

Inhibition of Th17 Cells Regulates Autoimmune Diabetes in NOD Mice

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OBJECTIVE—The T helper 17 (Th17) population, a subset of CD4-positive T-cells that secrete interleukin (IL)-17, has been implicated in autoimmune diseases, including multiple sclerosis and lupus. Therapeutic agents that target the Th17 effector molecule IL-17 or directly inhibit the Th17 population (IL-25) have shown promise in animal models of autoimmunity. The role of Th17 cells in type 1 diabetes has been less clear. The effect of neutralizing anti-IL-17 and recombinant IL-25 on the development of diabetes in NOD mice, a model of spontaneous autoimmune diabetes, was investigated in this study.

RESEARCH DESIGN AND METHODS AND RESULTS—Although treatment with either anti-IL-17 or IL-25 had no effect on diabetes development in young (<5 weeks) NOD mice, either intervention prevented diabetes when treatment was started at 10 weeks of age ($P < 0.001$). Insulinitis scoring and immunofluorescence staining revealed that both anti-IL-17 and IL-25 significantly reduced peri-islet T-cell infiltrates. Both treatments also decreased GAD65 autoantibody levels. Analysis of pancreatic lymph nodes revealed that both treatments increased the frequency of regulatory T-cells. Further investigation demonstrated that IL-25 therapy was superior to anti-IL-17 during mature diabetes because it promoted a period of remission from new-onset diabetes in 90% of treated animals. Similarly, IL-25 delayed recurrent autoimmunity after syngeneic islet transplantation, whereas anti-IL-17 was of no benefit. GAD65-specific ELISpot and CD4-positive adoptive transfer studies showed that IL-25 treatment resulted in a T-cell-mediated dominant protective effect against autoimmunity.

CONCLUSIONS—These studies suggest that Th17 cells are involved in the pathogenesis of autoimmune diabetes. Further development of Th17-targeted therapeutic agents may be of benefit in this disease. *Diabetes* 58:1302–1311, 2009

Type 1 diabetes is an autoimmune condition associated with the T-cell-mediated destruction of pancreatic β -cells. Detailed investigations using the NOD mouse, a model of spontaneous type 1 diabetes, have indicated that Th1 populations, which are associated with the transcription factor T-bet and the

secretion of cytokines, including γ -interferon (IFN- γ) and interleukin (IL)-2, are key mediators of β -cell autoreactivity (rev. in 1). Conversely, induction of Th2 populations, which are associated with the transcription factor GATA-3 and cytokines such as IL-4 and -10, results in a dominant protective effect against autoimmunity in this model (1). This paradigm is not specific to type 1 diabetes; in fact, the relative pathogenic contributions of Th1 cells and protective effects of Th2 cells have been described as a common feature of other organ-specific autoimmune diseases, including multiple sclerosis and rheumatoid arthritis (2–4).

More recently, a new subpopulation of CD4-positive T-cells has been characterized, the so-called Th17 cells, which are associated with the transcription factor ROR γ T and secretion of the proinflammatory cytokines IL-17 (IL-17A) and IL-17F (5,6). These cells appear to play a central role in early inflammation and eosinophil recruitment, and their common requirement for transforming growth factor (TGF)- β during activation suggests that this population has evolved to counteract the inhibitory properties of the regulatory T (Treg) cell population (7,8). Although the Th17 population contributes to the normal inflammatory response, it can become dysregulated in the presence of IL-23, which enhances the stability and survival of this subpopulation, a feature that has been implicated in the development of autoimmunity (8). Indeed, studies using IL-17 as a surrogate marker of Th17 activity have repeatedly associated high levels of IL-17 with most autoimmune conditions in humans and animal models, including rheumatoid arthritis (9), inflammatory bowel disease (10), and multiple sclerosis (11). The relative contribution of Th17 cells in type 1 diabetes has been less evident. High levels of the IL-17 transcript have been found within insulinitic lesions in NOD mice, and increasing levels of serum IL-17 were associated with the development of diabetes in a T-cell receptor transgenic NOD model with accelerated disease progression (12). More recently, studies have demonstrated that therapeutic intervention with an antigen-specific agent that protects against diabetes in NOD mice is associated with a decrease in Th17 populations (13). However, the specific contribution of Th17 cells to the natural progression of type 1 diabetes in the NOD mouse remains to be fully characterized.

In an effort to both understand the impact of and reduce the negative effects of the Th17 subpopulation, a number of studies have been carried out using neutralizing anti-IL-17 antibodies. A single injection of anti-IL-17 antibody prevented inflammation and bone erosion and reduced Th17 populations in experimental rheumatoid arthritis (14), whereas multiple doses of anti-IL-17 over 2 weeks dramatically reduced inflammatory lesions and neurological symptoms in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (15). Based on these studies, anti-IL-17 antibodies (AIN457 [Novartis]

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and AMG827 [Amgen]) are currently being investigated clinically in autoimmune diseases, including rheumatoid arthritis, psoriasis, and Crohn's disease (16). Another approach to interfere with Th17 populations involves the use of the cytokine IL-25 (IL-17E), a naturally occurring cytokine within the IL-17 family that has been shown to potently inhibit Th17 cells and instead promote the development of Th2 responses (17–19). IL-25 knockout mice are highly susceptible to autoimmunity, with a dramatic increase in Th17 cells using the EAE model (18). This study also demonstrated that both IL-25 knockout and wild-type animals could be protected from the deleterious effects of Th17 cells in EAE when recombinant IL-25 was administered.

Because Th17 subsets are increasingly considered to be a key mediator of all autoimmune disease, therapeutic strategies designed to inhibit these cells are likely to be applicable in type 1 diabetes. The purpose of the current study was to investigate the role of Th17 cells in type 1 diabetes using both neutralizing anti-IL-17 antibodies and recombinant IL-25 in the NOD mouse model.

RESEARCH DESIGN AND METHODS

NOD/LtJ and NOD-RAG^{-/-} mice (NOD.129S7-Rag1^{tm1Mom/J}) were obtained from Jackson Labs (Bar Harbor, ME). For remission and transplant studies, spontaneously diabetic NOD females were identified by monitoring our colony two to three times per week, with animals considered diabetic after two consecutive blood glucose readings >18 mmol/l or one reading >25 mmol/l with a One Touch Ultra Glucometer (LifeScan, Mississauga, ON, Canada). All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and ethical approval was obtained from the animal welfare committee at the University of Alberta.

Drug therapy. A mouse anti-IL-17 monoclonal antibody (clone 50104), mouse IgG2A isotype control antibody, and IL-25 (IL-17E) were all obtained from R&D Systems (Minneapolis, MN).

Diabetes prevention. For studies examining IL-17 neutralization, anti-IL-17 or isotype control was administered at 100 µg i.p. on alternating days over a 12-day period (six total injections). For studies using IL-25, the recombinant cytokine was administered at 1 µg s.c. daily for 25 days (an equivalent volume of saline was given to vehicle-administered animals).

Diabetes remission. Spontaneous diabetic mice were assigned randomly to one of the following treatment groups: anti-IL-17 (100 µg i.p. every other day), IL-25 (1 mg s.c. daily), or control (IgG at 100 µg i.p. for anti-IL-17 and vehicle for IL-25). Treatment was continued until eight consecutive daily readings >25 mmol/l were obtained, after which point the experiment was terminated.

Diabetes recurrence after syngeneic islet transplantation. For anti-IL-17 studies, transplanted animals received either anti-IL-17 or isotype control treatment (100 µg i.p.) on days 0, 4, 8, 12, and 16 posttransplant. For IL-25 studies, transplanted animals received either IL-25 (1 µg s.c.) or vehicle daily through day 15 posttransplant.

Insulinitis scoring. At 1 month after the completion of treatment, pancreatic tissue was harvested, fixed in formaldehyde, processed, and embedded in paraffin (20). Sections (10 µm) were stained using hematoxylin and eosin. All samples were blinded before being scored by a single pathologist using the scheme outlined by Yoon et al. (21). Representative islets were photographed using a Zeiss microscope at ×200 magnification.

Immunofluorescence. Before immunostaining, cryostat sections (10 µm) of pancreata were fixed in acetone and blocked using 20% goat serum. Rat anti-mouse CD4 (clone GK1.5; Ebioscience, Mississauga, ON, Canada), rat anti-mouse CD8 (clone 53–6.7; Ebioscience), rat anti-mouse Foxp3 (clone FJK-16s; Ebioscience), and polyclonal guinea pig anti-insulin (Dako, Mississauga, ON, Canada) were used as primary antibodies. To detect bound antibodies, biotinylated goat anti-rat antibodies (for CD4, CD8, and Foxp3; Cedarlane) and tetramethylrhodamine isothiocyanate-labeled goat anti-guinea pig antibodies (1:200, for insulin; Cedarlane) were used. Polyclonal rabbit anti-IL-17 (Cedarlane Labs, Mississauga, ON, Canada) was used to detect IL-17 on formalin-fixed sections. All slides were mounted using ImmunoGold mounting medium with 4',6-diamidino-2-phenylindole for nuclear counterstaining (Invitrogen, Mississauga, ON, Canada).

GAD65 autoantibody assays. Recombinant GAD65 was prepared as previously described and kindly provided by Dr. John Elliott (22). Serum was harvested via tail vein bleeds from treated animals, and GAD65 autoantibody

levels were determined using the method described by Ma et al. (23). Serum samples were diluted 1:50 in blocking buffer. Reactions were stopped after 20 min using 1 mol/l H₂SO₄ and immediately analyzed at 450-nm wavelength.

Islet transplantation studies. Mouse islets were isolated using established methods (24). Spontaneously diabetic NOD females were maintained on daily insulin injections for 2–3 weeks to collect a sufficient cohort to transplant, with insulin withdrawal the day before islet transplantation. A total of 500 NOD-RAG^{-/-} islets (syngeneic but without insulinitis) were transplanted under the left kidney capsule.

Flow cytometry, enzyme-linked immunosorbent assay, and enzyme-linked immunospot assays. An enzyme-linked immunosorbent assay (ELISA) for TGF-β1 and enzyme-linked immunospot (ELISpot) kits for IFN-γ, IL-4, and IL-17 were purchased from Ebioscience. At 1 month after the completion of treatment, animals were euthanized, and peripheral blood mononuclear cells were purified from spleen or pancreatic lymph nodes using Lympholyte-M (Cedarlane). An aliquot of cells was stained for Treg cell activity using the murine CD4/CD25/FoxP3 flow cytometry kit available from Ebioscience. A total of 1,000,000 splenocytes (or 2 × 10⁵ pancreatic lymph node cells) were incubated with 20 µg/ml GAD65 in RPMI medium supplemented with 10% FCS (Invitrogen) for 48 h. After this incubation period, ELISpot plates were washed and processed according to the manufacturer's protocols. Supernatants were harvested for TGF-β1 ELISA and processed according to the manufacturer's instructions. Developed plates were read using an ImmunoSpot ELISpot reader, and the total number of spots per well was quantified using ImmunoSpot 4.0.17 software (Cellular Technology, Shaker Heights, OH). Lymphocytes from individual animals in each experimental group were analyzed in triplicate. Four-color flow cytometric analysis was carried out using Cell Quest Pro (BD Biosciences, Mississauga, ON, Canada).

Adoptive transfer studies. At 1 month after the completion of treatment, splenocytes were harvested and purified with Lympholyte-M. CD4-positive T-cells were extracted from splenocyte suspensions using magnetic beads (negative selection; Miltenyi Biotec, Mississauga, ON, Canada) and confirmed to be >90% pure by flow cytometry (data not shown). Naïve NOD-RAG^{-/-} males received either 1 × 10⁷ splenocytes from a spontaneously diabetic NOD mouse ("diabetic splenocytes"), 1 × 10⁷ diabetic splenocytes combined with 2 × 10⁶ CD4-positive splenocytes from normoglycemic NOD mice previously treated with anti-IL-17, or 1 × 10⁷ diabetic splenocytes combined with 2 × 10⁶ CD4-positive splenocytes from normoglycemic NOD mice previously treated with IL-25. Blood glucose levels were monitored three times per week thereafter.

Statistics. All statistical analyses in this study were carried out using SigmaPlot 10 and SigmaStat 3.5 (Systat), and results are expressed as the means ± SE. Mann-Whitney rank-sum tests were used, and ANOVA performed on ranks with Bonferroni post hoc analysis was used to analyze multiple groups. Kaplan-Meier survival analyses were compared using the log-rank test.

RESULTS

Inhibition of Th17 cells prevents progression to diabetes in pre-diabetic animals. To explore the potential contribution of Th17 cells to the natural development of type 1 diabetes, either neutralizing anti-IL-17 antibodies or IL-25 were administered to 5-week-old NOD females to investigate the role of this population in the initiation phase of autoimmunity and to 10-week-old NOD females to investigate the role of Th17 cells during the effector phase of autoimmunity. Dosing regimens for anti-IL-17 (100 µg i.p. days on alternating days for 2 weeks) and IL-25 (1 µg s.c. each day for 25 days) were based on results obtained in the EAE model, which effectively controlled antigen-specific Th17 cells (15,18). As shown in Fig. 1A, IL-17 neutralization did not alter diabetes progression when treatment was initiated at 5 weeks of age, and a similar result was obtained using IL-25 beginning at 5 weeks of age (Fig. 1C). In contrast, both anti-IL-17 (Fig. 1B) and IL-25 (Fig. 1D) prevented diabetes development in the majority of treated animals by 6 months of age when treatment was initiated at 10 weeks of age ($P = 0.001$ by log-rank test for each group vs. controls; $n = 10$ per group). These data suggest that Th17 cells are involved in the natural progression of type 1 diabetes, particularly during the effector phase of disease development.

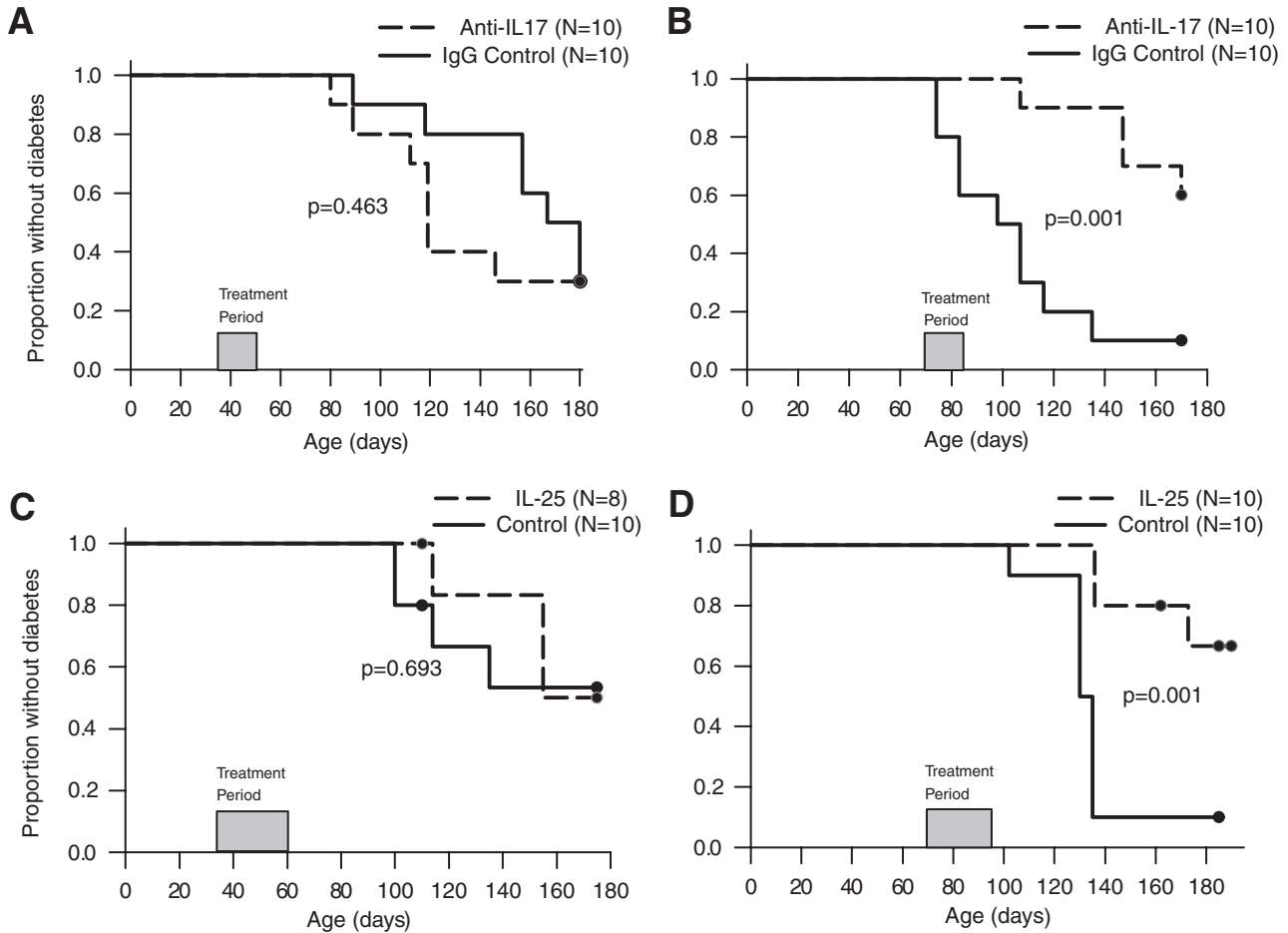


FIG. 1. Inhibition of Th17 cells with either neutralizing anti-IL-17 or IL-25 has no effect when treatment is initiated during the initiation stage of autoimmune diabetes, but treatment significantly reduces the incidence of diabetes when it occurs during the effector stage. The impact of a neutralizing anti-IL-17 antibody as well as the Th17-inhibitory cytokine IL-25 was investigated during two phases of diabetes development in NOD mice. Treatment was initiated at either 5 weeks of age, which represents the initiation stage of autoimmunity, as characterized by mild insulinitis, or at 10 weeks of age, when the effector phase of autoimmunity is occurring, and the majority of NODs will have invasive insulinitis. Anti-IL-17 treatment (100 μ g i.p. on days 0, 2, 4, 6, 8, and 10) did not alter the course of diabetes when it was initiated at 5 weeks of age (A), whereas treatment initiation at 10 weeks of age (B) resulted in a significant increase in the number of nondiabetic animals ($P = 0.001$ by log-rank). Similar results were obtained with recombinant IL-25, which had no effect when treatment was started at 5 weeks of age (C) but significantly increased diabetes-free survival at 10 weeks of age (D) ($P = 0.001$ by log-rank).

Anti-IL-17 and IL-25 treatment reduces islet inflammation. To further understand the mechanism of diabetes prevention using anti-IL-17 or IL-25 treatment during the effector phase of type 1 diabetes development, detailed histological analysis was carried out in normoglycemic animals 1 month after the completion of treatment (serial sections from six to eight animals per group). Insulinitis scoring by a pathologist using blinded samples indicated that both treatment strategies significantly reduced the degree of islet inflammation (Fig. 2). The majority of the pancreata in normoglycemic control animals had destructive insulinitis (mean score of 2.6 ± 0.3), whereas insulinitis was significantly reduced in both anti-IL-17-treated (mean score 2.0 ± 0.3) and IL-25-treated animals (1.5 ± 0.3 ; $P < 0.05$ for anti-IL-17, and $P < 0.02$ for IL-25 vs. controls; representative hematoxylin and eosin-stained sections from each cohort are shown in Fig. 2B–D). To determine the contribution of different T-cell populations within the peri-islet infiltrates in both anti-IL-17- and IL-25-treated animals, immunofluorescence staining for CD4, CD8, Foxp3, and IL-17 was carried out in combination with insulin staining and compared with control animals. As shown in Fig. 3, insulinitic lesions in

normoglycemic control NOD mice were composed primarily of CD4-positive cells, with a smaller proportion of CD8-positive lymphocytes and low Foxp3 staining. Examination of multiple sections of control NOD pancreata for IL-17 staining consistently showed a small number of IL-17-positive cells within the peri-islet infiltrates, regardless of the degree of insulinitis. Pancreatic sections from normoglycemic animals treated with either anti-IL-17 or IL-25 demonstrated a reduced level of insulinitis that was composed primarily of CD4-positive cells, a reduced number of CD8-positive cells, and enrichment in Foxp3-positive cells compared with control animals. Very few IL-17-positive cells were observed in anti-IL-17-treated animals, with only a small proportion of inflamed islets staining positive for IL-17 (representative sections in Fig. 3). Analysis of multiple sections from IL-25-treated animals for IL-17 staining did not reveal any IL-17-positive cells (representative section in Fig. 3). Overall, these data indicate that inhibition of Th17 cells with both anti-IL-17 and IL-25 treatment regimens significantly reduces islet-specific inflammatory T-cell infiltration and increases the proportion of Foxp3-positive cells around the islets.

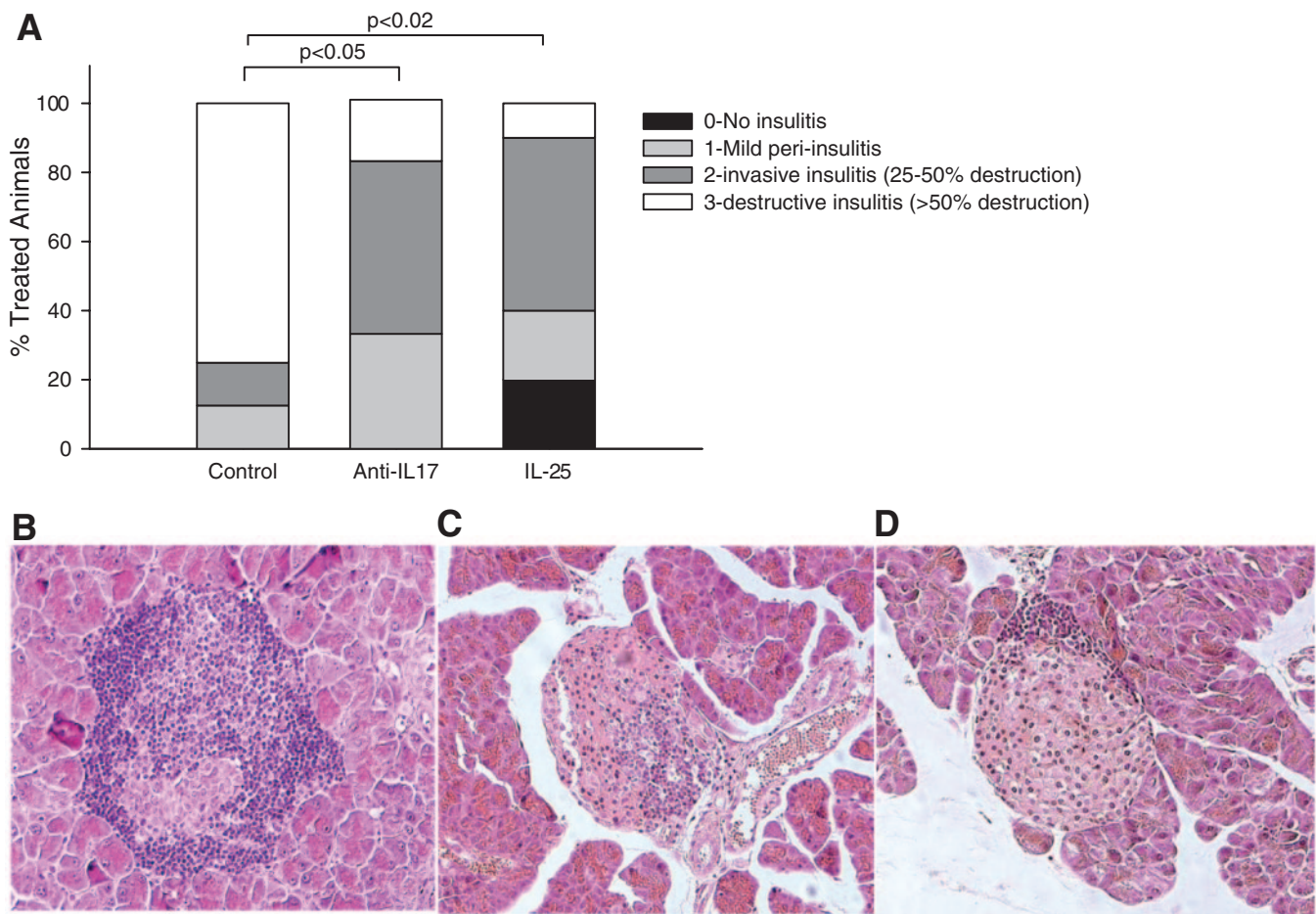


FIG. 2. Both anti-IL-17 and IL-25 reduced the degree of insulinitis in treated animals. **A:** Pancreata from anti-IL-17-treated, IL-25-treated, and control animals were harvested 1 month after the completion of treatment and stained with hematoxylin and eosin. The slides were blinded and scored by a pathologist according to the following scale (based on >50% of the islets in that section exhibiting the associated pattern): 0 (no infiltrate), 1 (mild peri-islet infiltrate), 2 (invasive insulinitis with 25–50% islet destruction), and 3 (destructive insulinitis with >50% islet destruction). Both anti-IL-17 (mean score 2.03 ± 0.33) and IL-25 (mean score 1.50 ± 0.33) treatment markedly reduced insulinitis compared with controls (mean score 2.63 ± 0.26 , $P < 0.05$ for anti-IL-17 vs. control and $P < 0.02$ for IL-25 vs. control by ANOVA). **B–D:** Representative hematoxylin- and eosin-stained islets from each cohort (control [B], anti-IL-17 [C], and IL-25 [D]) are presented at $\times 200$ magnification. (A high-quality digital representation of this figure is available in the online issue.)

Anti-IL-17 and IL-25 treatment prevents GAD65 autoantibody formation. Inhibition of Th17 populations in other models of autoimmunity has resulted in a measurable reduction in autoantibody formation (25,26). To determine the impact of anti-IL-17 and IL-25 therapies on autoreactive B-cells in NOD mice, serum samples were analyzed for anti-GAD65 autoantibodies, a later-stage marker of autoimmunity in this model (27). Initially, serum samples obtained from anti-IL-17- and IL-25-treated animals 1 month after the completion of treatment (effector phase) were analyzed, and a significant reduction in GAD65 autoantibodies was observed in both anti-IL-17-treated (mean optical density 1.11 ± 0.02) and IL-25-treated (1.03 ± 0.03) animals compared with controls (1.34 ± 0.08 ; $P < 0.02$ vs. anti-IL-17, and $P < 0.005$ vs. IL-25 by ANOVA) (Fig. 4A). Based on these data, we chose to prospectively monitor IL-25-treated animals for GAD65 autoantibody levels to determine whether this observation persisted over time (serum samples were not collected prospectively in anti-IL-17 studies and thus could not be analyzed). Animals treated with IL-25 exhibited a persistent reduction in GAD65 autoantibody formation up to 90 days after the completion of treatment ($P < 0.001$ vs. control by ANOVA). These data indicate that inhibition of

Th17 cells in type 1 diabetes can prevent autoantibody formation.

IL-25, but not anti-IL-17, treatment restores euglycemia in newly diabetic mice and delays recurrent autoimmunity after syngeneic islet transplantation. In the NOD mouse model, the initiation and effector phases of disease, before the onset of hyperglycemia, carry the lowest threshold for disease prevention (rev. in 28). However, once the autoimmune response has matured and resulted in hyperglycemia, reversal of diabetes and prevention of recurrent autoimmunity after β -cell replacement represent significant barriers, with only a few therapeutic strategies regulating type 1 diabetes in the NOD mouse at these late-stage disease time points (28). Thus, to understand the role of Th17 cells after the development of overt diabetes, a series of experiments were conducted in two different models: at the time of diabetes onset (attempt to reverse new-onset diabetes) and after a period of rest after diabetes onset with subsequent β -cell replacement via syngeneic islet transplantation (recurrent autoimmunity). Data in Fig. 5A illustrates that anti-IL-17 had no effect once diabetes was established, with all animals remaining persistently diabetic throughout the treatment period. However,

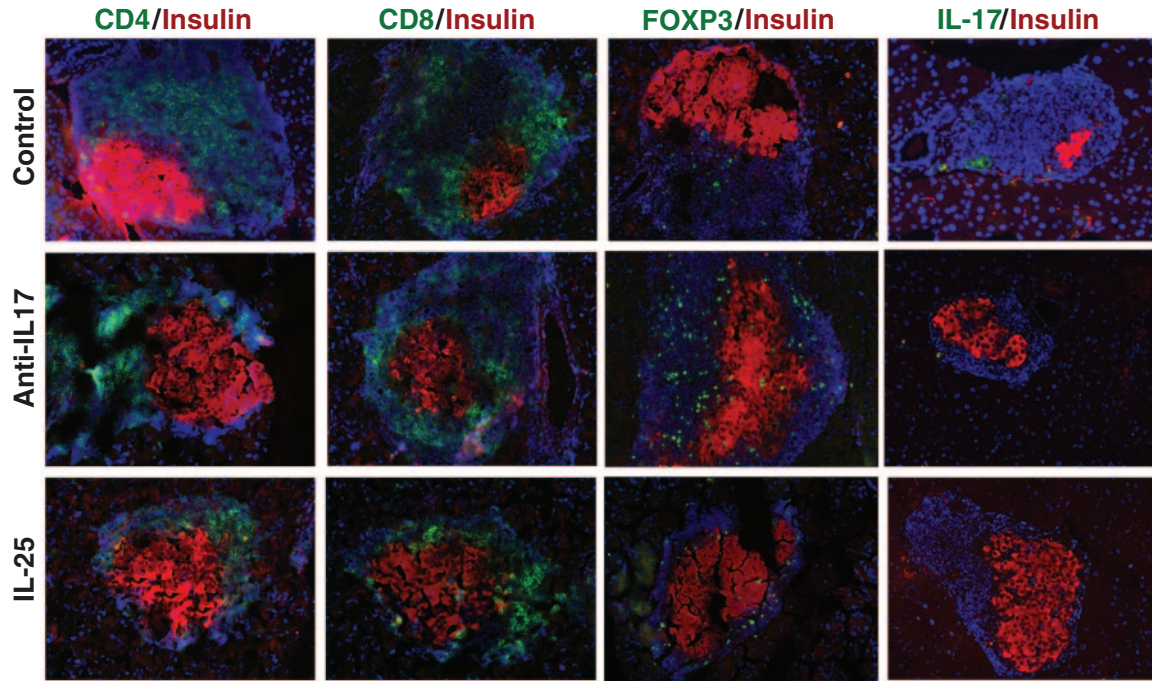


FIG. 3. Treatment with anti-IL-17 or IL-25 reduced the degree of peri-islet T-cell infiltration and was associated with an increase in the frequency of Foxp3-positive cells. Pancreata were collected from anti-IL-17-treated, IL-25-treated, and control animals 1 month after the completion of treatment. Tissue sections were stained for either CD4, CD8, Foxp3, or IL-17 (each in green) in combination with insulin (red) and nuclei (4',6-diamidino-2-phenylindole in blue). Treatment with either anti-IL-17 or IL-25 reduced the frequency of both CD4- and CD8-positive T-cells and increased the number of Foxp3-positive Treg cells in the peri-islet infiltrate compared with controls. IL-17-positive staining was only visible in a small proportion of cells present within the insulinitic lesion in control animals, and this frequency was further reduced after treatment with anti-IL-17 or IL-25. CD4, CD8, and Foxp3 staining was completed on cryosections, whereas IL-17 staining was completed on fixed sections, resulting in a difference in appearance on photography. Pancreata harvested from $n = 6-8$ normoglycemic animals from each treatment group were analyzed, and representative sections from each combination of staining are shown at $\times 200$ magnification. (A high-quality digital representation of this figure is available in the online issue.)

daily treatment with IL-25 resulted in remission in 90% of treated animals, versus none of the controls ($P < 0.0001$ by ANOVA, and $P = 0.002$ by Fisher's exact test) (Fig. 5B). Ultimately, most animals returned to hyperglycemia by 10 days after initiation of treatment, despite ongoing therapy, although one animal did exhibit persistent normoglycemia for >100 days even after IL-25 treatment withdrawal at day 30 (data not shown). This enhanced efficacy of IL-25 compared with anti-IL-17

was also observed in recurrent autoimmunity after syngeneic islet transplantation, where IL-25 nearly doubled islet graft survival time from 4.2 ± 0.8 days in control animals to 7.2 ± 0.2 days in treated animals ($P = 0.0013$ by log-rank test). These studies indicate that IL-25, which is known to directly inhibit Th17 populations, is superior to IL-17 neutralization in regulating a mature autoimmune response after the onset of hyperglycemia.

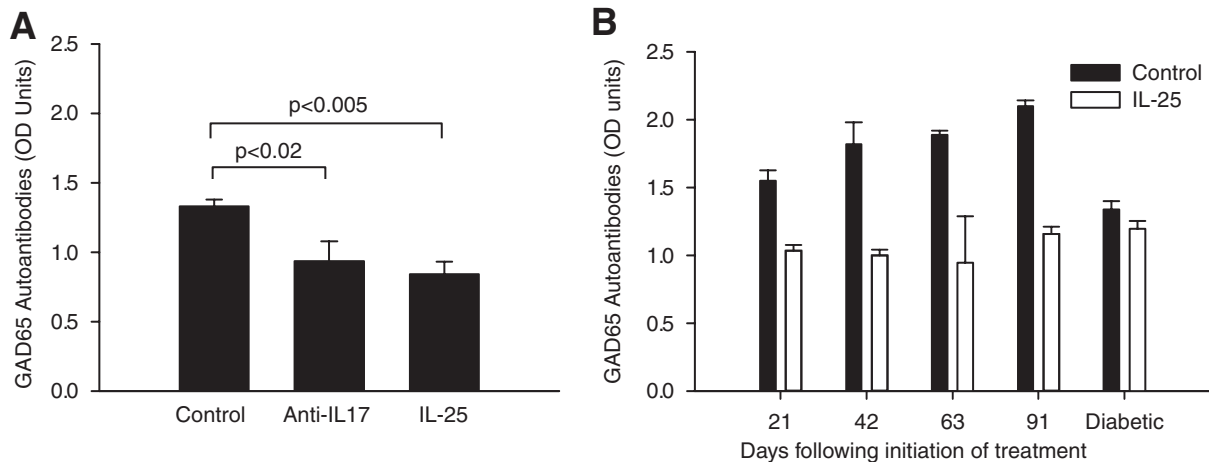


FIG. 4. Treatment with anti-IL-17 or IL-25 prevents GAD65 autoantibody formation. Serum samples were collected in anti-IL-17-treated, IL-25-treated, and control animals 1 month after the completion of treatment and analyzed for GAD65 autoantibodies using an ELISA. **A:** Both anti-IL-17 and IL-25 treatment regimens were associated with a significant reduction in the number of GAD65 IgG antibodies compared with control animals ($P < 0.02$ for anti-IL-17 vs. control and $P < 0.005$ for IL-25 vs. control by ANOVA). **B:** Animals treated with IL-25 were followed prospectively for GAD65 autoantibody development, and this treatment effectively prevented autoantibody formation >90 days after the initiation of treatment ($P < 0.001$ by ANOVA). A minimum of $n = 4$ animals were analyzed at each time point. OD, optical density.

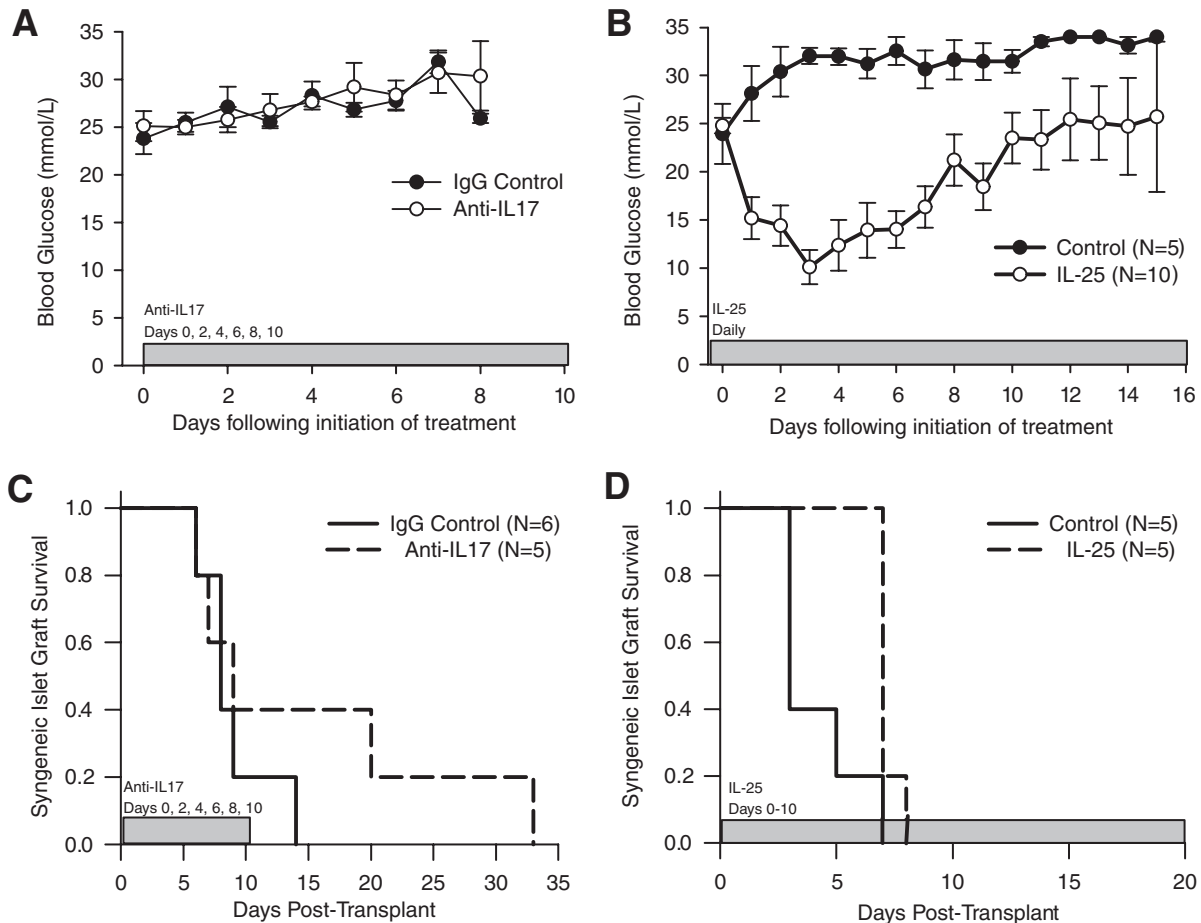


FIG. 5. Treatment with IL-25, but not anti-IL-17, can reverse new-onset diabetes and delay recurrent autoimmunity after syngeneic islet transplantation. Naïve spontaneously diabetic (blood glucose >18 mmol/l) NOD mice were randomly assigned to receive either anti-IL-17 (100 μ g i.p. every other day), IL-25 (1 μ g s.c. daily), or control (IgG for anti-IL-17, vehicle for IL-25). **A:** Treatment with anti-IL-17 did not reverse hyperglycemia after new-onset diabetes in NOD mice. **B:** Treatment with IL-25 resulted in a period of normoglycemia (mean 8.53 ± 2.77 days) in 9 of 10 animals, whereas none of the controls returned to normoglycemia ($P < 0.0001$ by ANOVA). One IL-25-treated animal experienced permanent remission beyond 100 days and after the discontinuation of IL-25 treatment at day 30 (data not shown). **C:** Although anti-IL-17 treatment did result in prolongation of syngeneic islet graft survival in 2 of 5 animals, no significant difference in recurrent autoimmunity was observed compared with IgG-treated controls. $P = 0.346$ by log-rank. **D:** Treatment with IL-25 delayed recurrent autoimmunity after syngeneic islet transplantation (mean survival time of 7.2 ± 0.2 days in IL-25-treated animals vs. 4.2 ± 0.8 days in vehicle-treated animals; $P = 0.0013$ by log-rank).

IL-25 treatment reduces the frequency of autoreactive Th2 and Th17 T-cells and results in the development of a Treg-enriched CD4-positive T-cell population that dominantly protects against disease transfer. Although both anti-IL-17 and IL-25 therapies were able to reduce the incidence of type 1 diabetes during the effector phase leading into type 1 diabetes, only IL-25 therapy was able to control diabetes once the disease was established. To further investigate the different mechanisms by which these two therapies function, splenocytes from normoglycemic treated animals in the prevention studies (Fig. 1) were examined ex vivo for autoreactive T-cell populations using GAD65-stimulated ELISpot assays at 1 month after the completion of treatment. Although no difference in IFN- γ -secreting GAD65-responsive splenocytes was observed compared with controls (Fig. 6A), a significant reduction in IL-4-secreting GAD65-responsive splenocytes was observed in IL-25-treated animals compared with both anti-IL-17-treated and control animals ($P < 0.02$) (Fig. 6B). Paradoxically, anti-IL-17 treatment resulted in an increased frequency of IL-17-secreting GAD65-responsive splenocytes, whereas the opposite occurred after IL-25 treatment, where a significant reduction

in this autoreactive Th17 population was observed ($P < 0.001$ for anti-IL-17 vs. control and IL-25, and $P < 0.05$ by ANOVA for IL-25 vs. control) (Fig. 6C).

Next, a series of adoptive transfer experiments was carried out using immunodeficient NOD-RAG^{-/-} recipients. In this model, transfer of 1×10^7 splenocytes from a recent-onset diabetic NOD mouse results in hyperglycemia in all recipients (control mean diabetes onset at 42.8 ± 2.3 days post-transfer) (Fig. 6D). To evaluate whether the protective effects of anti-IL-17 or IL-25 treatment could dominantly control autoreactive T-cell populations, 2×10^6 purified CD4-positive splenic T-cells, harvested from either anti-IL-17 or IL-25 treated animals 1 month after the completion of treatment, were coinjected with 1×10^7 diabetic splenocytes into naïve normoglycemic NOD-RAG^{-/-} recipients. In these experiments, cotransfer of CD4-positive splenocytes from animals previously treated with anti-IL-17 resulted in no delay in diabetes development (anti-IL-17 mean diabetes onset at 41.8 ± 4.8 days), demonstrating that anti-IL-17 treatment does not alter the CD4-positive T-cell compartment sufficiently to regulate effector diabetogenic splenocytes. In contrast, CD4-positive splenocytes harvested from animals previously

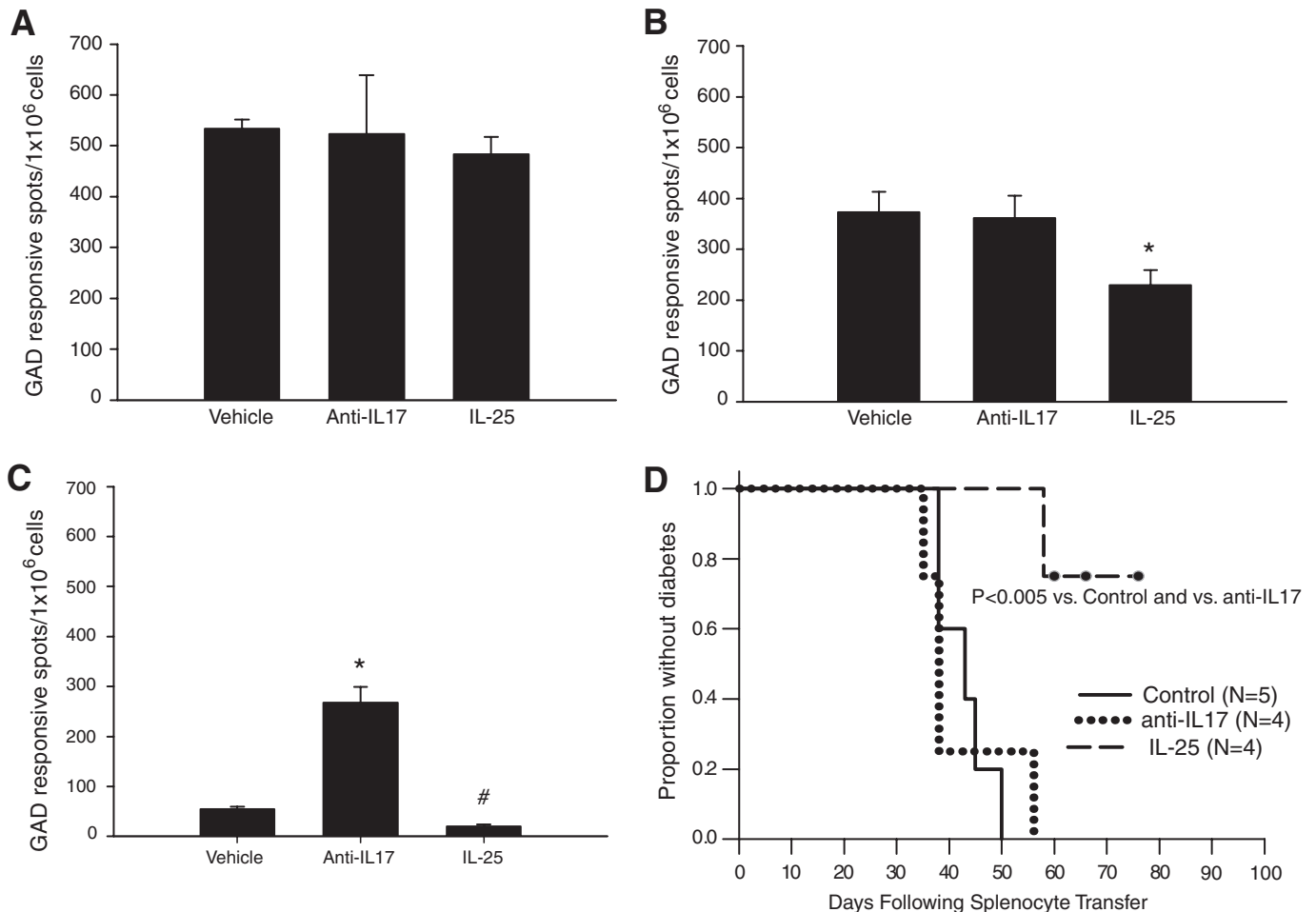


FIG. 6. IL-25 treatment reduces the frequency of autoreactive Th2 cells and Th17 cells and leads to the formation of a CD4-positive splenocyte population that can prevent type 1 diabetes development in an adoptive transfer model. At 1 month after the completion of treatment, splenocytes were harvested from either anti-IL-17-treated, IL-25-treated, or control animals ($n = 3-4$ animals per treatment group; all normoglycemic) that had been previously treated beginning at 10 weeks of age. Purified splenocytes were analyzed using cytokine ELISpot assays or adoptively transferred into NOD-RAG^{-/-} recipients. **A:** No difference in the frequency of GAD65-responsive, IFN- γ -secreting splenocytes was observed in either treatment group versus controls. **B:** IL-25 treatment resulted in a reduction in the number of GAD65-responsive, IL-4-secreting splenocytes compared with both anti-IL-17-treated animals ($*P < 0.02$ for IL-25 vs. anti-IL-17 and control by ANOVA), suggesting that IL-25 treatment can reduce the frequency of autoreactive Th2 cells. **C:** Whereas IL-25 treatment was associated with a reduction in the number of GAD65-responsive, IL-17-secreting splenocytes ($\#P < 0.05$ vs. control by ANOVA), anti-IL-17 treatment significantly increased the frequency of this population ($*P < 0.001$ by ANOVA vs. control and IL-25). **D:** CD4-positive lymphocytes were further purified from these splenocyte preparations using magnetic beads. Naïve NOD-RAG^{-/-} males received either 1×10^7 splenocytes harvested from spontaneously diabetic NOD mice (diabetic splenocytes) combined with 2×10^6 CD4-positive splenocytes isolated from mice previously treated with anti-IL-17, 1×10^7 diabetic splenocytes combined with 2×10^6 CD4-positive splenocytes from mice previously treated with IL-25, or 1×10^7 diabetic splenocytes with no CD4 supplementation (control). Animals were monitored three times per week thereafter for diabetes onset. Supplementation with CD4-positive cells harvested from mice previously treated with IL-25 resulted in a significant dominant protective effect, preventing diabetes development in 75% of the animals in this group ($P < 0.005$ vs. anti-IL-17 and control by log-rank). Supplementation with CD4-positive cells from animals previously treated with anti-IL-17 had no effect, with 100% of the animals becoming diabetic within 60 days post-transfer, which was comparable to the diabetes incidence observed in the control group.

treated with IL-25 exerted a dominant protective effect against diabetogenic splenocytes, resulting in diabetes prevention in 70% of reconstituted recipients by 80 days post-transfer (Fig. 6D).

Based on the results obtained in these adoptive transfer studies, further experiments were conducted to characterize the splenic- and pancreatic lymph node-derived lymphocyte populations in animals previously treated with either anti-IL-17 or IL-25. Flow cytometric analysis of these different lymphocyte sources revealed a significant increase in CD4/CD25/Foxp3-positive Treg cells compared with vehicle-treated controls (Fig. 7), whereas no difference in the relative frequency of CD8-positive T-cells was observed (data not shown). To further understand the function of these lymphocytes, splenic- and pancreatic lymph node-derived lymphocytes were incubated with

GAD65 for 48 h, and afterward the supernatant was assayed for TGF- β 1 activity, a key cytokine that drives expansion of the Treg cell population. Data in Fig. 7C demonstrate that prior treatment with either anti-IL-17 or IL-25 resulted in a significant increase in TGF- β 1 activity from pancreatic lymph node lymphocytes, with IL-25 treatment resulting in greater TGF- β 1 activity even when compared with animals previously treated with anti-IL-17. No difference in TGF- β 1 activity was observed among splenocyte populations within the three treatment groups. Taken together, these experiments indicate that IL-25 treatment alters the T-cell repertoire in NOD mice, reducing the frequency of autoreactive GAD65-responsive Th2 and Th17 cells and promoting the development of a dominantly protective CD4-positive T-cell population with a relative enrichment in Treg cell populations.

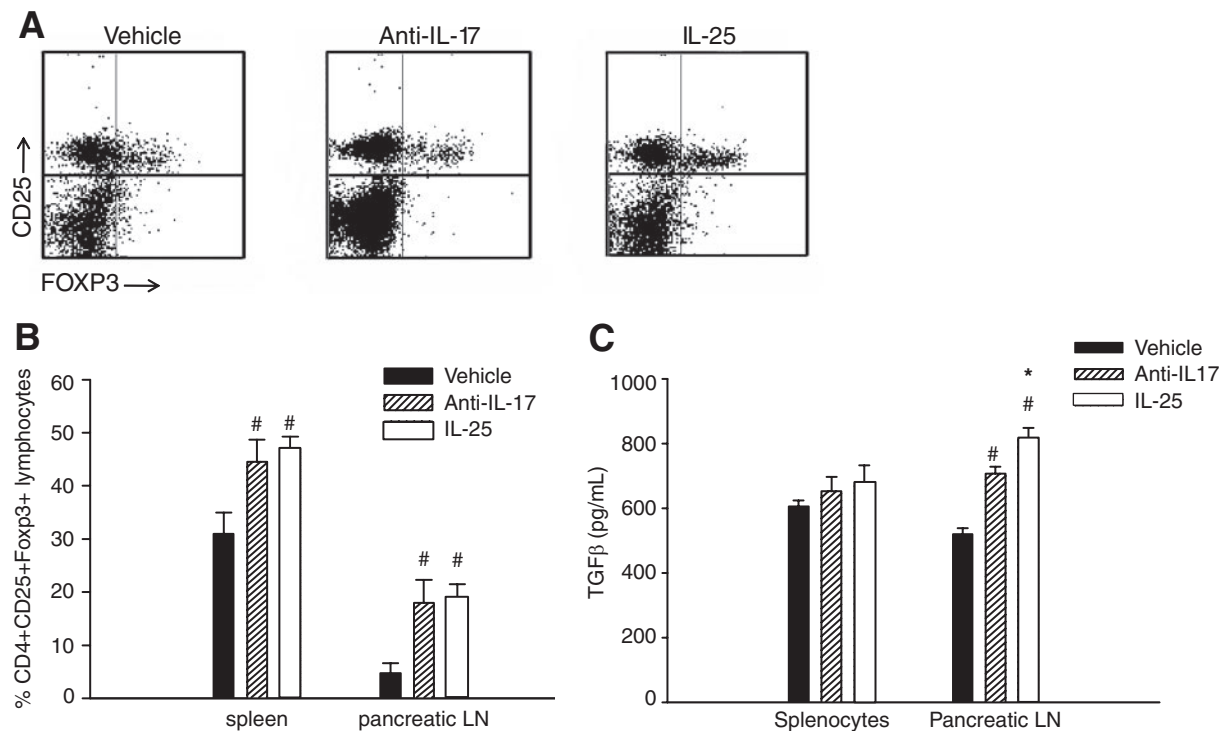


FIG. 7. Treatment with anti-IL-17 or IL-25 increases the frequency of Treg cells within pancreatic lymph nodes. At 1 month after the completion of treatment, pancreatic draining lymph nodes or spleens were harvested from either anti-IL-17-treated, IL-25-treated, or control animals ($n = 3-4$ animals per treatment group), and lymphocytes were extracted. Purified lymphocytes were analyzed using flow cytometry and TGF- β 1 ELISA to quantify the Treg cell activity present in each immune compartment. **A:** Representative flow cytometry panels from pancreatic lymph node cells harvested from each treatment group are shown. Each panel was first gated on lymphocytes and CD4-positive cells, and the dot plots shown compare CD25 staining (y -axis) versus FoxP3 staining (x -axis). **B:** The percentage of CD4-, CD25-, and FoxP3-positive Treg cells derived from spleen and pancreatic lymph nodes are shown. Treatment with either anti-IL-17 or IL-25 resulted in a significant increase in the proportion of Treg cells present in both the spleen and the pancreatic lymph node. $\#P < 0.05$ vs. vehicle. **C:** After purification, 2×10^5 cells from each animal in each treatment group were incubated with GAD65 for 48 h, and the supernatants were subsequently harvested for analysis using TGF- β 1 ELISA. Treatment with anti-IL-17 or IL-25 resulted in a significant increase in TGF- β 1 among pancreatic lymph node-derived lymphocytes compared with controls, whereas no difference was observed in splenic lymphocytes. $*P < 0.001$ vs. vehicle; $\#P < 0.005$ vs. anti-IL-17.

DISCUSSION

The recently characterized pathogenic Th17 population has been linked to a number of organ-specific autoimmune diseases (29) and is currently being investigated as a clinical therapeutic target in autoimmunity. The current study demonstrates for the first time that inhibition of Th17 cells, either with neutralizing anti-IL-17 or with recombinant IL-25, can impact the course of diabetes in NOD mice, indicating that the Th17 population is a major contributing factor in this model. Initially, the impact of these treatments was investigated in two age-groups that correlate with different stages in the development of autoimmunity. The impact on diabetes was most evident when treatment with anti-IL-17 or IL-25 was introduced during the active phase of autoimmunity preceding the development of overt disease. The observation that these treatments could reduce autoimmune β -cell destruction was confirmed by scoring of insulinitis and measurement of GAD65 autoantibodies, which was significantly reduced in both anti-IL-17- and IL-25-treated animals. Taken together, these data indicate that the Th17 population is less involved in the initiation of autoimmune diabetes and primarily contributes to the active phase of disease development in this model.

Further characterization of the peri-islet T-cell infiltrates revealed that both anti-IL-17 and IL-25 treatments reduced the degree of CD4- and CD8-positive infiltrates while increasing the proportion of Foxp3-positive cells, a marker of the Treg cell population (Fig. 3). These data were

confirmed by flow cytometric analysis of splenocytes and pancreatic lymph node lymphocytes, which revealed that either treatment resulted in a relative increase in the Treg cell population (Fig. 7). Interestingly, the frequency of Th17 cells within the pancreas was very low at all stages of disease, with only a small fraction of the peri-islet mononuclear cell infiltrate staining positive for IL-17, the characteristic marker of this population (Fig. 3). We observed that treatment with anti-IL-17 reduced the frequency of peri-islet IL-17-positive cells even further, whereas treatment with IL-25 resulted in no identifiable IL-17-positive infiltrating cells (Fig. 3). The relatively low frequency of Th17 cells within the insulinitic lesions suggests that this population is more involved in directing an immune response in the secondary lymphoid tissues rather than participating directly in β -cell destruction. This concept is supported by data using the EAE model, which demonstrates that pathogenic Th17 cells facilitate the development of autoreactive effector T-cells during antigenic priming within cervical draining lymph nodes and that the Th17 population remains dominant within this compartment throughout the course of disease (30,31). Our finding that inhibition of Th17 cells with anti-IL-17 or IL-25 leads to enrichment in peri-islet Foxp3-positive cells and pancreatic lymph node lymphocytes is also consistent with the Th17/Treg paradigm because it is known that these populations exert mutually opposing effects (7). Thus, our data suggest that inhibition of Th17 cells allows the Treg

cell population to expand and counteract the development of autoimmunity in this model.

To explore the impact of Th17 inhibition on the autoimmune response in more detail, B- and T-cell reactivity to the later-stage autoantigen GAD65 was analyzed (27). Previous treatment with either anti-IL-17 or IL-25 prevented GAD65 autoantibody formation, and this effect was durable over time (Fig. 4). This result is in keeping with data generated in a mouse model of inducible autoimmune myasthenia gravis, which demonstrated that IL-17 knockout animals are resistant to autoantibody formation primarily as a result of the loss of help signals from Th17 cells during the generation of autoreactive B-cells (32). Data in the current study also indicate that inhibition of the Th17 population resulted in a similar effect in autoreactive T-cells. Ex vivo analysis of splenocytes from animals previously treated with anti-IL-17 or IL-25 using ELISpot assays established that inhibition of Th17 cells alters the frequency of GAD65-responsive T-cell populations (Fig. 6). Surprisingly, no difference was observed in the frequency of GAD65-responsive IFN- γ -secreting splenocytes between both treatment groups and controls, despite the marked difference in disease outcomes as observed in Fig. 1. Our data indicate that treatment with these Th17-inhibitory therapies resulted in enrichment in Treg cell populations, which likely control the pathogenic effects of autoreactive Th1 cells in the vicinity of the islet.

As expected, treatment with IL-25 significantly reduced the frequency of GAD65-responsive IL-17-secreting cells (Fig. 6C), which is consistent with previous studies illustrating that this cytokine can inhibit formation of the Th17 population (18). The opposite effect was observed in splenocytes harvested from animals previously treated with anti-IL-17, which possessed a significant increase in the number of GAD65-responsive IL-17-secreting cells (Fig. 6C). The relative importance of this expanded Th17 population in these anti-IL-17-treated animals is unclear because this treatment prevented diabetes development (Fig. 1B). A possible explanation for these data are the findings that IL-17 secretion exerts autocrine and paracrine effects on Th17 cells, sending a negative feedback signal to inhibit proliferation (33). Thus, IL-17 neutralization could in theory remove that negative signaling event and enhance the development of Th17 cells. If this were the only effect of anti-IL-17 treatment, one would anticipate that autoimmunity would be accelerated in treated animals, rather than prevented, as shown in this study and others (9,14,15). Another possibility involves the "Spren effect," where anticytokine monoclonal antibody treatment paradoxically results in enhanced receptor signaling (34). Further investigation of the mechanism of anti-IL-17 therapy on autoimmunity in animal models and ongoing clinical trials will likely reveal the dominant pathway involved in modulation of autoimmunity using this approach.

The most intriguing finding in the current study relates to the potent effect of IL-25 treatment on the mature autoimmune response in NOD mice after the onset of hyperglycemia. It is well known that therapeutic interventions that can prevent diabetes in this model rarely mediate late-stage autoimmunity, resulting in reversal of disease (28). We explored the impact of both anti-IL-17 and IL-25 treatment using two different models of mature autoimmune responses, either immediately after disease onset or after a period of rest and subsequent syngeneic islet transplantation. As shown in Fig. 5, IL-25 therapy

restored normoglycemia in newly diabetic animals and significantly delayed recurrent autoimmunity after islet transplantation, whereas anti-IL-17 had no effect in either setting. These findings were particularly surprising given the short serum half-life of IL-25 after subcutaneous injection, with a peak concentration at 1 h and no detectable levels by 6 h postinjection (see supplemental Fig. A1, available in an online appendix at <http://diabetes.diabetesjournals.org/cgi/content/full/db08-1113/DC1>). It is likely that the therapeutic effect of IL-25 treatment persists longer because it inhibits Th17 populations and thus may result in a protracted immunological impact. Measuring this effect ex vivo after IL-25 treatment directly would be challenging; however, adoptive transfer studies using CD4-positive splenocytes harvested from animals previously treated with IL-25 indicated that IL-25 induces the formation of a dominant protective T-cell population that can control the mature autoimmune response, even after treatment withdrawal (Fig. 6D). Our data indicate that this result was likely due to a reduction in Th17 cells, expansion of Treg cells, and/or a combination of both effects. That anti-IL-17 had no effect on the mature autoimmune response in the current study is not all that surprising, given that it only neutralizes the effector molecule of the Th17 population without sending any direct negative signals. Thus, if the Th17 population has already developed and expanded, neutralizing the effector molecule IL-17 will have minimal impact on the course of disease. In fact, as mentioned previously, IL-17 has been shown to exhibit negative feedback to the Th17 population (33), so neutralizing this cytokine at later disease points may be disadvantageous.

In summary, the current study indicates that the Th17 population is involved in the pathogenesis of autoimmune diabetes in the NOD mouse and that intervention with treatments that inhibit this subset can alter the course of disease, even after the autoimmune response has evolved into overt diabetes. Based on these data, further exploration of this subset in the NOD model and in patients with type 1 diabetes is warranted. The current study also indicates that further ongoing development of agents like IL-25 that can directly inhibit the Th17 population will be superior to approaches using IL-17 neutralization in autoimmune diabetes. Whereas recombinant IL-25 may not represent the best therapeutic molecule because of its short half-life and potential role in allergic airway inflammation (35), this study and others provide proof of concept that this therapeutic target should be developed further for use in autoimmune disease (18).

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No potential conflicts of interest relevant to this article were reported.

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