Type 1 diabetes (T1D) results from the immune-mediated destruction of the insulin-producing β-islet cells of the pancreas. In patients with type 1 diabetes, the immune-mediated destruction of the pancreas is thought to begin years or even decades before metabolic dysregulation facilitates diagnosis (1). Therefore, the underlying environmental and genetic factors contributing to the initiation of T1D have been difficult to define. Indeed, at the time of T1D diagnosis, up to 80% of islet cell function has been lost because of immune destruction of the pancreas (2).

T cell-mediated destruction of the islets is clearly involved in T1D pathogenesis; in fact, in both animal models and human patients, the disease is strongly linked to polymorphisms in MHC class II (3, 4). However, despite the use of autoantibodies as both diagnostic and prognostic markers of T1D (5–7), the direct involvement of autoreactive B cells in disease pathogenesis remains controversial (8). Moreover, the mechanisms by which diabetogenic T cells escape control by Foxp3+ regulatory T (Treg) cells have not been defined but have important implications for the development of Treg cell-based therapies aimed at preventing or modulating disease (9).

RIPmOva mice express membrane-bound OVA under the control of the rat insulin promoter. We found that DORmO.RAG2−/− mice do not develop insulitis and are completely protected from diabetes, demonstrating that endogenous lymphocyte receptor rearrangement is required for disease development. Diabetes in DORmO mice is preceded by the development of OVA-specific autoantibodies and is delayed in B cell-deficient DORmO.JhD−/− mice, demonstrating that B cells contribute to disease progression. In addition, transfer of CD8+ T cells from diabetic animals into DORmO.RAG2−/− mice promotes insulitis by OVA-specific CD4+ T cells. Finally, although diabetes develops in DORmO mice in the presence of a significant population of Foxp3+ OVA-specific regulatory T cells, boosting regulatory T cell numbers by injecting IL-2 immune complexes dampens autoantibody production and prevents development of insulitis and overt diabetes. These results help define the events leading to diabetes in DORmO mice and provide new insights into the cellular interactions required for disease development in an Ag-specific model of T1D. The Journal of Immunology, 2010, 185: 4760–4768.

The online version of this article contains supplemental material.
Materials and Methods

Mice

All mice were bred and maintained under specific pathogen-free conditions, with free access to food and water, in the animal facility at the Benaroya Research Institute (Seattle, WA). DO11.10 transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred with BALB/c mice expressing RIPImOva (provided by Dr. A. K. Abbas, University of California, San Francisco, San Francisco, CA) to generate the DORMoO double-transgenic mice. BALB/c JbD-/- mice and BALB/c RAG2-/- mice were purchased from Taconic Laboratories (Hudson, NY) and bred with the DORMoO mice. All experiments and animal use procedures were approved by the Institutional Animal Use and Care Committee at the Benaroya Research Institute.

Diabetes monitoring

Beginning at 4 wk of age, DORMoO mice were bled weekly via the saphenous vein for the determination of their blood glucose level (BGL) using an Ascensia Contour blood glucose meter and blood glucose monitoring strips (Bayer Healthcare, Tarrytown, NY). When two consecutive blood glucose readings of >200 mg/dl were recorded, animals were considered diabetic. When two consecutive blood glucose readings of >300 mg/dl were recorded, animals were euthanized.

Lymphocyte isolation

The spleen and pancreatic lymph nodes were collected from each mouse. The tissues were pressed through a 100-μm filter and washed with HBSS supplemented with 2% FBS and prepared for flow cytometric and functional analyses.

Flow cytometric analysis

Prior to incubating cells with the specified fluorochrome-conjugated Abs, all cells were incubated in HBSS with 2% FBS and rat IgG (Sigma-Aldrich, St. Louis, MO) to block nonspecific binding. The following Abs were used for flow cytometry analyses were purchased from eBioscience (San Diego, CA): FITC-conjugated anti–CD62L, anti–IL-17, and anti–IFN-γ; Alexa Fluor 488-conjugated anti–Foxp3; PE-conjugated anti–B220; allophycocyanin-eFluor 780-conjugated anti–CD25; allophycocyanin-conjugated anti–CD25 and anti–CD62L; and, eFluor 450-conjugated Foxp3. Additional Abs were purchased from BioLegend (San Diego, CA): PE-conjugated Foxp3, PerCP-Cy5.5-conjugated anti–CD4, PECy7-conjugated anti–CD8a and anti–B220; Alexa Fluor 700-conjugated anti–CD4 and anti–CD44; and Pacific blue-conjugated anti–CD4 and anti–IFN-γ. Also, the following Abs were purchased from Caltag Laboratories (Burlingame, CA): Alexa Fluor 488-conjugated anti–CD4 and PE- and Alexa Fluor 647-conjugated anti–KJ1-26, PE- and PECy7-conjugated anti–CD45RB and PeCy7-conjugated anti–B220 were also purchased from BD Biosciences (San Jose, CA). Data were acquired on a BD Biosciences LSR II flow cytometer and analyzed using FlowJo software (Tree Star, Ashland, OR). For cell-sorting experiments, samples were first enriched for CD8+ cells, or depleted of CD4+ cells, using magnetic beads specific for CD8 or CD4, respectively, and magnetic columns (Miltenyi Biotec, Auburn, CA). Subsequently stained for flow cytometry.

In vitro stimulation

Splenocytes were plated in 96-well, flat-bottom culture plates at a concentration of 5 × 10^5–1 × 10^7/200 m/l well in DMEM supplemented with 5% FBS, PMA (50 ng/ml) and ionomycin (1 mg/ml) were added to the culture and incubated for 1 h at 37°C in 5% CO2. Monensin (10 mg/ml; eBioscience) was then added, and cells were cultured at 37°C for an additional 5 h. Following stimulation, the cells were stained with PerCP-Cy5.5-conjugated anti–CD4, PeCy7-conjugated anti–CD8, allophycocyanin-eFluor 780-conjugated anti–CD25, and PE- or Alexa Fluor 647-conjugated anti–KJ1-26 Abs in staining buffer (2% FBS-HBSS) prior to being fixed and permeabilized in eBioscience fixation/permeabilization buffer for 1 h at 4°C. Intracellular staining was performed using 0.5% saponin buffer supplemented with 2% FBS, containing labeled Pacific blue-conjugated anti–IFN-γ, allophycocyanin- or PE-conjugated anti–Foxp3, and FITC-conjugated anti–IL-17 for 30 min at 4°C.

Adoptive transfer of CD8+ T cells

Six- to 8-wk-old DORMoO.RAG2-/-, DO11.10.RAG2-/-, or RIPImOVA.RAG2-/- hosts were injected with 5 × 10^5–1 × 10^6 purified CD8+ cells via retro-orbital injection. Animals were monitored weekly thereafter for changes in BGLs. All mice were sacrificed at 15 wk posttransfer for analysis of the pancreatic inflammation and to examine lymphocytes in the pancreatic lymph nodes and spleen by FACs. Half of each pancreas was placed in optimal cutting temperature medium and snap frozen for immunofluorescence, and half was placed in neutral-buffered formalin for histological analysis. The lymph nodes and spleen were processed as described above and analyzed using flow cytometry.

IL-2+anti–IL-2 immune complexes

Purified rIL-2 and anti–IL-2 mAb (clone JES6-1A12) were purchased from eBioscience. IL-2 immune complexes (IL2Cs) contained 50 μg/mouse anti–IL-2 (clone JES6-1A12) and 1.5 μg/mouse purified IL-2 or 25 and 0.75 μg/mouse. The Ab was incubated with the purified IL-2 for 30 min at room temperature (RT) or overnight at 4°C. Prior to injection, sterile PBS was added to a final injection volume of 100 μl/mouse. All injections were given i.p. Control mice received 25 or 50 μg/mouse IgG as a control. Animals were injected weekly, beginning at 2 wk of age, until the control animals became diabetic. Prior to, throughout, and after treatment, all animals were monitored weekly for changes in their BGLs.

ELISA

Serum OVA-specific IgG1 was determined by ELISA. Serum samples were diluted 1/1000 for IgG1 ELISA using ELISA buffer (1× PBS plus 2% normal goat serum and 0.05% Tween 20). High-binding, 96-well plates were coated with 100 μl/well of 2 mg/ml OVA in distilled water and incubated overnight at 4°C. Plates were washed using the Skanwash 300 version B (Molecular Devices, Sunnyvale, CA) and PBS-Tween 20. To block nonspecific binding, 200 μl/well ELISA buffer was added to each well, and the plate was incubated at 37°C for 1 h in 5% CO2. Samples were plated in duplicate in the specified dilution and incubated overnight at 4°C. The IgG1 standard was prepared using eBioscience. After washing the plate with PBS-Tween 20, alkaline phosphatase-conjugated anti-IgG1 (1/2500; Jackson ImmunoResearch Laboratories, West Grove, PA) was added to each well. Plates were incubated at RT for 2 h, then washed with PBS-Tween 20. For the alkaline phosphatase substrate, para-nitrophenyl phosphate (Sigma-Aldrich) tablets were dissolved in a buffer containing 0.05 M Na2CO3 and 0.5 mM MgCl2, and 50 μl/well para-nitrophenyl phosphate buffer was added to each plate. The chromagen was allowed to develop for up to 15 min at RT before the plate was analyzed using the the SoftMax Pro 5 software VersaMax tunable microplate reader at λ = 405 nm (Molecular Devices).

Immunohistochemistry and immunofluorescence

For immunohistochemistry, tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin, and 5-μm sections were stained with H&E; bright-field images of the H&E-stained tissue were acquired using a Leica DM2500 microscope equipped with an Insight 4-megapixel color charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI). For immunofluorescence, tissues were placed in optimal cutting temperature, snap frozen, and stored at −80°C until sectioned. For each tissue, 6-μm sections were cut and placed on Superfrost Plus charged slides (VWR International, West Chester, PA). Slides were fixed in cold acetone for 10 min and allowed to air-dry at RT for 1 h. All slides were stored at −20°C until stained for microscopy. Prior to incubating the slides with the specified fluorochrome-conjugated Abs, slides were allowed to come to RT and then rehydrated with 1× PBS. Nonspecific binding was blocked by incubating the slides with PBS supplemented with 2% BSA and 2% normal goat serum for 30 min at RT. Then, sections were incubated with 1/200 guinea pig anti-insulin (Abcam), each with slides were incubated for 1 h at RT and washed with the staining buffer (1× PBS plus 2% BSA) three times for 5 min/wash. Sections were then incubated with Alexa Fluor 488- or Alexa Fluor 568-conjugated rabbit anti-guinea pig IgG (H+L) (Molecular Probes Invitrogen, Carlsbad, CA) and FITC-, Alexa Fluor 488-, Alexa Fluor 647-, or allophycocyanin-conjugated anti–KJ1-26, anti–CD4, anti–CD8, or anti–B220 for 1 h at RT. After 1 h, slides were washed as before. Images shown were visualized at ×40 magnification using Leica DMIRB inverted fluorescence microscope (Leica Microsystems, Bannockburn, IL) equipped with a Pursuit 4-megapixel cooled color/monochrome charge-coupled device camera (Diagnostic Instruments) or Bio-Rad MRC-1024 UV laser scanning confocal microscope system (Bio-Rad, Hercules, CA) attached to a Nikon Diaphot 200 inverted microscope (Nikon Instruments, Melville, NY). Images were acquired using a Windows 2000 PC and the Spot Advanced Software (SPOT Imaging Solutions; Diagnostic Instruments) for the Leica DM IRB microscope or the Laser Sharp 2000 acquisition software (Bio-Rad) for the laser scanning confocal microscope system.
**Statistical analysis**

The statistical differences were assessed by Student t test, one-way ANOVA (using Bonferroni posttests for pairwise comparisons), or log-rank test where appropriate. Statistical significance was determined by a p value of <0.05.

**Results**

**Autoantibody generation coincides with the onset of insulitis in DORmO mice**

Consistent with a recently published report (12), we found that >90% of DORmO animals are diabetic by 15 wk of age and that all mice became diabetic by 20 wk of age despite their high frequency of fully functional, islet Ag-specific Treg cells. In T1D, disease is thought to begin long before the loss of β-islet cells becomes severe enough to cause hyperglycemia. Therefore, to determine when the breakdown in tolerance occurs, we examined DORmO mice of various ages for development of insulitis and islet destruction. At 3 wk of age, islets from DORmO mice are indistinguishable from control DO11.10 littermates (Fig. 1A). However, in the majority of DORmO mice, progressively destructive insulitis began by 4 wk of age—at least 4–16 wk prior to detectable alterations in glucose metabolism (Fig. 1A). Correlating with insulitis induction, OVA-specific autoantibodies were readily detected in the serum of DORmO mice beginning at 3–4 wk of age, with titers peaking several weeks before the onset of diabetes (Figs. 1B, 2A). Immunofluorescence analyses demonstrated that, in addition to OVA-specific CD4+ T cells (identified by staining with the clonotypic Ab KJ1-26 [13]), the islet infiltrate also contained increasing numbers of B and CD8+ T cells (Fig. 1C). Notably, diabetes in DORmO mice occurs despite a large population of OVA-specific, KJ1-26 Foxp3+ Treg cells that coexist with effector T cells in inflamed islets of diabetic mice (Fig. 1D). Thus, the DORmO model of T1D recapitulates several features of disease observed in other animal models and in human patients, including a protracted period of asymptomatic autoimmunity accompanied by the production of prognostic autoantibodies, and islet infiltration by effector and regulatory CD4+ T cells, CD8+ T cells, and B cells.

**B cells accelerate T1D development in DORmO mice**

It is evident that both DO11.10 Tg CD4+ T cells and islet-expressed OVA are necessary for T1D development, because neither DO11.10 nor RIPmOva single transgenic mice develop insulitis or diabetes. However, rearrangement of endogenous TCR and Ig loci results in the development of polyclonal populations of B cells and CD8+ T cells that may also contribute to disease development. To determine whether these non-Tg lymphocyte populations were necessary for disease development in the DORmO model, DORmO animals were bred to RAG2−/− mice and monitored for disease development. Surprisingly, despite containing a full compartment of islet Ag-specific CD4+ T cells, DORmO.
RAG2−/− mice failed to develop diabetes and were fully protected from insulitis (Fig. 2A, 2B).

The lack of T1D in DORmO.RAG2−/− mice is not due to the absence of B cells, because DORmO.JhD−/− mice, which are B cell-deficient as a result of a targeted mutation in the IgH chain gene (14), still developed disease, albeit with a significant delay (Fig. 2A). Protection from diabetes in DORmO.RAG2−/− mice and the delay seen in the DORmO.JhD−/− mice were each associated with impaired effector differentiation of OVA-specific CD4+ T cells, evidenced by a decreased frequency of CD45RBlo CD62L− T cells among the pancreatic lymph nodes compared with wild-type DORmO animals (Fig. 3B). In addition, following ex vivo PMA/ionomycin stimulation, KJ1-26+ T cells from DORmO.RAG2−/− mice did not produce either IFN-γ or IL-17, and significantly fewer OVA-specific CD4+ T cells from DORmO.JhD−/− produced IFN-γ compared with wild-type DORmO controls (Fig. 3B). By contrast, the frequency of IFN-γ or IL-17 CD8+ T cells did not differ significantly between the DORmO and DORmO.JhD−/− mice. As expected, CD8+ T cells were not observed in DORmO.RAG2−/− animals. The impaired differentiation of IFN-γ-producing effector cells in DORmO.JhD−/− mice was not due to reduced access to Ag, because roughly equal fractions of CD4+KJ1-26+Foxp3+ T cells expressed the early activation marker CD69 in the pancreatic lymph nodes of DORmO and DORmO.JhD−/− animals (Fig. 4). Collectively, these data indicate that, in addition to producing islet Ag-specific autoantibodies, B cells contribute to the activation and functional differentiation of islet Ag-specific T cells, thereby accelerating T1D development in DORmO mice.

**CD8+ T cells promote insulitis when transferred into the DORmO.RAG2−/− mice**

Although B cells accelerate T1D development in DORmO mice, their absence cannot account for the lack of diabetes in DORmO.RAG2−/− animals. In DORmO mice, CD8+ T cells are also found in the pancreatic infiltrate of diabetic DORmO animals (Fig. 1C). Loss of CD8+ T cells may therefore contribute to disease protection in DORmO.RAG2−/− mice. To test this, we transferred 5 × 10^6 CD8+ T cells purified from prediabetic DORmO mice into 6- to 7-wk-old DORmO.RAG2−/−, RIPmOVA.RAG2−/−, or DO11.10.RAG2−/− recipients. These animals were monitored weekly for alterations in BGLs and the presence of transferred cells in circulation for up to 15 wk posttransfer (data not shown and Fig. 5A). All mice were subsequently sacrificed for analysis of pancreatic inflammation in the associated lymph nodes and spleen (Fig. 5B, 5C, and data not shown). Although one RIPmOVA.RAG2−/− recipient developed T1D during this period, we found that transfer of CD8+ T cells led to varying degrees of insulitis in all of the DORmO.RAG2−/− mice (Fig. 5B, 5C). By contrast, control DORmO.RAG mice displayed no islet infiltration by either H&E staining (Fig. 2B) or immunofluorescence (data not shown). Interestingly, no insulitis was observed in the DO11.10.RAG2−/− recipients, indicating that CD8+ T cells were not sufficient to cause insulitis in the absence of OVA expression in the pancreas. Rather, these data suggest that CD8+ T cells synergize with the OVA-specific KJ1-26+CD4+ T cells to promote insulitis. Indeed, transfer of CD8+ T cells resulted in substantial islet infiltration by endogenous CD4+KJ1-26+ cells (Fig. 5B).

**Expansion of Treg cells prevents autoantibody production and T1D development in DORmO mice**

DORmO mice contain a high frequency of OVA-specific Foxp3+ Treg cells, and Treg cells can be found within the pancreatic islets in diabetic animals (Fig. 1D). In addition, when DORmO mice were crossed with Foxp3-deficient scurfy mice, the absence of Treg cells led to severe insulitis by 4 wk of age (Supplemental Fig. 1). Clough et al. (12) have demonstrated that Treg cells in DORmO mice are functional and that Ab-mediated depletion of Treg cells...
in DORmO animals accelerates diabetes development. Thus, the suppressive activity of T_{reg} cells must be overcome during the development of diabetes, perhaps by production of proinflammatory cytokines such as IL-21 (12).

Modulating T_{reg} cell activity has been proposed as a cellular therapy in T1D. Therefore, to determine whether increasing the number of endogenous T_{reg} cells could prevent the development of T1D, we treated DORmO mice with IL2Cs (Fig. 6). Using the JES6-1A12 mAb, IL2Cs induces the specific expansion of T_{reg} cells in vivo with little impact on other IL-2–responsible immune cell populations (15). Indeed, weekly treatment with IL2Cs beginning between 1 and 2 wk of age led to robust expansion of both OVA (KJ1-26)+ and non–OVA-specific (KJ1-26−) T_{reg} cells and prevented diabetes development in DORmO mice (Fig. 6A, 6B). In addition, IL2C-treated mice developed less severe insulitis, and the majority of islet mass and function was preserved compared with control mice given rat IgG (Fig. 6D). Notably, protection from diabetes was also associated with a transient reduction in OVA-specific IgG autoantibodies, indicating that IL2C treatment also impaired the B cell response to autoantigen, most likely by limiting CD4+ T cell help (Fig. 6C). To determine whether long-term IL2C treatment beginning before insulitis or autoantibodies are evident would lead to development of durable immune tolerance in the absence of continued T_{reg} cell expansion, weekly IL2C treatment was stopped in one cohort of DORmO mice at ~15 wk of age, a time at which all of the rat IgG-treated mice had already progressed to overt diabetes. However, within 1–2 mo after treatment cessation, 80% (four of five) of the IL2C-treated animals developed T1D that was indistinguishable from that observed in control-treated DORmO mice (Fig. 6D, Supplemental Fig. 2). In addition, weekly IL2C treatment for 4 wk, beginning at the onset of autoantibody production and insulitis, failed to prevent or delay disease progression in any of the mice (Supplemental Fig. 2). Therefore, although IL2C treatment could prevent diabetes development, continued treatment was necessary for this protective effect to be maintained.

**Discussion**

The current report details the development of spontaneous diabetes in the DORmO double-transgenic mouse model of T1D. Novel aspects of our study include determining that B cells influence the activation and effector differentiation of islet Ag-specific CD4+ T cells, demonstrating that CD8+ T cells promote CD4+ T cell infiltration of the islets, and examining the impact of boosting regulatory T cell function on the short and long-term diseases development in DORmO mice.

The known Ag specificity of DORmO model makes it an attractive tool for delineating the relative contributions of different lymphocyte populations to diabetes progression and for examining the function of islet Ag-specific T_{reg} cells during disease development. The novelty of this model is further highlighted by the requirement for a nontransgenic, RAG-dependent lymphocyte population for diabetes development. This contrasts with the most commonly used TCR transgenic model of T1D, the BDC2.5 mouse on the NOD genetic background. In these animals, T1D development is dramatically accelerated when they are rendered tolerogenic for diabetes, and the protective effect to be maintained.

**FIGURE 3.** Reduced effector differentiation of OVA-specific CD4+ T cells in DORmO.RAG2−/− and DORmO.JhD−/− mice. A, Top panel, Representative flow cytometry analysis of CD45RB and CD62L expression by gated CD4+ KJ1-26+Foxp3+ cells from the pancreatic lymph nodes of 6- to 8-wk-old DORmO, DORmO.JhD−/−, and DORmO.RAG2−/− mice. Bottom panel, Quantitative analysis of the frequency of CD45RBlo/CD62Lhi T cells in DORmO, DORmO.JhD−/−, and DORmO.RAG2−/− mice. B, Top panels, Representative flow cytometry analysis of IFN-γ and IL-17 production by gated CD4+ KJ1-26+ and CD8+ splenocytes from 6- to 8-wk-old DORmO, DORmO.JhD−/−, and DORmO.RAG2−/− mice following in vitro stimulation with PMA/ionomycin. Bottom panels, Quantitative analysis of IFN-γ and IL-17 production by CD4+ KJ1-26+ and CD8+ splenocytes from 6- to 8-wk-old DORmO, DORmO.JhD−/−, and DORmO.RAG2−/− mice following in vitro stimulation with PMA/ionomycin. Each point represents the percent of cytokine positive cells from one animal, and the mean ± SEM for five to six animals per genotype is shown. Data are representative of four independent experiments.
RAG deficient. The enhanced disease likely stems from impaired development of various populations of Treg cells driven by endogenous TCR rearrangement in BDC2.5 mice (16, 17). By contrast, DORmO.RAG-2⁻/⁻ mice develop a population of OVA-specific, Foxp3⁺ Treg cells as a result of expression of mOVA in the thymus, and these cells are capable of controlling the potentially diabetogenic KJ1-26⁺Foxp3⁺CD4⁺ T cells (11, 18). Indeed, the lack of disease development in the DORmO.RAG-2⁻/⁻ was not due to an inability of Foxp3⁺CD4⁺ T cells to induce disease on their own, because insulitis rapidly develops when CD25⁺KJ1-26⁺CD4⁺T cells from DO11.10 mice are transferred into RIPmOva.RAG2⁻/⁻ hosts, and TID can be induced in these hosts by immunization with OVA (19). These data suggest that in DORmO mice, endogenous lymphocyte populations help CD4⁺ T cells overcome Treg cell-mediated suppression and promote overt autoimmunity.

The significant delay in disease progression in the DORmO.JhD⁺⁻/⁻ mice demonstrates that B cells influence the initiation and/or progression of TID in this model. The decreased activation of and reduced production of IFN-γ by OVA-specific T cells in the DORmO.JhD⁻⁻/⁻ animals suggest that B cells may have an important role as APCs, facilitating the activation and functional differentiation of diabetogenic T cells. Indeed, in the context of diabetes, B cells have been shown to be important in T cell activation (20–23). Moreover, autoantibodies produced by OVA-specific B cells may directly cause islet damage, increasing the amount of islet Ag available for presentation to autoreactive T cells. In addition, autoantibodies may directly induce the activation of dendritic cells and cross-presentation of pancreatic Ag-specific T cells and the immune destruction of the islets (24, 25). However, the fact that equal fractions of KJ1-26⁺Foxp3⁻/⁻ T cells express the early activation marker CD69 in the draining lymph node but rather represents a qualitative change in the immune environment that results in reduced differentiation of proinflammatory effector cells.

Our data clearly demonstrate that B cells accelerate disease development in DORmO mice; however, they are not ultimately required for diabetes. Although islet Ag-specific CD4⁺ T cells are sufficient to cause disease in other TCR transgenic models of TID, CD8⁺ T cells are thought to be important mediators of islet destruction in NOD mice and in TID patients (26, 27). Indeed,
DORmO.RAG2<sup>−/−</sup> mice given purified CD8<sup>+</sup> T cells from prediabetic DORmO donors developed insulitis by 15 wk post-transfer, supporting a role these cells in disease pathogenesis. As with tissue damage caused by autoantibodies, islet destruction by CD8<sup>+</sup> T cells may release large amounts of islet Ags, facilitating the activation and functional differentiation of autoreactive CD4<sup>+</sup> T cells and epitope spreading. In addition, CD8<sup>+</sup> T cells could expedite lymphocytic infiltration of the islets by altering the extracellular matrix surrounding the islets through secretion of matrix metalloproteinases, and such a mechanism for CD8<sup>+</sup> T cell function has been proposed in NOD mice (28). Consistent with a promoting role for CD8<sup>+</sup> T cells in islet inflammation, the islet infiltrate in DORmO.RAG2<sup>−/−</sup> mice given CD8<sup>+</sup> cells is composed largely of endogenous KJ1-26<sup>+</sup> T cells, with very few of the transferred CD8<sup>+</sup> cells evident. Interestingly, in at least one instance, the transferred CD8<sup>+</sup> T cells caused severe insulitis and T1D development in a RIPmOVA.RAG2<sup>−/−</sup> recipient, indicating that in the absence of OVA-specific CD4<sup>+</sup> T cells, these CD8<sup>+</sup> cells could themselves mediate disease. From these results, it is tempting to speculate that with only a small number of CD8<sup>+</sup> T cells present, the OVA-specific T<sub>reg</sub> cells found in DORmO.RAG2<sup>−/−</sup> mice may limit CD8<sup>+</sup> T cell-mediated islet destruction and act to prevent diabetes development in the 15-wk window analyzed in these experiments. Moreover, transfer of these CD8<sup>+</sup> T cells into DO11.10.RAG2<sup>−/−</sup> did not cause diabetes or insulitis, suggesting that recognition of OVA, perhaps through expression of the clonotypic KJ1-26 TCR, was required for these effects. DORmO mice develop T1D despite containing a large population of OVA-specific T<sub>reg</sub> cells. These T<sub>reg</sub> cells are functional, because they can suppress T cell proliferation ex vivo (11). Their failure to prevent diabetes is not due to their inability to access the target tissue as we found islet Ag-specific T<sub>reg</sub> cells even with the inflamed islets of diabetic DORmO mice. Moreover, either genetic or Ab-mediated depletion of T<sub>reg</sub> cells in DORmO mice dramatically accelerates insulitis and T1D development, indicating that these T<sub>reg</sub> cells do function in vivo to delay T1D development.

**FIGURE 6.** In vivo expansion of T<sub>reg</sub> cells prevents diabetes in DORmO mice. A, Representative flow cytometry analysis of Foxp3 and KJ1-26 expression by gated CD4<sup>+</sup> T cells from the spleens (top panels) or peripheral lymph nodes (PLNs) (bottom panels) of rat IgG- (left panels) or IL2C-treated (middle and right panels) DORmO mice at the indicated treatment times. B, BGLs monitored weekly in rat IgG- or IL2C-treated DORmO mice. Each point represents a value from an individual animal. C, ELISA analysis of OVA-specific IgG1 in the serum of rat IgG- or IL2C-treated DORmO mice. The mean ± SEM is shown for six to nine mice per treatment. D, Representative H&E staining of pancreatic islets from rat IgG- (left panels) or IL2C-treated (middle and right panels) DORmO mice at the indicated treatment times. Original magnification ×40.
in these animals (12). The processes underlying the failure of Treg cell suppression are not well understood. For instance, the cytokine IL-21 has been implicated in the inhibition of Treg cell function in DORMO mice (12). However, increased IL-21 production was first detected in the pancreatic lymph node at ~6 wk of age, whereas immune tolerance is breached at least 2–3 wk earlier, as evidenced by insulin and production of OVA-specific autoantibodies. Thus, although IL-21 may contribute to suppression of Treg cell function in the later stages of T1D development, its increased expression is unlikely to be the proximal cause of tolerance breakdown in DORMO mice. The proinflammatory cytokine IL-6 can both inhibit Treg cell function and prevent the de novo induction of Treg cells from Foxp3+ precursors in the periphery (29, 30), potentially contributing to the overall inhibition of Treg cell function during T1D development.

The protracted period of preclinical autoimmunity in T1D provides a unique opportunity for identification and therapeutic intervention in individuals before metabolic dysregulation begins. For instance, manipulating Treg cell activity in vivo during critical temporal windows in disease development may help restore immune homeostasis, leading to lasting immune tolerance (9). Indeed, expanding Treg cells with IL-2Cs has proven effective in preventing experimental autoimmune encephalomyelitis and blocking allograft rejection in mice (31). By contrast, IL2C treatment of 10-wk-old prediabetic NOD mice actually accelerated disease development, presumably through enhanced activation of CD25-expressing effector lymphocyte populations (32). We found that weekly injection of IL2Cs into DORMO mice beginning before any signs of detectable autoimmunity completely prevented insulins and diabetes and reduced autoantibody production. However, the expanded Treg cell population is not maintained once treatment is stopped, and all mice progressed to overt diabetes following cessation of treatment. Thus, despite greatly enhanced Treg cell activity during the temporal window in which loss of tolerance begins in DORMO mice, long-term IL2C treatment, on its own, did not result in the induction of durable, long-term tolerance. These results demonstrate that Treg cell-directed therapies have the potential to treat or manage diabetes and suggest that additional interventions, such as rapamycin treatment aimed at disarming effector T cells, may be necessary to induce full tolerance after Treg cell expansion (31).

Taken together, our results demonstrate that the disease process in the DORMO mouse model is a complex series of events that involves not only the transgenic OVA-specific CD4+ T cells but also multiple endogenous, non-Tg populations. Further examination of this model will be useful in delineating the mechanisms involved in the breakdown of T and B cell tolerance in a simplified, Ag-specific model of T1D. In addition, these findings highlight the potential value of modulating endogenous Treg cell activity for the prevention, treatment, or cure of autoimmune diseases.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. DORmO.scurfy mice develop severe insulitis by four weeks of age. Representative H&E staining of pancreatic islets from 3–4 week old Foxp3-deficient DO11.10.Scurfy or DORmO.Scurfy mice.
Supplemental Figure 2. Failure to induce long-term tolerance in IL-2C-treated DORmO mice. *Top*, Diabetes development in untreated DORmO mice or DORmO mice treated with IL2C weekly between 1 and 15 weeks of age. *Bottom*, Diabetes development in untreated DORmO mice or DORmO mice treated with IL2C weekly between 4 and 8 weeks of age. Treatment windows are represented by red boxes.