ T cells in vitro with CD11c⁺ MHC class II⁺ dendritic cells (DC) purified from draining pancLN or spleen of BDC2.5 or BDC.Idd3 mice, and in the presence of BDC2.5 mimotope. Our results show that splenic or pancLN DC from Idd3 congenic mice were as potent in inducing Ag-specific T cell proliferation as DC from NOD controls (Fig. 3C), suggesting that the suppressive environment conferred by the protective Idd3B6 locus is independent of more tolerogenic DC in Idd3 congenic mice.
The onset and severity of T1D (35, 36). We hypothesized that these important inflammatory mediators were suppressed by the presence of the protective Idd3<sup>B6</sup> allele. To this end, BDC2.5 or BDC. Idd3 CD4<sup>+</sup> T cells were injected into NOD or NOD.B6 Idd3 recipients, and 4 days post-transfer, distal and pannLN cells were harvested with mimotope-pulsed DC ev vivo and the production of IL-17 in T cells was assessed by ICS. Our results show that when BDC2.5 CD4<sup>+</sup> T cells were transferred into WT NOD recipients, a 6.5-fold enhancement in the frequency IL-17 producing CD4<sup>+</sup> T cells was observed (10.67 ± 4.2% vs 1.61 ± 0.5%; p ≤ 0.02) compared with BDC2.5 or BDC.Idd3 CD4<sup>+</sup> T cells transferred into NOD.B6 Idd3 recipient mice. This suggested that the presence of the Idd3<sup>B6</sup> locus in either donor T cells or recipient mice was sufficient to severely hamper the differentiation of IL-17 producing CD4<sup>+</sup> T cells (Fig. 4). Interestingly, IL-17 production was markedly hindered when the protective Idd3<sup>B6</sup> allele was present either exogenously within the donor cells or endogenously within the recipient animals (Fig. 4). These findings strongly suggest that the Idd3<sup>B6</sup> locus impacts the diabetogenic T cell pool by hindering its differentiation.

**The Idd3<sup>B6</sup> locus drives CD4<sup>+</sup>Foxp3<sup>+</sup> nTreg cell suppressive function and T1D protection**

We then sought to directly determine whether CD4<sup>+</sup>Foxp3<sup>+</sup> nTreg cells from Idd3<sup>B6</sup> congenic mice were intrinsically better inhibitors of diabetogenic T cells and T1D in vivo. To this end, we transferred CD4<sup>+</sup>CD25<sup>+</sup> T<sub>Teff</sub> cells from BDC2.5 or BDC.Idd3 mice into NOD.TCRα<sup>−/−</sup> recipients either alone or in combination with BDC2.5 or BDC.Idd3 CD4<sup>+</sup>CD25<sup>+</sup> nTreg cells at physiological 1:10 T<sub>Teff</sub>:nTreg cell ratios, and the onset of diabetes was monitored. Recipient mice transferred with BDC2.5 or BDC.Idd3 T<sub>Teff</sub> cells alone developed T1D simultaneously between days 11 and 14 and with similar incidence, suggesting that the Idd3<sup>B6</sup> allele did not affect the diabetogenic potential of T<sub>Teff</sub> cells in vivo (data not shown). Similarly, BDC2.5 and BDC.Idd3 T<sub>Teff</sub> cells accumulated with similar frequencies in the pancreas of recipient mice, further confirming that the Idd3<sup>B6</sup> allele does not directly impede the influx of islet-reactive CD4<sup>+</sup> T<sub>Teff</sub> cells in vivo (data not shown). Interestingly, 80% of the recipients receiving BDC2.5 T<sub>Teff</sub> and T<sub>nTreg</sub> cells developed diabetes by day 20 post-transfer, demonstrating that WT BDC2.5 T<sub>Teff</sub> cells are unable to maintain long-term self-tolerance (Fig. 5A). Intriguingly, BDC2.5 CD4<sup>+</sup>CD25<sup>+</sup> T<sub>nTreg</sub> cells were capable of significantly reducing T1D incidence (40%) when cotransferred with BDC.Idd3 T<sub>Teff</sub> cells, suggesting that the presence of the Idd3<sup>B6</sup> allele in islet-reactive CD4<sup>+</sup> T<sub>Teff</sub> cells is capable of potentiating the function of BDC2.5 CD4<sup>+</sup> T<sub>Teff</sub> cells in vivo. In stark contrast, NOD.TCRα<sup>−/−</sup> recipient mice receiving BDC.Idd3 CD4<sup>+</sup>CD25<sup>+</sup> T<sub>Teff</sub> cell populations, irrespective of the genotype of the T<sub>nTreg</sub> cell group, remained completely T1D-free for up to 20 days post-transfer, suggesting that BDC.Idd3 CD4<sup>+</sup>CD25<sup>+</sup> T<sub>nTreg</sub> cells are more efficient at controlling the onset of T1D (Fig. 5A).

Thus, these results show that Idd3<sup>B6</sup> allelic variants drive the development of intrinsically more potent CD4<sup>+</sup>Foxp3<sup>+</sup> nTreg cells, which, in turn, impact disease progression and resistance to T1D.

Studies in various mouse models of disease indicate that nTreg cells can localize in sites of inflammation to mediate their protective effect (30, 37–39). Given the increased suppression of T1D in mice receiving BDC.Idd3 nTreg cells, we then asked whether this protection was due to increased accumulation of CD4<sup>+</sup>Foxp3<sup>+</sup> nTreg cells in the pancreas. To this end, nondiabetic NOD. TCRα<sup>−/−</sup> mice receiving CD4<sup>+</sup> T<sub>Teff</sub> and nTreg cells from BDC2.5 or BDC.Idd3 donors were sacrificed on day 15 post-transfer, and the cellular frequency of nTreg and T<sub>Teff</sub> cells was analyzed in the pancreas and various lymphoid organs. Our results show that no significant differences are detected in the overall frequencies of

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** The expansion and accumulation of islet-reactive CD4<sup>+</sup> T cells is dampened in NOD.B6 Idd3 mice. CFSE-labeled CD4<sup>+</sup>CD25<sup>−</sup> T cells (3 × 10<sup>6</sup>) from BDC2.5 or BDC.Idd3 mice were adoptively transferred into NOD or NOD.B6 Idd3 recipients. The pannLN of recipient mice were harvested on day 4 post-transfer, and the proliferative capacity (A) and absolute numbers (B) of donor CD4<sup>+</sup>V<sup>4</sup> T cells were determined in recipient mice (n = 3–4 mice/group). Similar results were obtained in three independent experiments. Results represent the mean ± SD. *p < 0.02 difference between recipients receiving BDC2.5 T<sub>Teff</sub> cells or recipients receiving BDC.Idd3 T<sub>Teff</sub> cells. C. DC from draining pancreatic sites do not drive the Idd3-mediated effect on T<sub>Teff</sub> cell expansion. To examine the impact of DC on the proliferative capacity of CD4<sup>+</sup> T cells, CD11c<sup>+</sup>MHC II<sup>+</sup> DC were purified from draining pannLN and spleen of 3- to 4-wk-old BDC2.5 or BDC.Idd3 mice and plated at a 1:4 ratio with highly purified BDC2.5 CD4<sup>+</sup> T cells in the presence of BDC2.5 mimotope (100 ng/ml) for 72 h. Proliferation was assessed by thymidine incorporation. No significance (n.s.) was observed between groups.

The differentiation of diabetogenic, IL-17-producing CD4<sup>+</sup> T cells in pannLN is suppressed in NOD.B6 Idd3 congenic mice

Since the proliferative capacity of autoreactive CD4<sup>+</sup> T<sub>Teff</sub> cells was suppressed more efficiently in NOD.B6 Idd3 congenic mice than in control NOD mice, we wondered whether the differentiation of diabetogenic CD4<sup>+</sup> T cell pool would also be affected by protective Idd3 alleles. While inflammatory cytokines produced by Th1 cells and DC, like IFN-γ, IL-12, and TNF-α, are important mediators of β-islet destruction and play a pivotal role in the development of the insulitic lesions (34), recent studies show that IL-17-producing CD4<sup>+</sup> T (Th17) cells are important mediators of the pathogenesis in various autoimmune disorders (35, 36). Moreover, IL-17 mRNA transcripts have been shown to increase with the onset and severity of T1D (35, 36). We hypothesized that these
Recipient animals were collected and plated (1 recipient). Four days post-transfer, pancLN and distal mesenteric LN of re-
old donor mice were adoptively transferred to NOD or NOD.B6 Idd3 recipients. Days post-transfer, mice from each group were sacrificed, pancLN and pancreatic sites of mice receiving BDC.2.5 or BDC.Idd3 CD4+CD25+ nTreg cells and those receiving BDC.Idd3 Teff/BDC2.5 nTreg cells. B. Increased accumulation of CD4+Foxp3+ nTreg cells in PANritic sites of mice receiving BDC.2.5 or BDC.Idd3 nTreg cells. NOD.TCRα−/− recipient mice receiving BDC.2.5 Teff/BDC2.5 nTreg cells showed significantly higher frequencies of nTreg cells in the pancreas of recipient mice (n = 2–4 mice/group) were adoptively transferred as in (A), and on day 16 posttransfer, mice from each group were sacrificed, pancLN and pancreas were harvested, and the cellular frequency of islet-specific Foxp3+ nTreg cells within the CD4+Vβ4+ T cell compartment was determined by FACS. * p < 0.005 difference with control NOD.TCRα−/− mice receiving BDC.2.5 or BDC.Idd3 nTreg cells. C. Total frequency of islet-specific CD4+Vβ4+ T cells (left panel) and CD4+Vβ4+Foxp3+ nTreg cells (right panel) was determined in the pancLN and pancreas of 3- to 4-wk-old BDC2.5 and BDC.Idd3 mice (n = 10). Data are representative of the compilation of several independent experiments. Results represent the means ± SD. * p = 0.0005 difference with control BDC2.5 mice.

A significant greater frequency of intrapancreatic CD4+ cells transferred/genotype of recipient.

FIGURE 4. The differentiation of diabetogenic, IL-17-producing CD4+ T cells in pancreatic lymph nodes is suppressed in NOD.B6 Idd3 congenic mice. CFSE-labeled CD4+ T cells from BDC2.5 or BDC.Idd3 6- to 8-wk-
old donor mice were adoptively transferred to NOD or NOD.B6 Idd3 recipients. Days post-transfer, mice from each group were sacrificed, pancLN and pancreatic sites of mice receiving BDC.2.5 or BDC.Idd3 CD4+CD25+ nTreg cells and those receiving BDC.Idd3 Teff/BDC2.5 nTreg cells. B. Increased accumulation of CD4+Foxp3+ nTreg cells in pancreatic sites of mice receiving BDC.2.5 or BDC.Idd3 nTreg cells. NOD.TCRα−/− recipient mice receiving BDC.2.5 Teff/BDC2.5 nTreg cells showed significantly higher frequencies of nTreg cells in the pancreas of recipient mice (n = 2–4 mice/group) were adoptively transferred as in (A), and on day 16 posttransfer, mice from each group were sacrificed, pancLN and pancreas were harvested, and the cellular frequency of islet-specific Foxp3+ nTreg cells within the CD4+Vβ4+ T cell compartment was determined by FACS. * p < 0.005 difference with control NOD.TCRα−/− mice receiving BDC.2.5 or BDC.Idd3 nTreg cells. C. Total frequency of islet-specific CD4+Vβ4+ T cells (left panel) and CD4+Vβ4+Foxp3+ nTreg cells (right panel) was determined in the pancLN and pancreas of 3- to 4-wk-old BDC2.5 and BDC.Idd3 mice (n = 10). Data are representative of the compilation of several independent experiments. Results represent the means ± SD. * p = 0.0005 difference with control BDC2.5 mice.

FIGURE 5. The Idd3B6e locus potentiates CD4+Foxp3+ nTreg cell suppressive function and T1D protection in vivo. A. NOD.TCRα−/− recipient mice were adoptively transferred with FACS-sorted BDC.2.5 or BDC.Idd3 CD4+CD25+ Treg cells (5 × 10⁶) in the presence or absence (data not shown) of BDC.2.5 or BDC.Idd3 CD4+CD25+ (5 × 10⁴) nTreg cells from peripheral LN of 6- to 8-wk-old donor mice. Blood glucose levels in recipient mice (n = 7 recipients/group) were monitored for diabetes incidence every 48 h posttransfer. Results represent the means ± SD. p < 0.0001 difference between control NOD.TCRα−/− recipient mice receiving BDC.2.5 Teff/BDC2.5 nTreg cells and those receiving BDC.Idd3 Teff/BDC2.5 nTreg cells. p < 0.05 difference in diabetes onset between control NOD.TCRα−/− recipient mice receiving BDC.2.5 Teff/BDC2.5 nTreg cells and those receiving BDC.Idd3 Teff/BDC2.5 nTreg cells. B. Increased accumulation of CD4+Foxp3+ nTreg cells in pancreatic sites of mice receiving BDC.2.5 or BDC.Idd3 nTreg cells. NOD.TCRα−/− recipient mice (n = 2–4 mice/group) were adoptively transferred as in (A), and on day 16 posttransfer, mice from each group were sacrificed, pancLN and pancreas were harvested, and the cellular frequency of islet-specific Foxp3+ nTreg cells within the CD4+Vβ4+ T cell compartment was determined by FACS. * p < 0.01 and † p < 0.05 difference with control NOD.TCRα−/− mice receiving BDC.2.5 or BDC.Idd3 Treg cells along with BDC.2.5 Teff or those receiving BDC.2.5 or BDC.Idd3 Treg cells along with BDC.Idd3 Teff cells. C. Total frequency of islet-specific CD4+Vβ4+ T cells (left panel) and CD4+Vβ4+Foxp3+ nTreg cells (right panel) was determined in the pancLN and pancreas of 3- to 4-wk-old BDC2.5 and BDC.Idd3 mice (n = 10). Data are representative of the compilation of several independent experiments. Results represent the means ± SD. * p = 0.0005 difference with control BDC2.5 mice.

CD4+Vβ4+ T cells in the pancLN and pancreas of recipient mice regardless of T eff and nTreg cell origin, although frequencies of CD4+Foxp3+ T eff cells in the pancLN and pancreas of recipient mice were significantly reduced in the presence of BDC.Idd3 nTreg cells (data not shown). Strikingly, mice receiving BDC.Idd3 CD4+CD25+ nTreg cells showed increased frequencies of CD4+ Foxp3+ nTreg cells in the pancLN and pancreas compared with mice receiving BDC.2.5 CD4+Foxp3+ nTreg cells (Fig. 5B). Interestingly, a significantly greater frequency of intrapancreatic CD4+ Foxp3+ nTreg cells was observed in cotransfers with BDC.Idd3 T eff cells compared with BDC.2.5 T eff cells, indicating that Idd3B6e allelic variants in diabetogenic T cells on their own may not be sufficient to promote CD4+Foxp3+ nTreg cell activity and may act in a nTreg cell-intrinsic fashion (Fig. 5B). Thus, the decreased accumulation of CD4+Foxp3+ nTreg cells in the pancreas of BDC.2.5 mice may suggest that they may be impaired in their ability to delay disease progression due to reduced functional capacities in situ.

We next determined whether this preferential accumulation of BDC.Idd3 CD4+Foxp3+ nTreg cells in the pancreas also occurred in nonlymphopenic BDC.2.5 and BDC.Idd3 mice. While the percentage of CD4+Foxp3+ nTreg cells in nondraining lymphoid sites in BDC.2.5 and BDC.Idd3 mice did not differ, BDC.Idd3 mice show significantly higher frequencies of nTreg cells in the pancreas compared with BDC.2.5 mice (28.8 ± 11.1% vs 11.4 ± 3.9%; p <
Idd3 was that a lesser extent, in the pancLN (52.7% and the pancreas of prediabetic NOD and NOD.B6 sites. To this end, we examined by FACS various lymphoid organs apoptosis, consequently leading to their accumulation in pancreatic Idd3 and BDC mice (Fig. 6, left panel) or BDC2.5 and BDC.Idd3 (right panel) mice \((n = 3-5)\) were stained with CD4, Foxp3, and Bcl-2 and analyzed by FACS. Data are reported as the mean fluorescence intensity (MFI) values of individual animals.

Since the increased proportion of Treg cells in the pancreas of animals we hypothesized that an enhanced local expansion of Treg cells locally in the pancreas, correcting for the homeostatic "defect" in the BDC2.5, and consequently suppressing the function of diabetogenic T cells in situ. The Idd386 allele does not enhance resistance to apoptosis in \(T_{reg}\) cells

We show that TID protection in BDC.Idd3 mice correlates directly with an increased proportion of CD4\(^+\)Foxp3\(^+\) nTreg cells in the target organ of prediabetic mice. Recently, we showed that TID is not attributed to quantitative fluctuations in CD4\(^+\)Foxp3\(^+\) Treg cells but more so to a temporal loss in the capacity of CD4\(^+\)Foxp3\(^+\) Treg cells to expand in pancreatic sites, which in turn unleashes the diabetogenic potential of Teff cells (12). We hypothesized that Idd386 resistance alleles favor high frequencies of nTreg cells in the target organ, which suppress the accumulation of islet-specific CD4\(^+\) Eff cells and prevent the induction of T1D.

The Idd386 allele does not enhance resistance to apoptosis in \(T_{reg}\) cells

The Idd386 locus potentiates \(T_{reg}\) cell proliferation in vivo

Since the increased proportion of \(T_{reg}\) cells in the pancreas of protected BDC.Idd3 mice could not be attributed to prolonged survival, we hypothesized that an enhanced local expansion of \(T_{reg}\) cells could explain the protective phenotype observed in animals containing the Idd386 interval. To evaluate the impact of the Idd386 locus on \(T_{reg}\) cell expansion in draining pancLN, CFSE-labeled BDC2.5 or BDC.Idd3 CD4\(^+\) T cells were transferred into NOD or NOD.Idd3 recipients and proliferation was monitored. The greatest nTreg cell proliferation was observed when both the donor cells and recipient animals originated from Idd386 congenic mice (41.62 ± 10.1%), suggesting that both \(T_{reg}\) cell-intrinsic and -extrinsic factors cooperated to yield enhanced proliferative capacity (Fig. 7A). Interestingly, BDC.Idd3 CD4\(^+\)Foxp3\(^+\) Treg cells accumulated in greater numbers in draining pancreatic sites, irrespective of the genotype of the recipient animals, confirming that the Idd386 locus drives \(T_{reg}\) cell expansion and promotes their accumulation in pancLN (Fig. 7B). The presence of donor \(T_{reg}\) cells in this system did not impede the activation of the diabetogenic T cell pool, as \(T_{eff}\) cells from both genotypes exhibited similar proliferative profiles, irrespective of the origin of the recipient animal (data not shown). Interestingly, the accumulation of
CD4\(^+\)Foxp3\(^+\) nTreg cells were drastically reduced in NOD.B6 Idd3 recipients irrespective of the genotype of the donor T cells (Fig. 7C), demonstrating that the NOD.B6 Idd3 environment is tolerogenic and impedes the expansion of diabetogenic T eff cells. Thus, these data highlight the importance of the Idd3\(^{B6}\) locus in promoting Treg cell proliferation and restraining the expansion of diabetogenic T eff cells.

IL-2 allelic variants promote the cycling of CD4\(^+\)Foxp3\(^+\) nTreg cells directly in the pancreas

Since a greater proportion of Treg cells accumulated and proliferated in draining pancreatic sites of NOD.B6 Idd3 animals, we wondered whether the observed increased frequency of Idd3\(^+\) T reg cells within the target organ (Fig. 5C, right panel) was attributed to more efficient expansion in situ. To examine this possibility, we determined the cellular frequency of cycling CD4\(^+\)Foxp3\(^+\) T eff cells, as determined by the Ki-67 proliferation marker, in the spleen, nondraining mesenteric LN, pancLN, and pancreas of BDC2.5 and BDC.Idd3 mice. Our results show a marked decrease in the proportion of cycling CD4\(^+\)Foxp3\(^+\) T eff cells within the pancreas of BDC.Idd3 relative to WT BDC2.5 mice (17.4 ± 4.0% vs 28.0 ± 3.8%; \(p < 0.00001\)) (Fig. 8A), and similar cycling differences could not be detected in spleen and distal LN, suggesting that T1D protection in BDC.Idd3 mice correlates directly with the increased proportion of CD4\(^+\)Foxp3\(^+\) nTreg cells in the target organ. Additionally, although no significant differences were observed in non-draining and draining lymphoid sites between genotypes, the proportion of cycling nTreg cells was markedly enhanced within the pancreas of BDC.Idd3 mice relative to WT BDC2.5 mice, and correlated with the frequency of CD25-expressing cycling nTreg cells (13.4 ± 3.1% vs 6.5 ± 2.5%; \(p < 0.0001\)) (Fig. 8B). This suggests that an increase in IL-2 in the inflammatory milieu, generated by the diabetogenic T eff cell pool, drives the up-regulation of CD25, potentiating T reg cell suppressive functions, a finding consistent with the observed local expansion of CD4\(^+\)Foxp3\(^+\) nTreg cells within inflammatory sites seen in other mouse models (10, 37, 38, 41, 42). More importantly, the decline in cycling CD4\(^+\)Foxp3\(^+\) T eff cells in BDC.Idd3 mice correlates directly with an increased proportion of cycling CD4\(^+\)Foxp3\(^+\) nTreg cells (12.0 ± 0.8% vs 4.4 ± 1.9%; \(p = 1.1 \times 10^{-8}\)), suggesting that the proliferative potential of nTreg cells correlates directly with their functional potency, and is strongly indicative that nTreg cells are actively suppressing autoreactive CD4\(^+\)Foxp3\(^+\) T eff cells within the target organ (Fig. 8). Collectively, our data strongly suggest that a regulatory feedback loop initiated by IL-2-producing self-reactive Idd3\(^{B6}\) CD4\(^+\) T cells favors the preferential expansion and function of CD4\(^+\)Foxp3\(^+\) nTreg cells within the target organ, in turn increasing the T reg/T eff cell ratio and tipping the balance to self-tolerance.

Discussion

CD4\(^+\)Foxp3\(^+\) nTreg cells have been implicated as a central control point in the pathogenesis of TID in NOD mice (30, 43). TID resistance also correlates with the expansion of CD4\(^+\)CD25\(^+\) T cells within pancLN and insulitic lesions, and with a consequential decrease in the priming, expansion, or differentiation of Treg cells in these sites (30, 41, 44, 45). Developmental or functional defects of CD4\(^+\)Foxp3\(^+\) nTreg cells in autoimmune-prone hosts may represent a major predisposition factor for spontaneous TID (46, 48).

Fine mapping studies have established Idd2 as the primary genetic determinant of disease protection operative in the Idd3\(^{B6}\) locus (22, 31). Considering the critical role of IL-2 in nTreg cell function, we hypothesized that Idd3\(^{B6}\) protective alleles impart potent resistance to TID by potentiating nTreg cell-mediated regulation of diabetogenic T cells. We found that the protective Idd3\(^{B6}\) allele, relative to the NOD allele, augments the amount of IL-2 mRNA and protein produced by diabetogenic CD4\(^+\) T eff cells and affords resistance to spontaneous and CD4\(^+\) T cell-induced TID. While the frequency of CD4\(^+\)Foxp3\(^+\) nTreg cells is not affected, their functional potency is increased in Idd3\(^{B6}\) congenic mice, we make the novel finding that Idd3\(^{B6}\) protective alleles primarily favor TID disease resistance by heightening the cycling and function of CD4\(^+\)Foxp3\(^+\) nTreg cells within the inflammatory environment of the pancreas. Collectively, we show that the TID-protective Idd3\(^{B6}\) allele variants dictate the amount of IL-2 production by diabetogenic CD4\(^+\) T eff cells, which initiates a regulatory feedback loop driving the functional homeostasis of CD4\(^+\)Foxp3\(^+\) nTreg cells in the target organ.

IL-2 is now viewed as an important signal for the development, function, and competitive fitness of nTreg cells in vivo (23, 26). As CD4\(^+\)Foxp3\(^+\) nTreg cells fail to make IL-2, their primary source of IL-2 in vivo is likely CD4\(^+\) T eff cells (29, 50, 51). Indeed, mice deficient for B7/CD28, CD40/CD40L, IL-2, IL-2Rα/β, or STAT5A/B have drastically reduced nTreg cell numbers and suffer from severe autoimmune (24, 25, 52–57). Consistently, in vivo neutralization of IL-2 in NOD mice actually precipitates the onset and incidence of TID (26). Interestingly, NOD T cells respond normally to TCR activation until 4 wk of age, at which point they become anergic and sustain a drastic reduction in IL-2 production,
of the Ig-like domain 3 (Idd3) allele would likely result in higher IL-2 levels, particularly in the local environment of inflammation, which in turn would affect nTreg cell homeostasis and function to assure disease protection. Interestingly, recent evidence has demonstrated that IL-2 is a potent inhibitor of Th17 cell differentiation in vitro and in vivo (58). Our data would also suggest that the increased production of IL-2 by T cells from Idd3 mice might promote protection to T1D by hindering Th17 cells while simultaneously promoting CD4⁺ Foxp3⁺ Treg cells. Thus, our model would suggest that the presence of protective Idd3 alleles permits diabetogenic CD4⁺ T cells to produce sufficient IL-2 to optimally promote the expansion and function of CD4⁺ Foxp3⁺ nTreg cells in the panLN and pancreas, and in turn blocking the diabetogenic process in the target organ. Consistently, administration of low-dose IL-2 promoted Treg cell survival and protected NOD mice from developing T1D (28).

Studies have shown that the “susceptible” and “resistant” IL-2 differ in their N-terminal sequence, correlating with putative, differential glycosylation states, suggesting that IL-2 variants may be functionally distinct, potentially affecting the synthetic rate, protein folding/half-life, binding affinity, or signaling of IL-2 (32). Yamanouchi et al. recently demonstrated that several single nucleotide polymorphisms within the 5’ region of the NOD haplotype of Idd2 promoter collectively influence the competency of activated CD8⁺ T cells to initiate IL-2 transcription or secrete IL-2 protein in activated self-reactive T cells (22). This increased IL-2 expression by the Idd3 allele in activated T cells may be related to improved assembly/activation of the transcription machinery during T cell activation (59, 60), in turn enabling more efficient IL-2 transcription or secretion in T cells with the downstream effect of increasing the cellular frequency of IL-2 secreting CD4⁺ T cells in a given immune response. More importantly, the sole presence of Idd3 allele in diabetogenic CD4⁺ T cells is able to partially correct the “defective fitness” of CD4⁺ Foxp3⁺ nTreg cells in NOD hosts (Fig. 5A), indicating that Idd3 allele variants in diabetogenic T cells are important contributors in self-tolerance mechanisms. These results do not exclude a possible CD4⁺ nTreg cell-intrinsic role for protective Idd3 alleles.

The capacity of nTreg cells to localize directly within inflamed tissues to dampen immune responses has been shown in various models of infectious disease, inflammatory bowel disease, and tumors (37, 39, 40). Similarly, CD4⁺ Foxp3⁺ nTreg cells block the diabetogenic process, in part, by localizing within insulitic lesions, where they suppress the function of T eff cells (12, 30, 45, 61). We show that nTreg cells from BDD.Idd3 mice preferentially expand within the pancreas of T1D-protected mice, where they control the effector functions of infiltrating diabetogenic CD4⁺ Foxp3⁺ T cells. This would suggest that the loss of Ag-driven homing, activation, or expansion of nTreg cells in pancreatic sites may represent an essential checkpoint in the T1D progression, and that the protective Idd3 alleles correct for this defect in NOD.B6 Idd3 mice. It is unknown whether Idd3 engages unique transcription factors in infiltrating nTreg cells, particularly with regard to genes affecting metabolism, cell cycle, homing, and survival of nTreg cells.

In conclusion, we link the T1D-protective affect of Idd3 with a more potent CD4⁺ Foxp3⁺ nTreg cell compartment, particularly with regard to its ability to promote regulatory function in the local inflammatory environment of the pancreas. Our results are potentially relevant to human TID considering that some studies have suggested a reduction of nTreg cell number and/or function in individuals with T1D (62). In human T1D, the genes for CTLA-4, insulin, and PTPN22 (protein tyrosine phosphatase, nonreceptor type 22) map to TID susceptibility, and although an association with IL2 in human T1D has not been made, recent studies identified two single nucleotide polymorphisms in IL2ra/CD25, which correlate with T1D susceptibility in humans (63–66). Thus, the control of organ-specific autoimmunity is critically dependent on the dominant regulation of self-reactive T cells, and that in genetically susceptible subjects with a defective CD4⁺ Foxp3⁺ nTreg cell compartment, an increase in IL-2R signaling may diminish T1D risk. This study illustrates that some T1D susceptibility genes may alter the balance between pathogenic and nTreg cell populations and ultimately contribute to T1D pathogenesis.

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