Prevention of Autoimmune Diabetes by Ectopic Pancreatic β-Cell Expression of Interleukin-35

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Interleukin (IL)-35 is a newly identified inhibitory cytokine used by T regulatory cells to control T cell–driven immune responses. However, the therapeutic potential of native, biologically active IL-35 has not been fully examined. Expression of the heterodimeric IL-35 cytokine was targeted to β-cells via the rat insulin promoter (RIP) II. Autoimmune diabetes, insulitis, and the infiltrating cellular populations were analyzed. Ectopic expression of IL-35 by pancreatic β-cells led to substantial, long-term protection against autoimmune diabetes, despite limited intraislet IL-35 secretion. Nonobese diabetic RIP-IL35 transgenic mice exhibited decreased islet infiltration with substantial reductions in the number of $CD4^+$ and $CD8^+$ T cells, and frequency of glucose-6-phosphatase catalytic subunit–related protein-specific $CD8^+$ T cells. Although there were limited alterations in cytokine expression, the reduced T-cell numbers observed coincided with diminished T-cell proliferation and G1 arrest, hallmarks of IL-35 biological activity. These data present a proof of principle that IL-35 could be used as a potent inhibitor of autoimmune diabetes and implicate its potential therapeutic utility in the treatment of type 1 diabetes. ***Diabetes*** **61:1519–1526, 2012**

Type 1 diabetes is an autoimmune chronic disorder in which a self-reactive immune response leads to the targeted destruction of insulin-producing β-cells (1,2). The main sign of disease, a rise in blood glucose, manifests only after the majority of β-cells already have been destroyed. The late diagnosis limits the types of therapies that can be implemented to restore β-cell function and euglycemia. Although daily administrations of insulin are sufficient for disease management, a cure is likely to involve the transplantation of cadaver-derived islets or glucose-responsive, insulin-producing cells. However, the survival of the transplants will require either deleterious, long-term immunosuppression or the development of therapeutic approaches to establish long-term graft tolerance to prevent autoimmune and allogeneic destruction.

Nonobese diabetic (NOD) mice have been the best available model for human type 1 diabetes research (3,4). Several immunoregulatory and antiapoptotic molecules already have been tested for their efficacy in the prevention of diabetes onset and islet graft protection in the NOD mouse by restricted transgenic expression in pancreatic β-cells via the rat insulin promoter (5). Most of these failed to prevent autoimmune diabetes, whereas some unexpectedly exacerbated disease. A notable exception was decoy receptor 3, which prevents FasL- and LIGHT (lymphotoxin-like, exhibits inducible expression and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes)-induced β-cell apoptosis and provides 100% protection on the NOD background (6). Although interleukin (IL)-4 and transforming growth factor (TGF) β did reduce diabetes onset on the NOD background (7,8), significant complications were observed with TGFβ disrupting islet development, whereas crossing IL-4 transgenic to BDC2.5 T-cell receptor (TCR) transgenic resulted in exacerbated disease (8,9). Thus, it remains to be determined whether an immunoregulatory mediator can be identified that is potent enough to protect β-cells without causing severe complications.

IL-35 is a recently discovered immunoregulatory cytokine that is secreted by $CD4^+Foxp3^+$ T regulatory cells (T_{regs}) or iTr35, a regulatory T-cell population induced by IL-35 (10,11). IL-35 has been shown to have regulatory and therapeutic potential in mouse models of several inflammatory disorders, including inflammatory bowel disorder and multiple sclerosis. IL-35 acts primarily by inhibiting T-cell proliferation and can further amplify its effects by inducing a regulatory population capable of suppressing immune responses via IL-35 (10). We hypothesized that the selective but potent immunoregulatory properties of IL-35 will result in protection against autoimmune diabetes without causing adverse complications. To test the therapeutic potential of IL-35, we generated NOD transgenic mice with restricted expression of IL-35 in β-cells and assessed the development of autoimmune diabetes and the mechanism underlying the disease resistance observed.

RESEARCH DESIGN AND METHODS

The NOD.RIP-IL35 transgenic mice were generated by John Stockton at the Manipulated NOD Mouse Core at the Joslin Diabetes Centre (Harvard Medical School, Boston, MA). The transgene vector was made by cloning mouse IL-35 (p35 and EBI3 chains joined by cleavable P2A sequence) into a pBR322 vector, downstream of the rat insulin promoter sequence and a rabbit globin intron (a kind gift from Christophe Benoist, Joslin Diabetes Center, Harvard Medical School). Plasmid DNA was purified using the Endo-Free Maxi-Prep kit (Qiagen, Valencia, CA) and cut using the restriction enzymes *Bam*HI and *Bgl*II. The repurified DNA was dialyzed and used for microinjection. NOD.*scid*, NOD/ShiLtJ, and NOD.129S2(B6)-Ins2tm1Jja/GseJ (NOD insulin 2 knockout mice, referred to as NOD.$Ins2^{-/-}$ hereafter) mice were obtained from The Jackson Laboratories. All mice were bred and housed at the St. Jude Animal Resources Center (Memphis, TN) in a *Helicobacter*-free specific pathogen-free facility following state, national, and institutional mandates. The St. Jude Animal Resources Center is accredited by the American Association for the Accreditation of Laboratory Animal Care. All animal experiments followed animal protocols approved by the St. Jude Institutional Animal Care and Use Committee.

Immunofluorescent analysis. Antigen-retrieval of tissue slides was achieved by microwaving in citrate buffer (10 mmol/L citric acid, 0.05% Tween-20, pH 6) for 5 min. Sections were probed with anti-EBI3 polyclonal rabbit antibody (M-75; Santa Cruz) or anti-p35 rat antibody (45806; R&D Systems) in combination with guinea pig anti-insulin antibody (Novus Biologicals, Littleton, CO) and mouse anti-glucagon antibody (Sigma-Aldrich, St. Louis, MO), followed by staining with anti-host secondary antibodies conjugated to Alexa Fluor-488,

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-555, or -647 (Invitrogen). Images were captured using Slidebook (Intelligent Imaging Innovation, Denver, CO).

Enzyme-linked immunosorbent assay. Whole islets obtained from individual NOD.*scid*.RIP-IL35^{A}, NOD.*scid*.RIP-IL35^{B}, or NOD.*scid* mice were incubated at 100 islets per 150 μL complete RPMI media with 11 mmol/L glucose for 24 h. Supernatant was analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA) with anti-p35 antibody (clone 8G7; provided by Jacques Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium) as the capture reagent and biotinylated antibody DV1.4H6.29 as the detection reagent, followed by streptavidin-conjugated horseradish peroxidase (GE Healthcare) and tetramethylbenzidine substrate (Sigma). As a control, supernatant from 293T cells transfected with p35-P2A-EBI3 construct (as previously described in 11) was used. The reaction was terminated with 1 N H_2SO_4 and read on plate reader at 450 nm.

Assessment of insulitis and diabetes. Pancreata of NOD mice were harvested at the indicated age, placed into 10% buffered formalin, and embedded in paraffin; 4-μm-thick sections were cut at 150-μm step sections and stained with hematoxylin and eosin at the St. Jude Histology Core Facility. Islets (90–100 per mouse) were scored in a blinded manner using the method outlined in *Current Protocols in Immunology* (12). Diabetes incidence was monitored weekly by testing for the presence of glucose in the urine by Clinistix (Bayer, Elkhart, IN). Mice testing positive by Clinistix then were tested with a Breeze2 glucometer (Bayer, Elkhart, IN) for elevated blood glucose levels and were considered diabetic if their blood glucose was >400 mg/dL.

Islet isolation. Pancreata were perfused by injecting 3 mL collagenase 4 (Worthington, Lakewood, NJ) (400 units/mL in Hanks' balanced salt solution [HBSS] and 10% fetal bovine serum [FBS]), harvested, and placed in 3–5 mL collagenase 4. The pancreata then were incubated at 37°C for 25 min, after which they were washed three times with 7 mL 5% FBS/HBSS and resuspended in 10 mL 5% FBS/HBSS. Islets were handpicked and incubated at 37°C for 15 min in 1 mL cell dissociation buffer (Invitrogen, Carlsbad, CA) and then further dissociated by vortexing and pipetting. Cells were then washed in 10 mL 5% FBS/HBSS, counted, and analyzed by flow cytometric analysis.

Flow cytometric analysis, sorting, and tetramer enrichment. Flow cytometric analysis was performed on BD LSRII flow cytometer system, and analyzed by FlowJo analysis software. Flow cytometric–based cell sorting was performed on an iCyt Reflection Highly Automated Parallel Sorter. Antibodies to CD4, CD8a, CD25, CD45RB, IL-2, interferon-γ, tumor necrosis factor (TNF) α, IL-10, and Foxp3 were obtained from Biolegend. Antibodies to Ki67 and IL-17 were obtained from BD Pharmingen. Glucose-6-phosphatase catalytic subunit–related protein (IGRP)-specific CD8^{+} tetramer (NRP-V7) KYNKANVFL/H-2K(d) was obtained from the National Institutes of Health Tetramer Core. Surface antigen and tetramer staining was conducted on ice for 30 min. An intracellular cytokine staining kit was obtained from BD Pharmingen. Intracellular cytokine staining was conducted after 5 h stimulation at 37°C with 10 ng/mL phorbol myristic acid (PMA) (Sigma) and 1 μmol/L ionomycin (Sigma) in the presence of brefeldin A (BD Pharmingen). Foxp3 staining buffers were obtained from eBioscience and also were used for Ki67 staining. NRP-V7 tetramer enrichment was performed on pooled peripheral lymphoid organs (excluding pancreatic lymph nodes [PLNs]) as described previously (13). In brief, pooled organs were labeled with NRP-V7 (KYNKANVFL/H-2K[d]) tetramer, followed by anti-PE magnetic-activated cell sorting (MACS) bead (Miltenyi Biotec) purification, and the positively selected fraction was counted and analyzed by flow cytometry.

Induction of diabetes by adoptive transfer. Diabetes was adoptively transferred by injecting NOD.*scid* or NOD.*scid*.RIP-IL35^{B} mice intravenously with 10^{7} 6- to 7-week-old female NOD wild-type splenocytes or 10^{5} NY4.1 sorted retrogenic cells. NY4.1 retrogenic mice were generated as described previously (14,15), and spleens were harvested 5–6 weeks after bone marrow transfer, sorted for CD4^{+}GFP^{+} T cells, and injected into NOD.*scid* or NOD.*scid*.RIP-IL35^{B} mice intravenously. In some experiments, CD4^{+} T cells were sorted from the islets of 10-week-old NOD or NOD.RIP-IL35^{B} mice and adoptively transferred intraperitonially at 5,000 cells per NOD.*scid* recipient in combination with an intravenous injection of 10^{7} CD4^{+} T cell–depleted splenocytes from 7-week-old wild-type NOD mice.

In vivo proliferation. In vivo proliferation was assessed with a BD Pharmingen BrdU allophycocyanin kit. In brief, 2 mg of BrdU were injected in 200 μL PBS i.p. into 10-week-old female mice; 4 h after BrdU injection, organs were harvested and processed. Cells were stained with antibodies to cell surface CD4 or CD8 and nuclear BrdU, as per manufacturer's instructions.

Statistical analysis. Time to the onset of diabetes was analyzed using the Kaplan-Meier method, and groups were compared using the log-rank test. All other group comparisons were made using the Mann-Whitney nonparametric test. All statistical tests were two tailed, and P values <0.05 were considered statistically significant. All statistically significant differences are noted in figures and figure legends. Statistical analysis was performed using Prism software.

RESULTS

IL-35 expression in β-cells protects NOD mice against autoimmune diabetes. To investigate the therapeutic potential of IL-35 in preventing autoimmune diabetes, transgenic NOD mice expressing IL-35 under the rat insulin

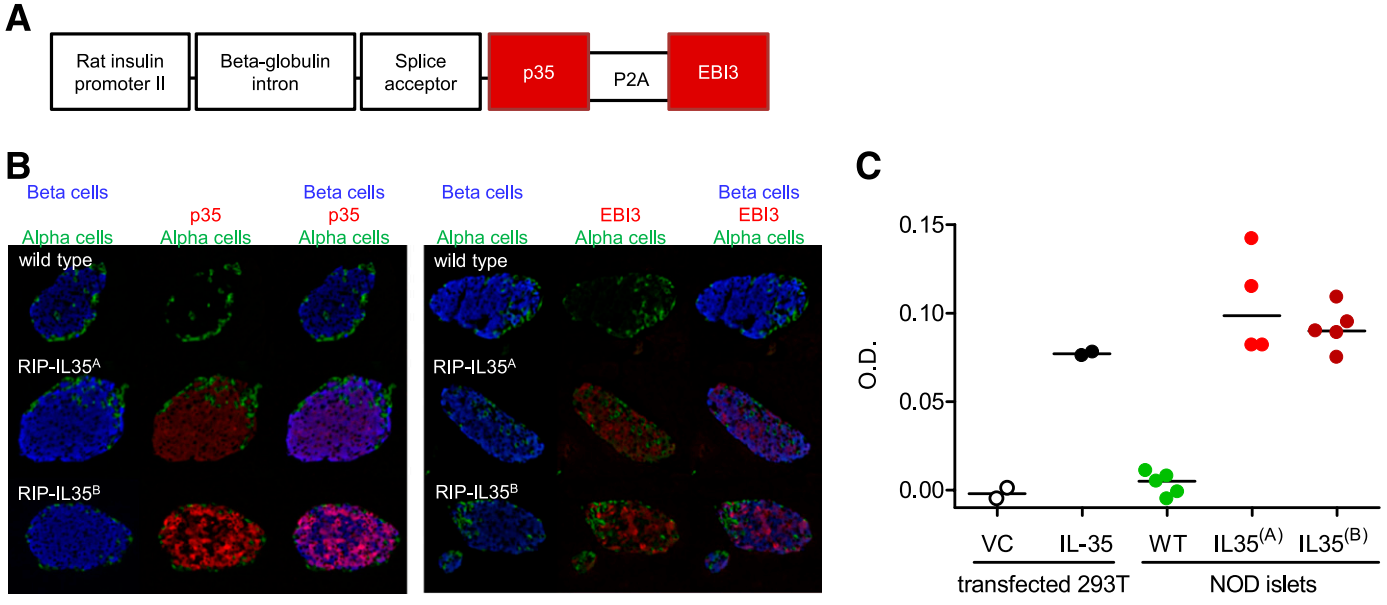

FIG. 1. Generation of the NOD.RIP-IL35 transgenic mouse. *A*: Schematic diagram of transgene construct. *B*: Immunofluorescent detection of p35 and EBI3 expression in the pancreatic sections of NOD.RIP-IL35^{A} and NOD.RIP-IL35^{B} mice. *C*: Detection of IL-35 in supernatant collected from cultured transgenic islets isolated from NOD.*scid* (green circles), NOD.*scid*.RIP-IL35^{A} (red circles), and NOD.*scid*.RIP-IL35^{B} (dark red circles) mice. IL-35 was measured by sandwich ELISA with a coating of primary anti-p35 and secondary biotenylated anti-EBI3. Supernatant from the 293T-cell line transfected with p35-P2A-EBI3 construct (IL-35, black circles) or vector alone (VC, white circles) was used as a control. A representative of two independent experiments is shown; each dot represents islets from a single mouse (n = 4–5). Horizontal bars represent the median. (A high-quality digital representation of this figure is available in the online issue.)

promoter (RIP) were generated. The two chains of the heterodimeric IL-35 protein (Ebi3 and p35) were connected by a self-cleaving P2A peptide that allows for translation and stoichiometric expression of two separate cistrons (Fig. 1*A*) (16,17). Of six founders positive for IL-35 expression, two were selected for further analysis based on differential expression levels and designated NOD.RIP-IL35^{A} (low copy transgenic) and NOD.RIP-IL35^{B} (high copy) (Supplementary Fig. 1). Expression of p35 and EBI3 in the two transgenic lines was verified by immunofluorescent analysis of pancreatic sections and colocalized with β-cells, confirming restricted expression under the RIP promoter (Fig. 1*B*). Whereas p35 and EBI3 expression in the NOD.RIP-IL35^{A} was uniform among all the β-cells, NOD.RIP-IL35^{B} mice exhibited higher but variegated expression (Fig. 1*B*). Secretion of heterodimeric IL-35 was confirmed by ELISA, following ex vivo islet isolation and in vitro culture (Fig. 1*C*). This approach has been previously used to verify cytokine production (e.g., TNFα and IL-4) following ectopic expression under the RIP promoter (18,19). Of interest, the amount of protein secreted by both transgenic lines was similar, and this correlated with the level of *Il12a* and *Ebi3* mRNA expression in islet β-cells (Fig. 1*C* and Supplementary Fig. 2). Of importance, the size and number of islets were indistinguishable between transgenic mice and littermates, suggesting that IL-35 expression had not affected β-cell and islet development (data not shown).

Diabetes incidence and onset was monitored in both transgenic lines, compared with littermate controls, to control for possible transgene integration effects. Both transgenic lines exhibited significant protection against spontaneous autoimmune diabetes. Although the wild-type mice exhibited ~80% diabetes onset by 30 weeks of age, both the NOD.RIP-IL35^{A} and NOD.RIP-IL35^{B} transgenic lines exhibited significant reduction in diabetes onset with only 20 and 15% diabetes incidence, respectively (Fig. 2*A* and *B*). Furthermore, the time of disease onset in the transgenic mice that did develop autoimmune diabetes was substantially delayed.

We next analyzed the level of insulitis in these mice and observed a reduction of invasive insulitis in transgenic mice at 10 weeks of age compared with wild-type littermates, although this was only significant with the NOD.RIP-IL35^{A} line (Fig. 2*C*–*F*). It is possible that the more uniform expression of IL-35 in the β-cells of NOD.RIP-IL35^{A} mice results in better and/or more consistent protection against invasive insulitis. Although, there was a marked decrease in progression to diabetes, the transgenic islets still exhibited significant monocytic infiltration and insulitis, which persisted through 55 weeks of age (Fig. 2*D* and *F*).

We next examined the potency of IL-35 protection by crossing the NOD.RIP-IL35^{B} mice onto the insulin 2–deficient (*Ins2*$^{-/-}$) NOD strain, which develops accelerated and exacerbated autoimmune diabetes (20,21). Deletion of the *Ins2* gene, which is expressed in both the thymus and pancreas, unlike the *Ins1* gene, which is only expressed in the pancreas, results in increased susceptibility to diabetes presumably as a result of inefficient deletion of insulin-reactive clones during thymic selection (21). The majority of the *Ins2*$^{-/-}$ NOD mice (86%) developed diabetes by 16 weeks, with 100% incidence by 25 weeks of age, compared with only 27 and 59% of wild-type NOD littermates, respectively (Fig. 3*A*). In contrast, *Ins2*$^{-/-}$ NOD.RIP-IL35^{B} mice developed a significantly reduced incidence of diabetes (25% at 16 weeks of age and 58% by 30 weeks of age) (Fig. 3*A*).

We next tested whether transgenic expression of IL-35 could protect mice from diabetes induced by adoptively transferred splenocytes from NOD mice or a monoclonal, autoantigen-specific TCR retrogenic population (15). Limited protection occurred when splenocytes from 6- to 7-week-old NOD mice were adoptively transferred into NOD.*scid*.RIP-IL35^{B} mice, resulting in 36% of NOD.*scid*.RIP-IL35^{B} mice developing diabetes compared with 77% of NOD.*scid* littermate recipients (Fig. 3*B*). Partial protection also was observed after transfer of islet antigen–reactive NY4.1 CD4^{+} retrogenic T cells. NOD.*scid*.RIP-IL35^{B} mice were protected with 67% diabetes incidence compared with 100% in wild-type NOD.*scid* littermates (Fig. 3*C*). It is conceivable that prior to transfer, NY4.1 islet antigen–reactive T cells have been exposed to their cognate antigen in the peripheral organs of retrogenic mice, since they exhibit early islet infiltration (15). Furthermore, splenocytes from 6- to 7-week-old polyclonal NOD mice can potentially include some recirculating primed T cells. Thus, it is possible that a higher frequency of islet-reactive T cells and/or potential exposure to antigen prior to transfer may render these cells less sensitive to IL-35 regulation than a naïve polyclonal T-cell population present in a naturally developing autoimmune response in NOD.RIP-IL35 mice.

We next assessed whether continuous exposure to IL-35 in the islets was necessary for regulation of the infiltrated T-cell population and whether this protection could be transferred. CD4^{+} T cells were sorted from the islets of 10-week-old NOD.*scid*.RIP-IL35^{B} mice and littermate controls and adoptively transferred into NOD.*scid* recipients together with CD4^{+} T cell–depleted splenocytes from wild-type NOD mice as a source of CD8^{+} T cells and antigen presenting cells (APCs). As expected, control mice that received CD4^{+} T cell–depleted splenocytes alone did not develop diabetes. However, addition of CD4^{+} T cells from islets of either wild-type or transgenic mice resulted in accelerated autoimmune diabetes development, albeit slightly delayed in recipients of NOD.RIP-IL35^{B}–derived cells (Fig. 3*D*). This result suggests that protection seen in transgenic mice is not a result of a permanent conversion of the infiltrating T cells into regulatory populations, such as iTr35, and that constant exposure to IL-35 is necessary for continued suppression of the infiltrating cells and protection from diabetes. However, we cannot exclude the possibility that upon transfer into lymphopenic NOD.*scid* recipient mice, CD4^{+} T cells lose their suppressed phenotype as a result of homeostatic expansion or an absence of other inhibitory factors present in the islets of transgenic mice.

Because IL-35 expression in the islet β-cells is driven by the *Ins2* promoter, it is conceivable that low-level expression of IL-35 in medullary thymic epithelial cells could cause enhanced tolerance of diabetogenic T cells during development. However, *Il12a* and *Ebi3* mRNA expression in NOD.RIP-IL35 transgenic mice and wild-type controls was not statistically different (Supplementary Fig. 2). In addition, there was no difference in the onset or incidence of autoimmune diabetes following adoptive transfer of splenocytes from NOD.RIP-IL35 transgenic versus littermate control mice into NOD.*scid* recipients (data not shown). Although these data argue against a role for thymic transgene expression, this possibility cannot be completely ruled out.

Reduced T-cell infiltration and proliferation in the islets of NOD.RIP-IL35 mice. Our histological analysis of NOD.RIP-IL35^{B} mice over a course of 55 weeks showed slightly reduced, but persistent, insulitis (Fig. 2*D* and *F*).

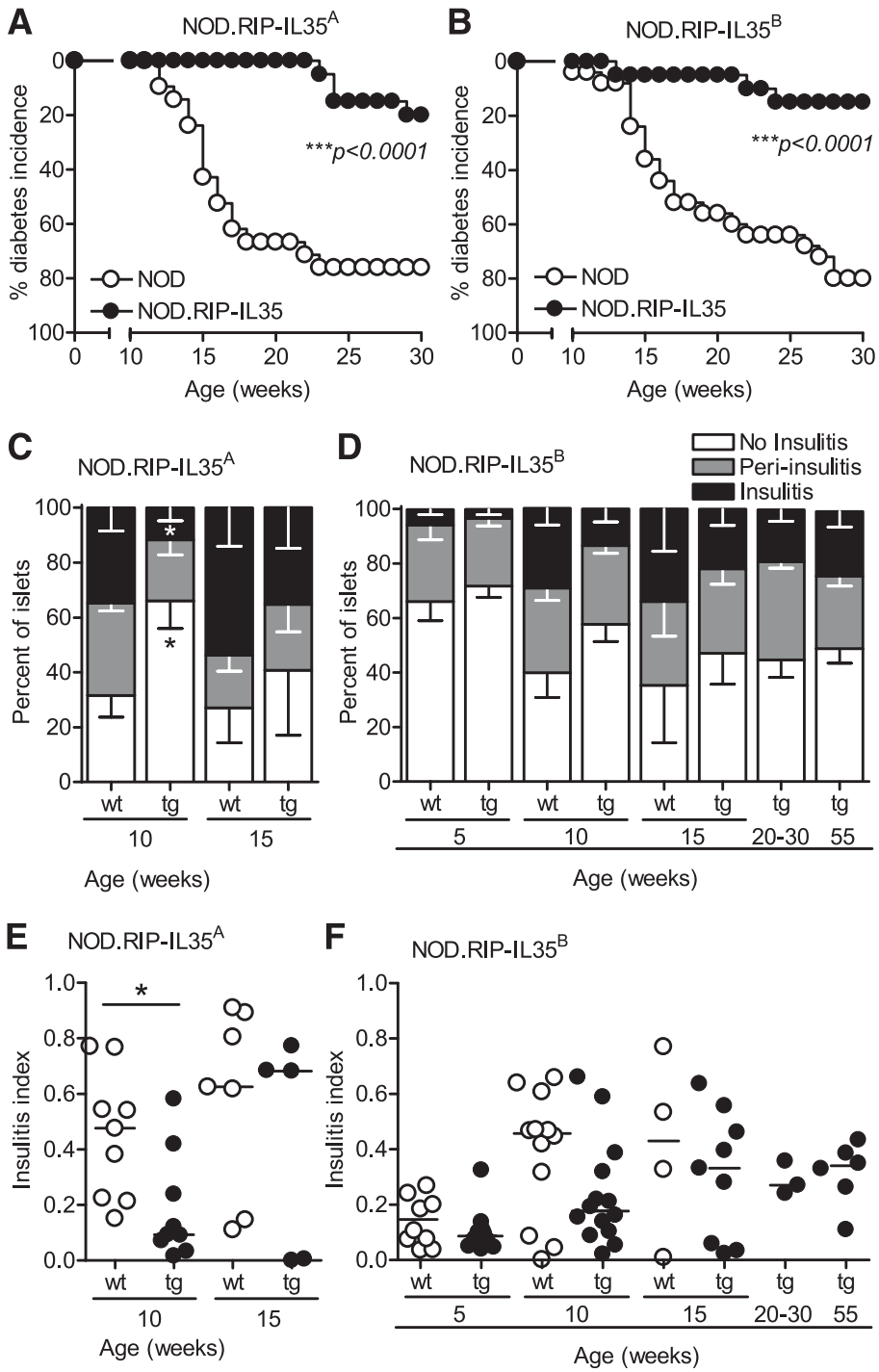

FIG. 2. NOD.RIP-IL35 mice are protected from diabetes. NOD.RIP-IL35^{A} (*A*) and NOD.RIP-IL35^{B} (*B*) transgenic strains were monitored for diabetes development (n = 20–25) (*P < 0.0001, Kaplan-Meier). Insulitis and insulitis index were assessed in NOD.RIP-IL35^{A} (black circles) (*C* and *E*) and NOD.RIP-IL35^{B} (black circles) (*D* and *F*) transgenic strains and compared with wild-type NOD littermate controls (white circles). At least nine mice per group were analyzed at 5 and 10 weeks of age, and 3–9 mice per group were analyzed at 15–55 weeks of age (*P < 0.02, Mann-Whitney). *D* and *F*: Horizontal bars represent the median.**

This observation of chronic insulitis in the absence of symptomatic diabetes has been previously observed in NOD mice (7) and suggests a controlled balance between regulatory and proinflammatory responses in the islets rather than a lack of response. We analyzed the composition of the islet infiltrate to evaluate if there were differences associated with protection in transgenic mice. At 10 weeks of age, there was a significant decrease in total cell number infiltrating the islets of female transgenic mice (Supplementary Fig. 3), despite a more modest change in the frequency of infiltrated islets (Fig. 2*C*–*F*). There also was a substantial reduction in the number of islet-infiltrating CD4^{+} and CD8^{+} T cells in transgenic islets compared with islets obtained from wild-type littermates (Fig. 4*A* and *B*). However, no significant differences were observed in the pancreatic lymph node, nonpancreas draining lymph nodes (ndLNs), and spleen,

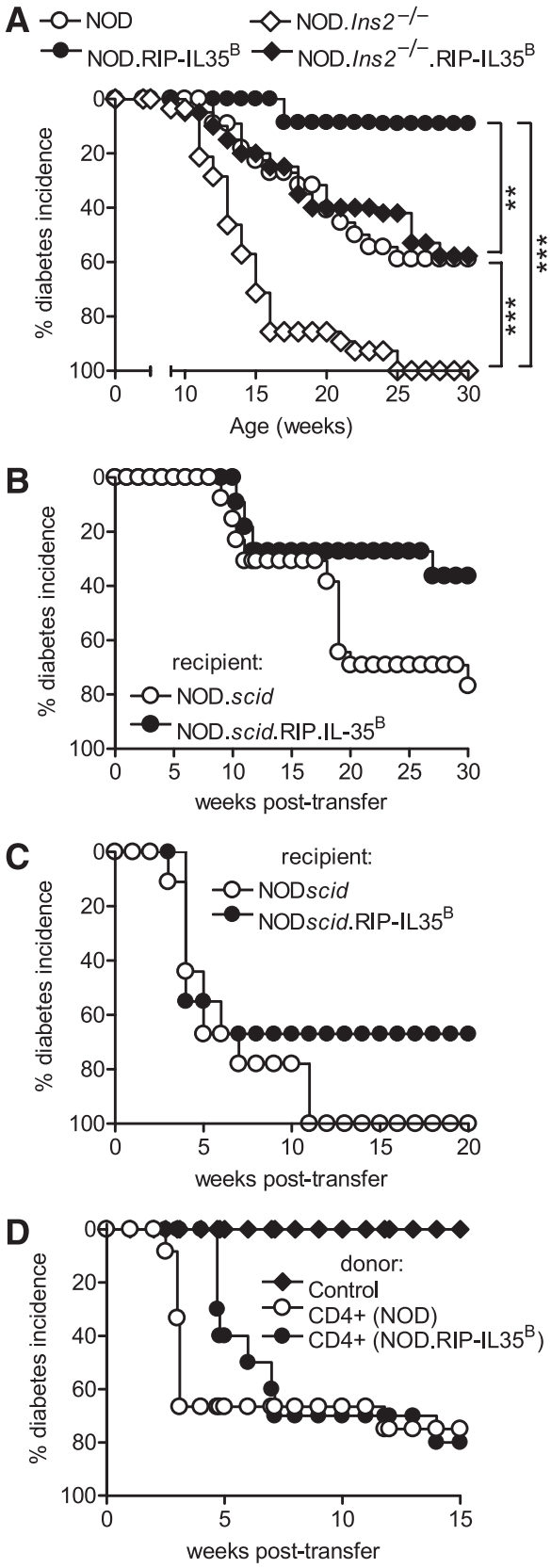

FIG. 3. Transgenic expression of IL-35 confers protection against diabetes under stringent conditions. *A*: *Ins2*$^{-/-}$ mice were crossed with NOD.RIP-IL35^{B} mice and monitored for diabetes onset (n = 20–28) (P < 0.002; ***P < 0.0001, Kaplan-Meier). *B*: Diabetes was adoptively transferred to NOD.*scid*.RIP-IL35^{B} mice by injecting 10 million splenocytes from 6- to 7-week-old female wild-type NOD mice (three separate experiments, n = 11–14). *C*: Diabetes was transferred by injecting 10^{5} NY4.1 retrogenic T cells intravenously into NOD.*scid*-RIP-IL35^{B} (n = 9). *D*: Diabetes was transferred with 5,000 CD4^{+} T cells sorted from the islets of 10-week-old mice in combination with CD4-depleted splenocytes from 7-week-old mice. Control group received splenocytes only (three separate experiments, n = 8–12).**

demonstrating that the effect of β-cell–derived IL-35 is restricted to the islets.

IL-35 normally is produced by CD4^{+}Foxp3^{+} T_{regs} (11). However, it is unknown whether IL-35 has a positive feedback on T_{reg} number and homeostasis, as described for TGFβ (22). Frequencies of CD4^{+}Foxp3^{+} T cells and levels of Foxp3 and CD25 expression were not altered in NOD.RIP-IL35^{B} mice, which suggests that factors other than increase in T_{reg} number are responsible for protection (Fig. 4*C* and data not shown).

Previous studies have suggested that the primary effect of IL-35 on T cells is suppression of proliferation (11). Furthermore, our in vitro analysis of the effect of IL-35 on cell-cycle progression suggested that IL-35 inhibits T cells by inducing cell-cycle arrest at the G1 phase (V. Chaturvedi and D.A.A. Vignali, unpublished observations). To address the possibility that the reduced number of infiltrating CD4^{+} T cells is attributed to a block at G1, we assessed CD4^{+} T-cell proliferation by Ki67 expression, a cell-cycle protein that is expressed in all phases of the cell cycle, including G1, and by BrdU incorporation into the DNA, which occurs during the S phase. We chose a short 4-h BrdU pulse in order to limit analysis to local cell proliferation. Indeed, there was negligible BrdU incorporation in the PLN and ndLNs, with no differences observed between transgenic and littermate controls. In contrast, there was a substantial reduction in CD4^{+} T-cell, CD8^{+} T-cell, and T_{reg} proliferation in the islets of transgenic mice (40, 60, and 56%, respectively) (Fig. 4*D–F*). Of interest, there was no difference in the frequency of Ki67^{+} T cells in the islets (Fig. 4*G–I*), suggesting that the proportion of infiltrating T-cell subsets that enter G1 is comparable, but they are blocked from progression through the S phase by exposure to islet-derived IL-35.

β-Cell–restricted expression of IL-35 did not seem to have a dramatic effect on cytokine expression because there was no difference in the frequency of IL-2^{+} or TNFα^{+} CD4^{+} T cells in the islets of transgenic mice and littermate controls (Fig. 5*A*). Likewise, frequencies of IL-17^{+}– or IL-10^{+}– producing CD4^{+} T cells were not affected by the transgene, and both populations were present at low levels (Fig. 5*A* and and data not shown). Of interest, there was a small but significant reduction in the percentage of interferon γ^{+} CD4^{+} T cells in the islets of transgenic mice compared with littermate controls (Fig. 5*A*). Because this analysis reflects the percentage of cytokine-expressing cells following ex vivo PMA/ionomycin stimulation, we cannot rule out the possibility that difference in cytokine production may exist in the local islet microenvironment. It is possible that IL-35 secreted by islets in the transgenic mice also is inducing a regulatory population iTr35 that exerts its effects via IL-35 (10). Although no differences were observed in endogenous *Ebi3* mRNA in the CD4^{+}CD25^{-} T_{eff} cells isolated from the islets of transgenic versus littermate controls, arguing against significant iTr35 conversion, we cannot rule out the possibility that limited conversion does occur and is not detectable via this approach (Supplementary Fig. 4).

Last, we asked if there were alterations in the frequencies of islet antigen–specific T-cell populations by tetramer analysis of IGRP-reactive T cells that are associated with

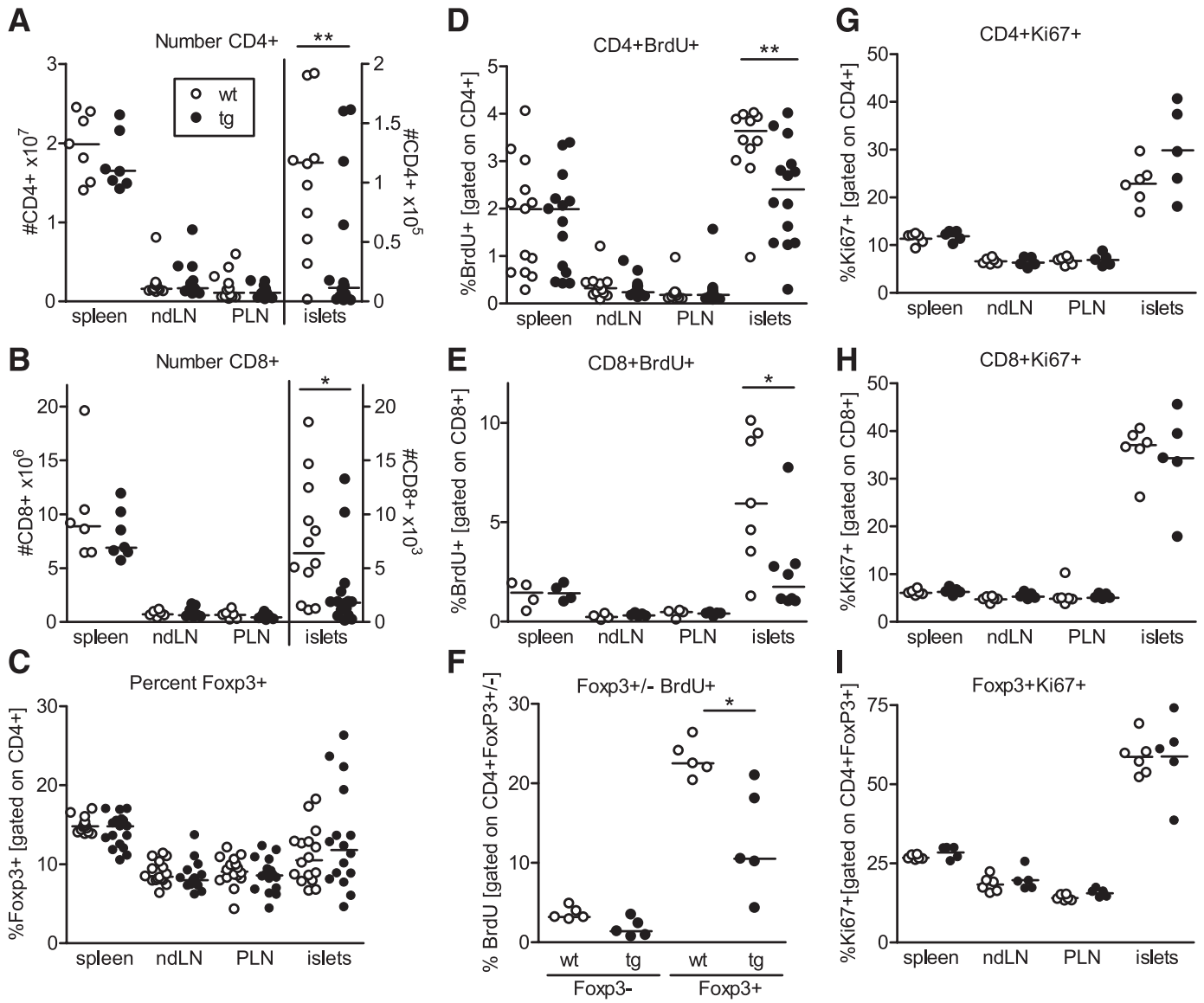

FIG. 4. Reduced T-cell infiltration and proliferation in NOD.RIP-IL35^B mice. Numbers of CD4$^+$ (*A*) and CD8$^+$ (*B*) T cells were calculated based on flow cytometric analysis in spleens, ndLNs, PLNs, and purified islets of 10-week-old female mice (n = 7–16) (*P < 0.05; **P < 0.01, Mann-Whitney). *C*: Frequency of Foxp3$^+$ cells was assessed in NOD.RIP-IL35^B mice and compared with littermate controls (n = 15–17). In vivo CD4$^+$ (*D*), CD8$^+$ (*E*), and Foxp3$^+$ (*F*) T-cell proliferation was assessed via BrdU incorporation after 4 h in vivo BrdU pulse (CD4$^+$: n = 12–14; CD8$^+$: n = 7–8; Foxp3$^+$: n = 5) (*P < 0.02; **P < 0.001, Mann-Whitney). Ki67 staining of CD4$^+$ (*G*), CD8$^+$ (*H*), and Foxp3$^+$ (*I*) T cells in organs of 10-week-old female wild-type and transgenic mice (n = 5–6). Horizontal bars represent the median.

progression to disease (23). Of interest, the frequency of IGRP$^+$ CD8$^+$ T cells in the islets and PLNs of transgenic mice was significantly reduced compared with littermate controls (Fig. 5*B*). This was somewhat unexpected given the β-cell–restricted expression of IL-35. Although the mechanism behind a decrease in tetramer$^+$ cells in the PLN is unclear, we would not expect significant amounts of IL-35 to reach the draining lymph nodes. It is possible that the frequency of IGRP$^+$CD8$^+$ T cells in the PLN is lower as a result of recirculating activated (CD44^{hi}) T cells that had previously been in the islets (24) and thus would have been subjected to the effects of IL-35 expression by β-cells in the transgenic mice. It also is possible that IL-35 suppresses the activation, maturation, or trafficking of APCs from the islets to the PLNs, which in turn altered T-cell activation and expansion, although the activation status of F4/80$^+$ and CD11c$^+$ populations obtained from draining lymph nodes and islets did not reveal any significant differences between transgenic and wild-type littermates, as was assessed based on CD80, CD86, and major histocompatibility complex class II expression (data not shown). We also considered the possibility that the precursor frequency of naïve IGRP-reactive T cells could be reduced in NOD.RIP-IL35 mice as a result of *Ins2* promoter activity, and thus IL-35 expression, in the thymus, even though there was no significant increase in the expression of *Il12a* and *Ebi3* message in medullary thymic epithelial cells above wild-type controls (Supplementary Fig. 2). We used a previously published protocol utilizing MACS bead enrichment of tetramer$^+$ cells from all peripheral lymphoid organs (13) to assess the precursor frequency of naïve IGRP-reactive T cells. There were no differences in the numbers of IGRP$^+$CD8$^+$CD44^{lo} precursor T cells in either peripheral lymphoid organs or single positive thymocytes when we

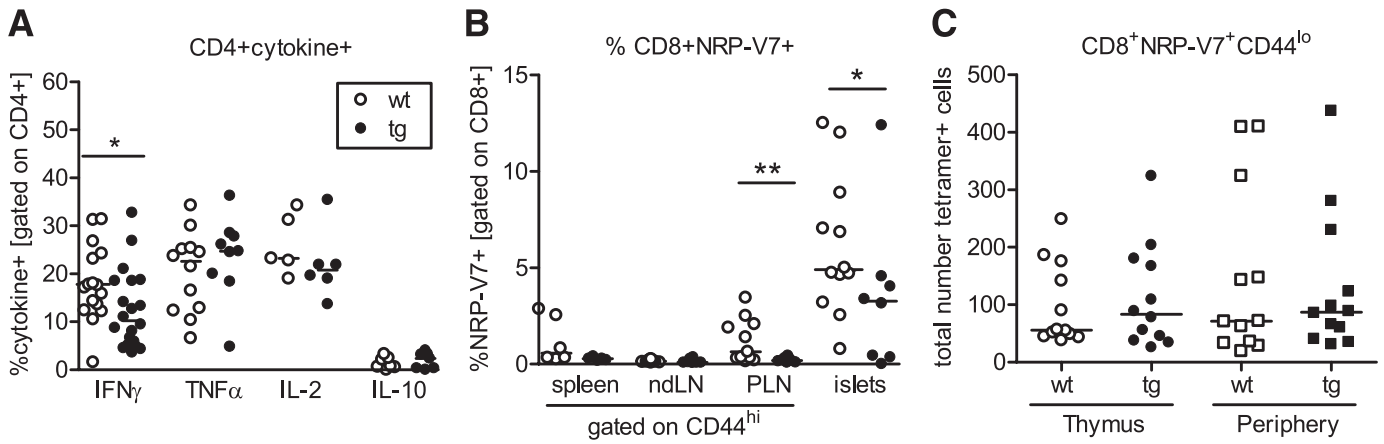

FIG. 5. Phenotypic analysis of islet infiltrating CD4$^+$ and CD8$^+$ T cells in NOD.RIP-IL35^B mice. *A*: Cytokine production by CD4$^+$ T cells was analyzed after 5 h in vitro restimulation with PMA and ionomycin (n = 5–16) (*P < 0.04, Mann-Whitney). *B*: Frequency of IGRP tetramer (NRP-V7)-positive CD8$^+$ T cells in 10-week-old female transgenic mice and littermate controls (n = 5–12) (*P < 0.05; **P < 0.009, Mann-Whitney). *C*: Numbers of naive IGRP-reactive CD8$^+$ T cells in the thymus and peripheral lymphoid organs of 5-week-old female transgenic mice and littermate controls (n = 12). Horizontal bars represent the median.

compared NOD.RIP-IL35^B mice and littermate controls (Fig. 5*C*). In combination with transfer experiments, these data suggest that continuous local production of IL-35 in the islets is responsible and necessary for inhibition of infiltrating antigen-specific T cells.

DISCUSSION

This study demonstrates that targeted expression of IL-35 in pancreatic β-cells results in protection against spontaneous and accelerated autoimmune diabetes in NOD mice. Local islet expression of IL-35 is sufficient in controlling both CD4$^+$ and CD8$^+$ T-cell responses and did not seem to have any systemic effects on T cells or developmental abnormalities of the islets.

Expression of heterodimeric IL-35 under the RIP promoter resulted in specific targeted expression by β-cells in the islets, and there seemed to be no alteration of β-cell number, size, or function. Previous studies using immunoregulatory cytokines to affect diabetes onset reported either altered T-helper phenotype and/or induced immunological and developmental complications as a result of their pleiotropic activity (8,25). For instance, targeted expression of IL-4 in the β-cells resulted in protective Th2-type response rather than pathogenic Th1, leading to protection of NOD mice from diabetes (7). Surprisingly, limiting the TCR repertoire by crossing these mice onto the BDC2.5 TCR transgenic background resulted in exacerbated disease, seemingly because of an increase in antigen presentation by IL-4–activated APCs (9). These findings underline the complicated and often opposing effects of pleiotropic cytokines. In contrast to many other regulatory or anti-inflammatory–type cytokines, IL-35 seems to be unique in having a very restricted mode of action: inhibition of T-cell proliferation (10,11). IL-35 production by β-cells resulted in substantial reductions in the number of islet-resident CD4$^+$ and CD8$^+$ T cells and a reduced frequency of islet-antigen–specific T cells. The effect of β-cell–secreted IL-35 was limited to a reduction in T-cell proliferation, whereas alterations in cytokine expression were relatively modest. Unlike TGFβ, which can boost T_{reg} accumulation in the islets (26), ectopic expression of IL-35 resulted in reduced proliferation and accumulation of both effector and regulatory T-cell populations. However, it is unknown whether IL-35 had a positive effect on the function of Foxp3$^+$ T_{regs}.

It is unclear whether IL-35 secreted by β-cells induced the generation of an iTr35 inhibitory population in the islets (10). Although we could not detect *Il12a* and *Ebi3* transcripts associated with this population by conventional RT-PCR, it is possible that their frequency is low and below the limit of detection. Furthermore, it is unknown whether iTr35 exists naturally as a part of the NOD islet infiltrate, and secretion of IL-35 by iTr35 or CD4$^+$Foxp3$^+$ T_{regs} is contributing to the regulation of the autoimmune response in the islets. Our previous studies suggest that Foxp3$^+$ T_{reg}-derived IL-10 contributes to IL-35–driven iTr35 conversion (10). It is possible that levels of IL-10 present in the islets are not sufficient for optimal in vivo iTr35 generation in contrast to other in vivo models, where we have observed their generation. Although, macrophage and dendritic cell populations did not seem to be affected by IL-35 in the islets of transgenic mice based on cell-surface activation markers or cell frequency, we cannot rule out the possibility that there are functional alterations.

Our study represents, to our knowledge, the first proof of principle that native, biologically active IL-35 has the capacity, under certain circumstances, to substantially protect β-cells from autoimmune attack. These studies raise three possible avenues of future study. First, because IL-35 can impact autoimmune diabetes and can be induced in high amounts during inflammatory reactions, there may be a role of endogenously generated IL-35 or a lack thereof during the development of type 1 diabetes. Second, IL-35 could be specifically targeted to the pancreas to limit autoimmune diabetes and provide immunoregulatory cover for β-cell neogenesis in new-onset patients. Third, IL-35 could be used during islet or β-cell transplantation in diabetic patients to protect the graft from autoimmune destruction and/or induce infectious tolerance to the graft. Given the limited stability and/or poor secretion of IL-35 (27), it is likely that mutant forms of IL-35 that improve these limitations could result in even greater efficacy. Last, our data suggest that IL-35 may have substantial therapeutic utility not only for the treatment of type 1 diabetes but also other autoimmune and inflammatory diseases and supports further investigation of the role and therapeutic potential of IL-35.

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D.A.A.V. has submitted patents that are pending and is entitled to a share in net income generated from licensing of these patent rights for commercial development. No other potential conflicts of interest relevant to this article were reported.

M.B. researched data and wrote the manuscript. A.H.C., G.P.L., and A.R.B. researched data. D.A.A.V. conceived and directed the project and revised and edited the manuscript. D.A.A.V. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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