

Neutralization of Interleukin-16 Protects Nonobese Diabetic Mice From Autoimmune Type 1 Diabetes by a CCL4-Dependent Mechanism

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OBJECTIVE—The progressive infiltration of pancreatic islets by lymphocytes is mandatory for development of autoimmune type 1 diabetes. This inflammatory process is mediated by several mediators that are potential therapeutic targets to arrest development of type 1 diabetes. In this study, we investigate the role of one of these mediators, interleukin-16 (IL-16), in the pathogenesis of type 1 diabetes in NOD mice.

RESEARCH DESIGN AND METHODS—At different stages of progression of type 1 diabetes, we characterized IL-16 in islets using GEArray technology and immunoblot analysis and also quantitated IL-16 activity in cell migration assays. IL-16 expression was localized in islets by immunofluorescence and confocal imaging. In vivo neutralization studies were performed to assess the role of IL-16 in the pathogenesis of type 1 diabetes.

RESULTS—The increased expression of IL-16 in islets correlated with the development of invasive insulinitis. IL-16 immunoreactivity was found in islet infiltrating T-cells, B-cells, NK-cells, and dendritic cells, and within an insulitic lesion, IL-16 was derived from infiltrating cells. CD4⁺ and CD8⁺ T-cells as well as B220⁺ B-cells were identified as sources of secreted IL-16. Blockade of IL-16 in vivo protected against type 1 diabetes by interfering with recruitment of CD4⁺ T-cells to the pancreas, and this protection required the activity of the chemokine CCL4.

CONCLUSIONS—IL-16 production by leukocytes in islets augments the severity of insulinitis during the onset of type 1 diabetes. IL-16 and CCL4 appear to function as counterregulatory proteins during disease development. Neutralization of IL-16 may represent a novel therapy for the prevention of type 1 diabetes. *Diabetes* 59:2862–2871, 2010

Insulinitis and type 1 diabetes are mediated by the regulated homing of leukocytes to pancreatic islets by chemoattractants (1–3). Unlike chemokines that bind chemokine receptors, the interleukin-16 (IL-16) cytokine binds to CD4 and selectively recruits CD4⁺ T helper one (Th1) cells due to the functional relationship

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between CD4 and the CCR5 and CXCR3 chemokine receptors (4,5). Mature IL-16 is secreted after caspase-3 mediated cleavage of either neuronal IL-16 or precursor protein (pro-IL-16) (6–13). IL-16 also regulates T-cell activation, CD25 and major histocompatibility complex class II expression, IL-2 production (14), antigen-induced upregulation of CD95 expression (14), and chemokine-induced chemoattraction (15,16). Binding of IL-16 to CD4 selectively desensitizes CCL4/CCR5-induced chemotaxis, and pretreatment of T-cells with CCL4 blocks chemotaxis triggered by IL-16 binding to CD4 (15). CD4 and CCR5 may therefore share a unique relationship mediated by either IL-16 and/or CCL4 binding, and IL-16 and CCL4 may cross-regulate each other's activity. Thus, IL-16 is an immunomodulatory and proinflammatory cytokine that influences the recruitment and activation of CD4⁺ T-cells in vivo.

Given that CD4⁺ T-cells mediate the development of type 1 diabetes, we examined whether IL-16 influences CD4⁺ T-cell recruitment to pancreatic islets. We show that IL-16 potentiates insulinitis and type 1 diabetes in NOD mice, and that anti-IL-16 antibody therapy protects against type 1 diabetes even when administered at the mid-late stages of disease progression. This protection results from reduced trafficking of CD4⁺ T-cells to islet lesions and requires CCL4 activity. Thus, IL-16 may represent a novel therapeutic target for the prevention of type 1 diabetes.

RESEARCH DESIGN AND METHODS

NOD/Del, NOD.Scid, and NOD.RAGKO mice were housed at the Robarts Research Institute (London, ON, Canada). C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The NOD-BDC2.5.Thy1.1 mice were a kind gift of the Bluestone laboratory at the University of California San Francisco. Mice were maintained in a specific pathogen-free barrier facility according to Institutional guidelines.

Reagents. The 14.1 (mouse IgG2a[κ]) and 17.1 (mouse IgG1) anti-IL-16 monoclonal antibodies (mAbs), which react with mouse and human IL-16, were purified from ascites on a Protein A affinity column (15).

GEArray technology. Changes in gene expression were analyzed using GEArray technology according to the manufacturer's directions (Super-Array Biosciences). A detailed protocol is presented in the supplementary Material and Methods, available in an online appendix at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-0131/DC1>.

In vivo treatment of mice. NOD mice were injected intraperitoneally 3 times weekly with the 14.1 anti-IL-16 neutralizing mAb as follows: either 0.1 mg from 3–16 weeks of age or 0.2 mg from 3 to 7, 9 to 16, or 17 to 22 weeks of age. Control mice received isotype-matched mouse IgG2a(κ) (UPC 10, 200 μg, Sigma) (17). To determine whether CCL4 mediates anti-IL-16 induced protection from type 1 diabetes, 0.2 mg of anti-IL-16 (14.1) and 0.1 mg of anti-mouse CCL4 mAb (R&D Systems) were coadministered 3 times weekly from 9 to 16 weeks of age. Control mice received isotype-matched control IgG2a(κ) (UPC 10) and rat IgG (Sigma). A blood glucose level (BGL) of ≥11.1 mmol/l on two consecutive readings indicated the onset of type 1 diabetes.

Histopathology. Pancreata were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 7 μm intervals. The incidence and severity of

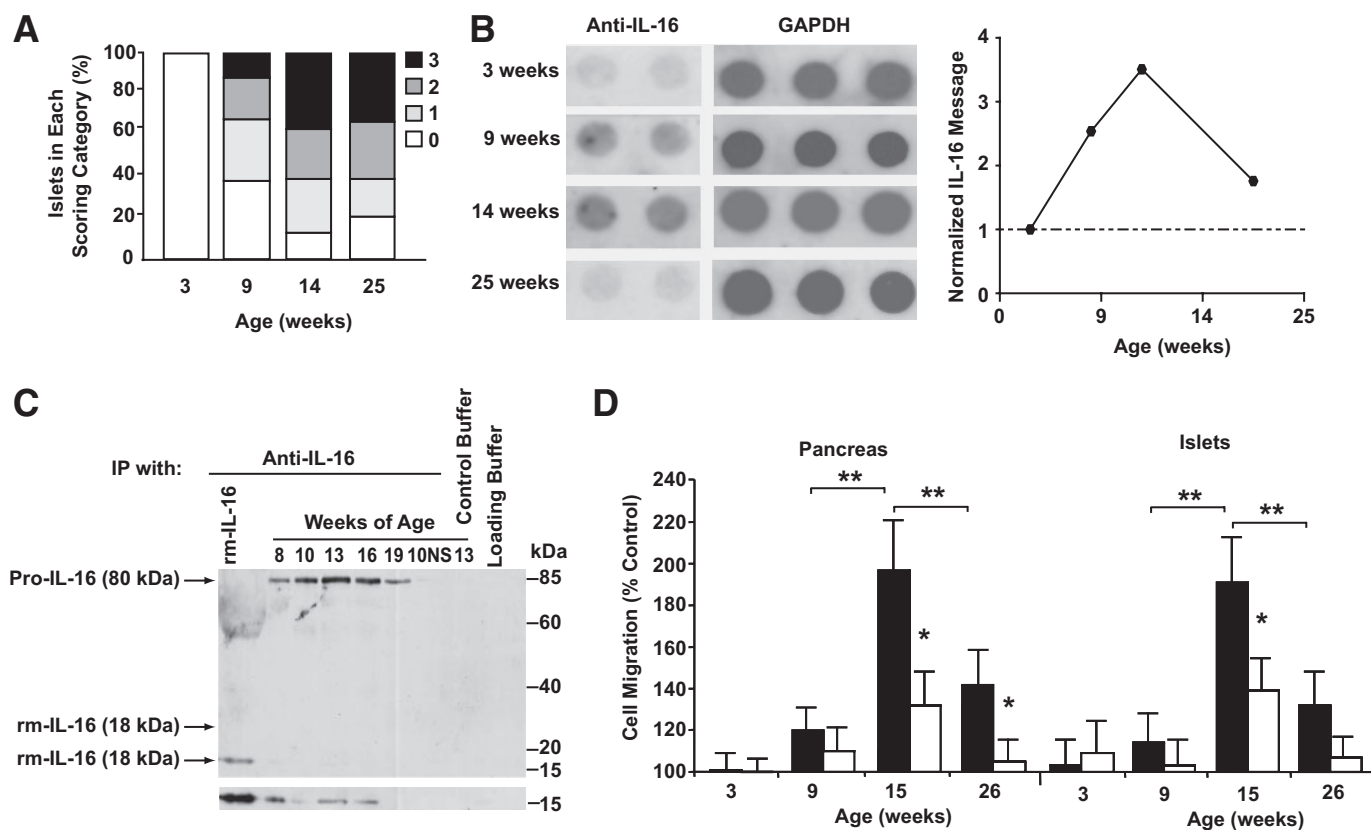


FIG. 1. Quantitation of IL-16 mRNA and chemotactic activity in the pancreas and islets. **A:** Severity of insulinitis in female NOD mice at the indicated ages. **B:** GEArray analysis of intra-islet IL-16 mRNA in total islet RNA isolated at the indicated ages. The relative amount of IL-16 transcript at each age was estimated by comparing its average signal intensity with the average signal derived from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized to 3 weeks of age. One of two representative experiments is shown. **C:** Immunoprecipitation of IL-16 isoforms in pancreata from NOD and NOD.Scid (NS) mice. Lysate aliquots were immunoprecipitated as described in RESEARCH DESIGN AND METHODS. All blots were initially developed by electrochemiluminescence. Visualization of mature IL-16 was enhanced by stripping, reblotting, and detecting with the more sensitive ECL-Plus reagent (*lower panel*). Stripping and reblotting with nonspecific isotype-matched biotinylated control failed to detect IL-16. Results are from one of three representative and reproducible experiments. **D:** Production of bioactive IL-16 in the pancreas and islets. IL-16 chemoattractant activity was analyzed at the indicated ages using a modified Boyden chamber assay. For the indicated ages, purified splenic T-cells (5×10^6) were incubated with either immunoaffinity-isolated IL-16 samples from islets and pancreas (black bars) or a neutralizing anti-IL-16 mAb ($5 \mu\text{g/ml}$; white bars). *Indicates IL-16 induced chemotaxis was significantly neutralized by anti-IL-16, $P < 0.05$. **Denotes significant IL-16-induced chemotaxis at the indicated ages, $P < 0.05$. Similar results were obtained in two independent and reproducible experiments.

insulinitis were examined after hematoxylin–eosin (H&E) staining. At least 10 nonadjacent sections were analyzed per pancreas, and average percentages of insulinitis were determined from ≥ 100 islets per treatment group.

Adoptive T-cell transfer. NOD.Scid mice (5–7 weeks old, $n = 8/\text{group}$) were injected intraperitoneally with spleen T-cells (4×10^6 per mouse) isolated at 16 weeks of age from NOD mice treated 3 times weekly from 9 to 16 weeks of age with $200 \mu\text{g}$ of anti-IL-16 (14.1) or isotype control IgG2a(κ).

Statistical analysis. Significant differences were determined using the Student *t* test for unpaired samples, the Mantel-Haenszel log-rank test, and the Mann-Whitney nonparametric test where indicated. Statistical comparisons were performed using GraphPad Prism (www.graphpad.com; San Diego, CA). A probability value of $P \leq 0.05$ was considered significant.

A supplementary Research Design and Methods section is available in the online appendix.

RESULTS

IL-16 expression during development of insulinitis. In our NOD female mice, nondestructive peri-insulinitis develops from 6 to 10 weeks of age and is followed by an invasive and destructive insulinitis in 15–25-week-old mice (Fig. 1A). Onset of diabetes is observed as early as 13 weeks of age, progressing to a cumulative disease incidence of 80–90% by 30 weeks of age. Cytokine GEArray analyses of changes in islet cytokine expression revealed that, relative to 3-week-old mice, IL-16 mRNA was increased >2 -fold in 9-week-old mice and reached a maxi-

mum by 15 weeks of age (Fig. 1B). IL-16 mRNA expression decreased between 15 and 25 weeks of age, even though insulinitis persists during this period. This may reflect a reduced number of total islet infiltrating leukocytes resulting from the destruction of a majority of islets by this age.

The above-described analyses of intra-islet IL-16 mRNA detected expression of all IL-16 isoforms. We therefore characterized the IL-16 protein isoforms in the pancreas and islets at progressive stages of insulinitis, noting that only the mature IL-16 isoform is secreted and chemotactic for CD4^+ cells. Initially, IL-16 was immunoprecipitated from islet and pancreas lysates between 3 and 19 weeks of age. Intrapancratic pro-IL-16 (80 kDa) expression increased during this time-frame and peaked near 16 weeks of age (Fig. 1B). Similarly, mature IL-16 (18 kDa) was detected between 8 and 16 weeks of age. Because a very low level of 80 kDa pro-IL-16 was detected in the pancreas of NOD.Scid mice (Fig. 1C), IL-16 is expressed in the pancreas in the absence of insulinitis and increases with disease progression. To more accurately quantitate the levels of mature IL-16, IL-16 was enriched from pancreas and islet lysates by immunoaffinity chromatography and assayed for its ability to induce CD4^+ T-cell chemotaxis. This enabled

the quantitation of only mature IL-16 in contrast to other approaches, e.g., ELISA, that due to the limited specificities of commercially available antibodies do not differentiate among the various IL-16 isoforms. For each lysate, IL-16 was enriched using an equivalent amount of total protein. IL-16 chemotactic activity in the islets and pancreas increased significantly among 3 and 15 weeks of age, with the largest increase detected between 9 and 15 weeks (Fig. 1D). Note that IL-16 activity was reduced appreciably by 26 weeks of age, indicative of the destruction of the majority of islets at this time of islet isolation. The specificity of IL-16-induced T-cell chemotaxis was demonstrated by the capacity of the 14.1 neutralizing anti-IL-16 mAb to abrogate this chemotactic response. Thus, levels of mature IL-16 in islets increase in an age-dependent manner, suggesting that mature IL-16 is associated with proinflammatory responses against islets.

In situ localization of IL-16 in islets. To examine which cells produce IL-16 in an insulinitic lesion, immunohistochemistry and confocal imaging was used. Because IL-16 is synthesized as pro-IL-16 and cleaved only by activated caspase-3 to produce mature IL-16 (10), cells secreting IL-16 were identified by triple immunostaining for IL-16, active caspase-3, and various cell surface markers of immune cells. IL-16 immunoreactivity was found in a majority of mononuclear cells in insulinitic lesions, including Thy1⁺ T-cells, B220⁺ B-cells, CD4⁺ and CD8⁺ T-cells, p46⁺ NK-cells, and CD11c⁺ dendritic cells (Fig. 2 and supplementary Fig. 1, available in an online appendix). For all experiments, the appropriate isotype controls were used to rule out nonspecific staining (supplementary Fig. 2A, available in an online appendix). As determined by colocalized staining for activated caspase-3, Thy1⁺ T-cells, B220⁺ B-cells, and both CD4⁺ and CD8⁺ T-cells produce mature IL-16. Colocalization of IL-16 and activated caspase-3 occurred mainly in a polarized manner. While many lymphocytes constitutively express pro-IL-16, only a low frequency of lymphocytes stained for activated caspase-3, in support of our immunoprecipitation studies that displayed a low level of mature IL-16 in the pancreas. Because IL-16 expression was not detected in insulin-producing islet β -cells, IL-16 production appears to be restricted to immune cells in an islet lesion in support of our finding that IL-16 is not expressed in islets from NOD.RAGKO mice (supplementary Fig. 2B). Thus, in an insulinitic lesion, mature IL-16 is secreted by several types of immune cells, and this may enable these cells to recruit additional CD4⁺ T-cells to an islet lesion and augment an ongoing inflammatory response.

Neutralizing anti-IL-16 mAb prevents insulinitis and type 1 diabetes. Having determined that intra-islet expression of mature IL-16 increases with the severity of insulinitis, we next examined whether blockade of endogenous IL-16 can prevent type 1 diabetes. NOD mice were treated with a neutralizing anti-IL-16 mAb or isotype-matched control antibody from 3 to 16 weeks of age when intrapancreatic levels of IL-16 are increased. This treatment reduced the overall severity of insulinitis and prevented the onset of destructive insulinitis as insulin expression was preserved (Fig. 3A). In comparison to 33-week-old NOD mice treated with isotype control antibody (group 1), the percentage of islets (14%) displaying severe insulinitis (insulinitis score = 3) in anti-IL-16 treated mice (group 2) was significantly less than that observed (53%) in control mice. Anti-IL-16 treated mice still pos-

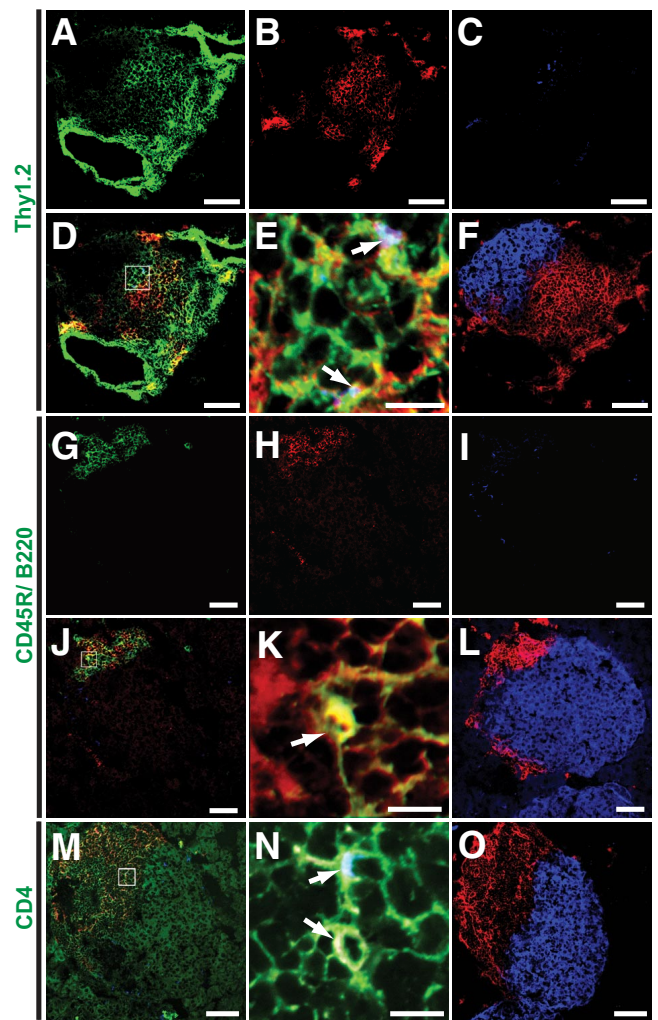


FIG. 2. In situ localization of IL-16 to lymphocytes in islets of 10-week-old NOD mice. Pancreas sections were stained with anti-IL-16 (red; all panels), anti-activated caspase-3 (blue; C–E, I, J, M, and N), anti-insulin (blue; F, L, and O), and various cell surface markers in green (Thy1.2 in A, D, and E; B220 in G, J, and K; CD4 in M and N). Magnified images of areas indicated by the white boxes in merged images (D, J, and M) are presented in E, K, and N. Colocalized signals of IL-16, activated caspase-3, and Thy1.2 (E) or B220 (K) or CD4 (N) are denoted by arrows. Adjacent serial sections stained for IL-16 and insulin are shown in F, L, and O. Scale bars represent 50 μ m for all images except for the magnified images (E, K, N), where the scale bar represents 10 μ m. (A high-quality digital representation of this figure is available in the online issue.)

sessed 52% normal healthy islets (insulinitis score = 0), whereas only 20% of islets were healthy in the control-treated mice. In addition, the incidence of type 1 diabetes was reduced from 88% (16/18 mice) in control-treated mice to 44% (8/18 mice; $P = 0.004$) in anti-IL-16 treated mice at 33 weeks of age (Fig. 3B). These data show that neutralization of IL-16 protects against insulinitis and type 1 diabetes.

Because intrapancreatic IL-16 expression peaks between 9 and 16 weeks of age, we reasoned that neutralization of IL-16 may prevent type 1 diabetes more effectively during this time than at a younger age when intra-islet levels of mature IL-16 are low. To test this possibility, anti-IL-16 or control antibody was administered during either 3–7 weeks of age, 9–16 weeks of age, or after the onset of invasive insulinitis. At 33 weeks of age, a similar incidence of type 1 diabetes was initially observed

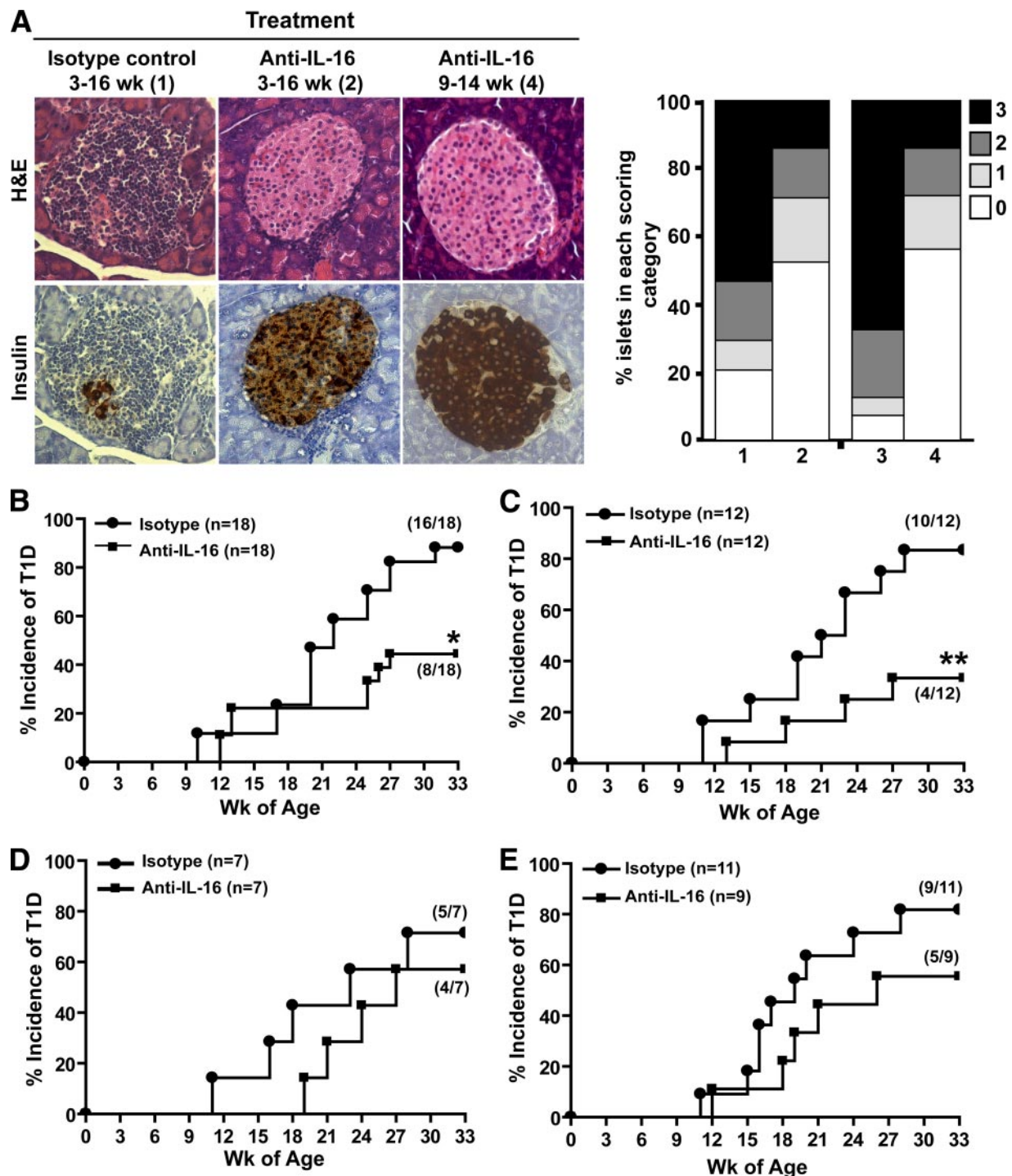


FIG. 3. Neutralization of IL-16 prevents destructive insulinitis and type 1 diabetes in NOD mice in a time- and dose-dependent manner. **A:** Representative H&E- and insulin-stained pancreas sections from NOD mice at 33 weeks of age previously treated three times weekly from 3 to 16 weeks of age with 100 μ g of isotype control (1) or anti-IL-16 (2), or from 9 to 16 weeks of age with 200 μ g isotype control (3) (note: H&E images are not shown) or anti-IL-16 (4). At 33 weeks, the severity of insulinitis was similar for mice treated with isotype control antibody from 9 to 16 (3) or 3 to 16 weeks of age (1). Bar graphs represent the percent of islets in each insulinitis scoring category for each denoted treatment group. A representative islet of the most prevalent insulinitis score observed for each treatment group at 33 weeks of age is presented. More than 100 islets were analyzed for each treatment group. NOD mice treated three times weekly with anti-IL-16 from either 3 to 16 (**B**) ($P = 0.005$) or 9 to 16 weeks of age (**C**) ($P = 0.015$), but not from 3 to 7 (**D**) ($P = 0.45$) or 17 to 22 weeks of age (**E**) ($P = 0.19$), are protected against type 1 diabetes compared with control-treated mice. (A high-quality digital representation of this figure is available in the online issue.)

for NOD mice treated between 3 and 14 weeks of age with either 100 or 200 μ g of anti-IL-16 mAb per injection (8/18 = 44%, Fig. 3B; and 3/8 = 37.5%, data not shown), respectively. Thus, a dose range of 100–200 μ g per injection is most effective for neutralization of IL-16. As short treatment regimens were used, mice were injected with a

two-fold higher dose of antibody than that used for longer treatments (3–16 weeks of age). Compared with control mice treated from 9 to 16 weeks of age (group 3; H&E images not shown), anti-IL-16 treatment prevented the development of destructive insulinitis (group 4) with protection being equally effective to that in mice treated with

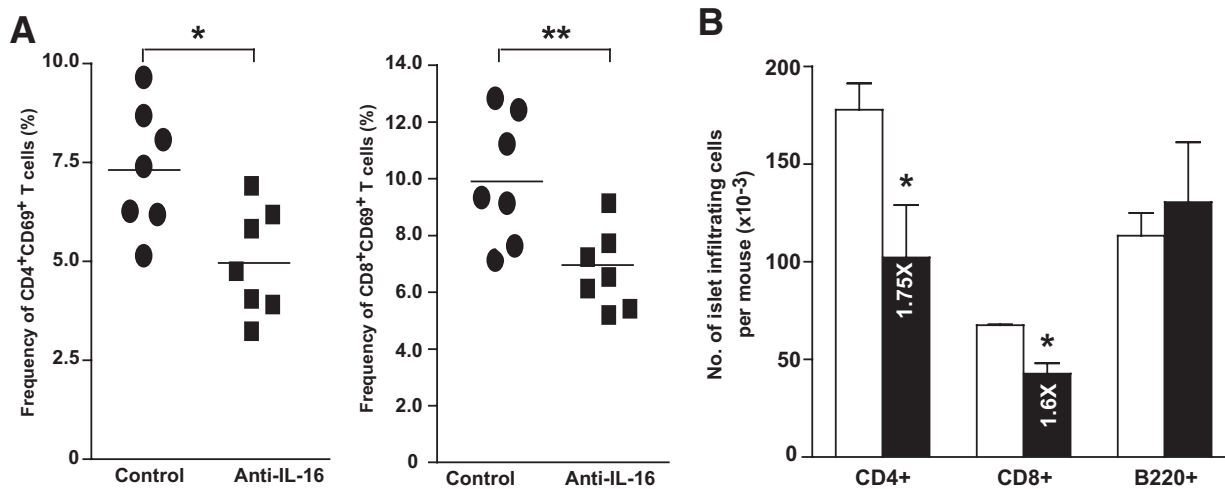


FIG. 4. Anti-IL-16 protective therapy inhibits T-cell activation in the pancreas and islet infiltration by T-cells. **A:** Independent groups of NOD mice were treated with anti-IL-16 (■) or control antibody (●) 3 times per week from 9 to 16 weeks of age, at which time PLN-derived T-cells were pooled from seven groups of three mice immediately after treatment. Surface CD69 expression was then determined using flow cytometry ($*P \leq 0.003$; $**P < 0.02$). **B:** NOD mice ($n = 6-8$ /group) were treated as above and the frequencies of islet-infiltrating lymphocytes were determined by flow cytometry, allowing for lymphocyte cell numbers per pancreas to be calculated. The numbers of islet infiltrating lymphocytes per mouse obtained in three experiments were [(2.5, 2.8, 3.1) $\times 10^5$] for anti-IL-16 and [(3.4, 3.8, 2.9) $\times 10^5$] for control-treated mice. Numbers in the bars indicate the fold change for the particular T-cell subset ($*P < 0.03$).

anti-IL-16 from 3 to 16 weeks of age (group 2). Importantly, the incidence of type 1 diabetes was reduced ($P = 0.015$) by 50% in the 9–16-week-old anti-IL-16 treated mice (4/12 mice = 33%) relative to control-treated mice (10/12 mice = 83%) (Fig. 3C). In contrast, anti-IL-16 did not prevent type 1 diabetes ($P = 0.451$) when administered between 3 and 7 weeks of age (Fig. 3D). In addition, anti-IL-16 treatment from 17–22 weeks of age (after disease onset when severe insulinitis predominates) was ineffective ($P = 0.194$) at protecting against type 1 diabetes (Fig. 3E). Note that the median survival time of 26 weeks in anti-IL-16 treated mice exceeded that of 19 weeks in control-treated mice. Therefore, IL-16 activity is not critical for the initiation of disease but is important for the transition from noninvasive to destructive insulinitis. Neutralization of IL-16 during this transition elicits maximum protection against type 1 diabetes.

Anti-IL-16 treatment decreases T-cell activation in the pancreatic lymph nodes and diminishes T-cell recruitment to islets. Because IL-16 recruits CD4⁺ T-cells to sites of inflammation and mature IL-16 levels increase more than threefold in the islets and pancreas of 3–15-week-old NOD mice, we analyzed whether anti-IL-16 treatment from 9 to 16 weeks of age protects from type 1 diabetes by blocking the recruitment to and activation of CD4⁺ T-cells in the pancreas. Consistent with our result that anti-IL-16 therapy from 9–16 weeks of age prevents invasive insulinitis and type 1 diabetes, CD69 expression on CD4⁺ ($P = 0.010$) and CD8⁺ T-cells ($P = 0.012$) from the pancreatic lymph nodes (PLN) of 16 week-old mice was significantly lower in anti-IL-16 than control-treated mice (Fig. 4A). Thus, T-cells in anti-IL-16 treated mice are less activated at the time of destructive insulinitis. Moreover, a lower percentage of CD3⁺ T-cells and higher percentage of B220⁺ B-cells was detected in islets of anti-IL-16 treated versus control-treated mice (supplementary Fig. 3A, available in an online appendix), decreasing the CD3⁺/B220⁺ ratio of islet-infiltrating cells from 2.2 to 1.1. Although B220⁺CD11c^{neg} immunosuppressive dendritic-like cells can prevent type 1 diabetes (18), we did not detect an increased frequency of this cellular subset in the pancreas of anti-IL-16 treated mice (supplementary Fig. 3B). In

addition, a significant decrease occurred in the total number of islet-infiltrating CD4⁺ ($P = 0.021$) and CD8⁺ ($P = 0.001$) T-cells in anti-IL-16 treated mice without any significant change in the number of B220⁺ B-cells ($P = 0.464$) (Fig. 4B), suggesting a preferential inhibitory effect on T-cells. Because CD4 can function as a chemotactic receptor for IL-16 on co-stimulated CD8⁺ T-cells (19), neutralization of IL-16 may diminish the recruitment of activated CD8⁺ T-cells to islets. Interestingly, a significantly altered chemokine profile was also observed in the PLN of anti-IL-16 treated mice (supplementary Table 1, available in an online appendix). Comparative analyses of changes in gene expression of 96 different cytokines and chemokines and their receptors performed using GEArray technology revealed that the expression of several transcripts, including CXCR1, CXCR3, CCR4, CCR5, CXCL9, CXCL10, CXCL12, CCL22, and macrophage migration inhibitory factor (MIF), was decreased >2-fold in anti-IL-16 versus control-treated mice. The activities of MIF, CCL22, CXCL10, CXCL12, ligands for CCR4 (CCL22), CCR5 (CCL5), and CXCR3 (CXCL10) have been associated with the development of type 1 diabetes (2,3,20–24). Only CCL4 and IL-10 mRNA expression was upregulated in the PLN of anti-IL-16 versus control-treated mice, which supports our later result that T-cell production of CCL4 is enhanced in the PLN of mice protected from type 1 diabetes. Note that, while a detectable increase in frequency of CD4⁺ IL-10⁺ T-cells in the PLN of anti-IL-16 treated mice was not detected, the mean fluorescent intensity (MFI) was increased, indicating that CD4⁺ T-cells produce more IL-10 on a per cell basis. Moreover, in contrast to the spleen, diminished Th1-like responses were observed in the PLN of anti-IL-16 treated mice (supplementary Fig. 4, available in an online appendix). Thus, neutralization of IL-16 is associated with the decreased expression of several inflammatory gene transcripts in the PLN, where autoreactive T-cells may encounter antigen before migrating to islets and eliciting their destruction (25).

Diabetogenic potential of spleen T-cells is reduced in anti-IL-16 treated mice. Adoptive transfer studies were conducted to investigate whether a decrease in the diabe-

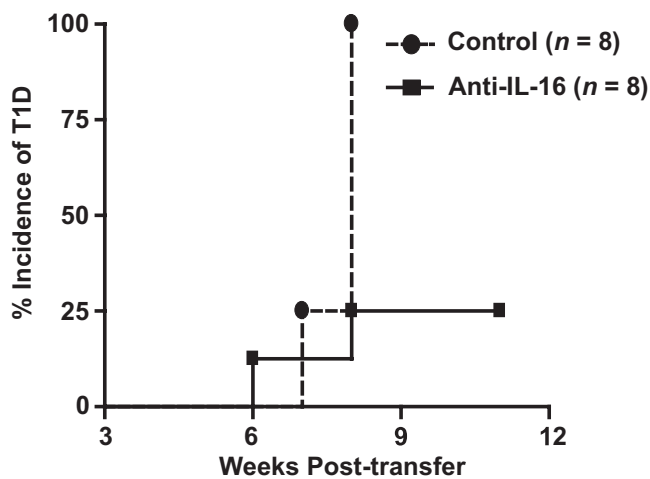


FIG. 5. Diabetogenic potential of spleen T-cells is decreased in anti-IL-16 treated NOD mice. NOD mice were treated intraperitoneally with anti-IL-16 or isotype control antibody three times per week from 9 to 16 weeks of age, and their spleen T-cells were pooled at 16 weeks of age and transferred intraperitoneally to NOD.*Scid* recipients (4×10^6 cells per mouse, eight mice per group). BGL were monitored until 11 weeks after transfer, and two consecutive BGLs ≥ 11.1 mmol/l were indicative of diabetes onset ($*P = 0.011$). Results are representative of three independent and reproducible experiments.

togenic potential of spleen T-cells mediates anti-IL-16-induced protection against type 1 diabetes. Transfer of type 1 diabetes was significantly prevented ($P = 0.011$) in NOD.*Scid* recipients of spleen T-cells from anti-IL-16 treated NOD mice (16 weeks old) (Fig. 5). Whereas only 25% (2/8) of recipients of spleen T-cells from anti-IL-16 treated mice developed type 1 diabetes by 12 weeks after transfer, 100% (8/8) of NOD.*Scid* recipients of spleen T-cells from control-treated mice developed type 1 diabetes by 8 weeks after transfer. Thus, the diabetogenic potential of splenic T-cells is diminished appreciably in anti-IL-16-treated mice at 16 weeks of age. Because we did not detect a significant increase in the number of regulatory T-cells in the spleen of anti-IL-16 treated mice (our unpublished data), another mechanism(s) may con-

trol the diabetogenic potential of T-cells in anti-IL-16 treated mice.

Anti-IL-16 therapy elicits Th1-like immune responses and susceptibility of T-cells to apoptosis. Because Th2-type cytokine responses can prevent type 1 diabetes (26), we considered whether a heightened Th2-type immune response develops in anti-IL-16 treated mice. Unexpectedly, activated spleen T-cells from anti-IL-16 treated mice produced significantly more IL-2 ($P = 0.008$), IFN- γ ($P = 0.001$), and CCL4 ($P = 0.004$) than T-cells from control-treated mice (Fig. 6A). However, no changes in the level of IL-4 were detected (our unpublished data). Similarly, activated T-cells secreted elevated levels of CCL4, but not IL-4, in the PLN of anti-IL-16 treated mice (Fig. 6B). Administration of CCL4 directly prevents type 1 diabetes in NOD mice (27). Moreover, elevated levels of IFN- γ in the spleen diminish the autoimmune response in NOD mice by promoting the activation-induced cell death (AICD) of T-cells (28). Considering that IL-16 regulates susceptibility of CD4⁺ T-cells to apoptosis (7), we determined whether anti-IL-16 enhances the apoptosis of T-cell receptor (TCR)-stimulated spleen cells. At 48 h poststimulation, significantly higher levels of apoptotic NOD spleen CD4⁺ T-cells ($P = 0.008$) were detected in anti-IL-16 than in control-treated mice (Fig. 6C). Therefore, anti-IL-16 may protect from type 1 diabetes by enhancing CD4⁺ T-cell susceptibility to apoptosis in response to TCR stimulation.

Anti-IL-16 therapy inhibits the activation of autoreactive T-cells in the pancreas. To further test the hypothesis that anti-IL-16 therapy regulates the recruitment and activation of islet-specific CD4⁺ T-cells in the pancreas, we compared the levels of antigen-induced T-cell proliferation in the pancreas of anti-IL-16 and isotype control-treated mice. NOD mice were treated with anti-IL-16 or isotype control antibody between 3 and 15 weeks of age. At 14 weeks of age, both groups of NOD mice received CD4⁺CD25^{neg} T-cells from NOD.BDC2.5 Thy1.1⁺ mice. At 7 days posttransfer, 73.5% of islet antigen specific Thy1.1⁺BDC2.5 T-cells were dividing in the pancreas of isotype-treated mice compared to only 0.01% of the corresponding T-cells in anti-IL-16 treated

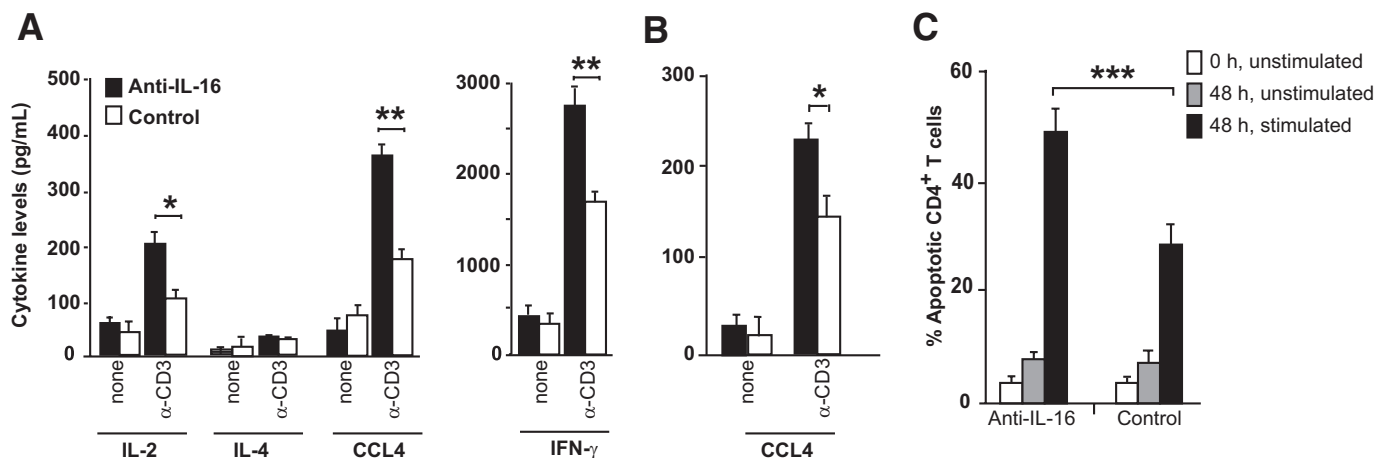


FIG. 6. Anti-IL-16 treatment enhances Th1-type cytokine secretion and apoptosis of spleen T-cells. Spleen T-cells (A) or PLN-derived T-cells (B) (10^6 /ml) purified from mice treated three times per week from 9 to 16 weeks of age with anti-IL-16 or control antibody were activated for 48 h in vitro with plate-bound anti-CD3 ϵ . Cytokine and chemokine accumulation in supernatants from triplicate cultures were determined by ELISA. Mean values \pm SD are shown, and similar results were obtained in two independent and reproducible experiments ($*P < 0.01$; $**P < 0.005$). C: Apoptosis of activated CD4⁺ T-cells is augmented in anti-IL-16 treated mice. Spleen T-cells (2×10^6 per ml) from anti-IL-16 or isotype Control-treated mice were stimulated with plate-bound anti-CD3 ϵ , and at the indicated time points, the percentage of CD4⁺ T-cells undergoing apoptosis was determined by transferase-mediated dUTP nick-end labeling staining. The results of triplicate cultures are expressed as the mean \pm SEM ($***P = 0.005$). Results are representative of two independent and reproducible experiments.

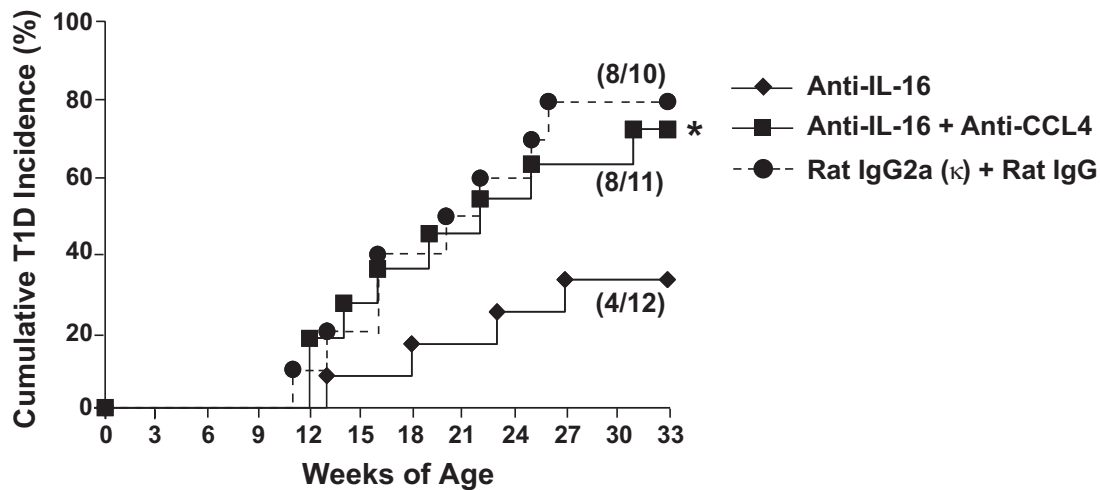


FIG. 7. Anti-IL-16-induced protection from type 1 diabetes in NOD mice is CCL4-dependent. NOD mice were treated with 200 μ g of the 14.1 anti-IL-16 mAb ($n = 12$), 200 μ g of anti-IL-16 plus 100 μ g of anti-CCL4 ($n = 11$), or 200 μ g of isotype control IgG2a(κ) plus 100 μ g control rat IgG ($n = 10$) three times per week from 9 to 16 weeks of age. Mice were monitored for the development of type 1 diabetes until 33 weeks of age, and two consecutive BGLs of ≥ 11.1 mmol/l were indicative of onset of type 1 diabetes (* $P \leq 0.05$ relative to anti-IL-16 treated mice).

recipients (supplementary Fig. 5, available in an online appendix). Minimal proliferation was observed in antigen-deficient inguinal lymph nodes (IGLN) in either isotype control or anti-IL-16 treated recipient mice. These results indicate that anti-IL-16 therapy inhibits the proliferation of islet antigen-specific T-cells in the pancreas.

Neutralization of CCL4 abrogates anti-IL-16-induced protection against type 1 diabetes. Because CCL4 protects from type 1 diabetes (27) and spleen- and PLN-T-cell production of CCL4 is elevated in anti-IL-16 mAb treated mice, we determined whether anti-IL-16-induced protection from type 1 diabetes occurs in a CCL4-dependent manner. The effect of coadministration of neutralizing anti-CCL4 and anti-IL-16 antibodies to female NOD mice from 9 to 16 weeks of age on the incidence of type 1 diabetes was evaluated. This combination treatment abrogated ($P = 0.05$) the ability of anti-IL-16 to protect against type 1 diabetes (Fig. 7). Thus, anti-IL-16 therapy augments CCL4 production by T-cells, and anti-IL-16 mediated protection against type 1 diabetes is CCL4-dependent.

DISCUSSION

In this study, we demonstrate that progression to the onset of type 1 diabetes in NOD mice is associated with an increased expression of IL-16 in pancreatic islets and identify IL-16 as a novel potential target for protection against the development of type 1 diabetes. Our studies localized mature IL-16 to B-cells and T-cells in islet lesions and detected IL-16 only during the development of invasive and destructive insulinitis up to 15–16 weeks of age. Importantly, the secretion of IL-16 by several cell types in islet lesions can potentially augment the autoimmune response by recruiting additional CD4⁺ cells, including various T-cell populations and dendritic cells, to sites of islet damage. Consistent with this idea, treatment of pre-diabetic NOD mice with a neutralizing anti-IL-16 mAb beginning at the outset of mature IL-16 expression in islets resulted in lower numbers of activated CD4⁺ T-cells in the pancreas and provided optimum protection against destructive insulinitis and type 1 diabetes. Consequently, we have demonstrated for the first time that neutralization of endogenous IL-16 activity may have therapeutic value for

the treatment of type 1 diabetes because of its ability to arrest the transition from peri-insulinitis to an invasive/destructive insulinitis and onset of disease. This notion builds on the relationship between therapeutic efficacy and stage of disease treatment recommended for translational efficiency in clinical trials of type 1 diabetes (29). Furthermore, this relationship may apply for other successful modes of anti-IL-16 mediated protection, e.g., inflammation and disease in the experimental allergic encephalomyelitis (EAE) mouse model of multiple sclerosis and in a mouse model of inflammation-mediated ischemia-reperfusion injury of the kidney (30–32).

Although our study is the first to characterize the function of IL-16 during the development of type 1 diabetes, earlier reports have described how administration of a nondepleting anti-CD4 mAb also prevents type 1 diabetes (33,34). Similar to anti-IL-16 immunotherapy, this nondepleting anti-CD4 mAb prevents type 1 diabetes when administered after the development of invasive insulinitis (33). The latter studies agree with our results considering that such an anti-CD4 mAb may block IL-16/CD4 interactions, and the efficacy of a nondepleting anti-CD4 mAb may be due in part to the inhibition of IL-16 function.

IL-16 can suppress CD4⁺ T-cell activation and AICD (7), and such mechanisms may promote inflammation and/or autoimmunity by regulating T-cell homeostasis and limiting the clonal expansion of autoreactive T-cells (35,36). In NOD mice, the development of type 1 diabetes is influenced by defects in central and peripheral tolerance including TCR-stimulated CD4⁺ T-cell resistance to apoptosis (37–41). Here, we showed that neutralization of IL-16 enhances TCR-induced susceptibility of spleen CD4⁺ T-cells to apoptosis. Although the mechanism that regulates apoptosis of CD4⁺ T-cells in anti-IL-16 treated mice is unknown, previous studies predict that CD95 may play a role (7). In addition, IFN- γ may also regulate T-cell apoptosis and homeostasis in anti-IL-16 treated mice given that we found an increased production of IFN- γ by spleen T-cells from anti-IL-16 versus control-treated mice. The latter finding is consistent with reports that IFN- γ induces the apoptosis of effector Th1 cells (42), triggers CD4⁺ T-cell AICD (43,44), and protects from type 1 diabetes by

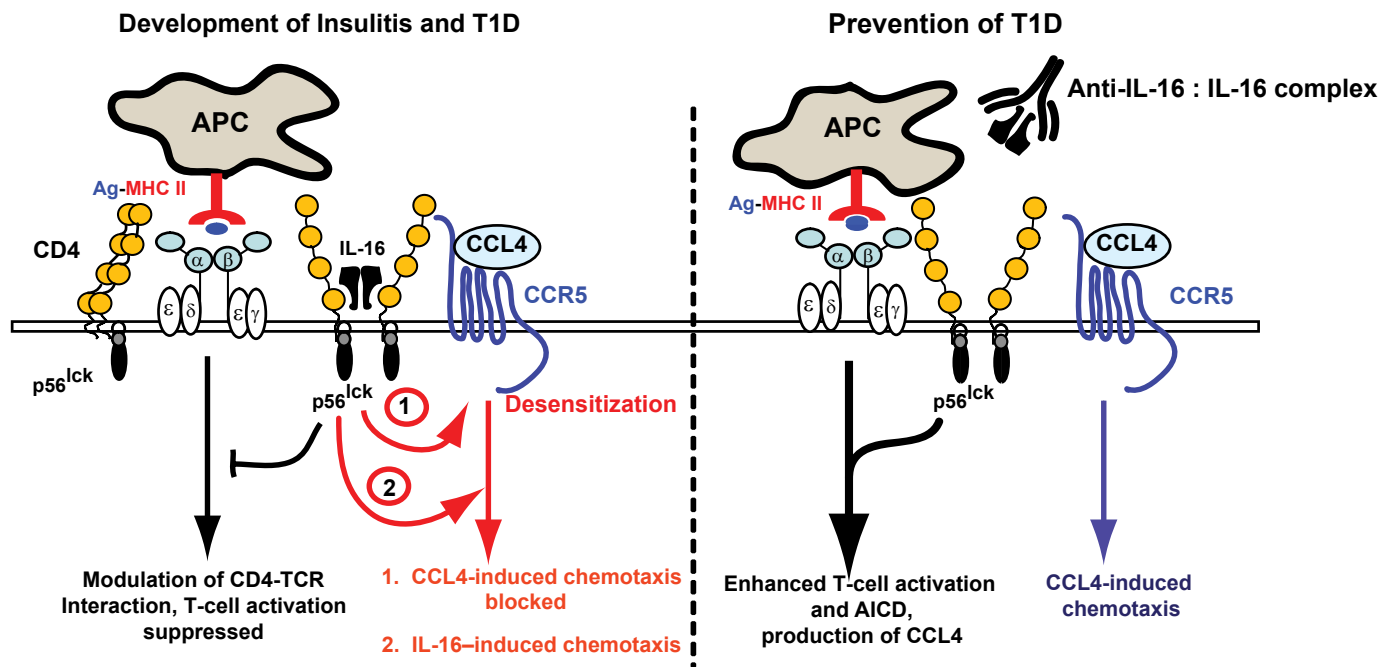


FIG. 8. IL-16 and CCL4 may function as counterregulatory cytokines during the development of type 1 diabetes. Upon binding of IL-16 to CD4, interactions between CD4 and TCR are modulated as CD4 self-association is prevented and antigen-induced T-cell activation is suppressed. Concomitantly, a $p56^{lck}$ dependent signal desensitizes CCR5 and blocks the activity of CCL4 and other CCR5 ligands. A second non $p56^{lck}$ -dependent signal mediates IL-16-induced chemotaxis, which is enhanced by CCR5. Upon neutralization of IL-16, CD4 facilitates T-cell activation and CCL4 production is enhanced, resulting in the prevention of type 1 diabetes.

enhancing the apoptosis of autoreactive spleen T-cells in NOD mice (28). Furthermore, we observed that spleen T-cells from anti-IL-16 treated mice were deficient in their ability to transfer type 1 diabetes to NOD.*Scid* mice. Thus, by enhancing susceptibility of $CD4^+$ effector T-cells to apoptosis together with neutralization of IL-16 chemotactic activity, anti-IL-16 therapy may reduce $CD4^+$ T-cell trafficking to islets by different mechanisms. Moreover, the reduced severity of islet infiltration by $CD4^+$ and $CD8^+$ T-cells in anti-IL-16 treated NOD mice is also accompanied by the decreased expression of several other inflammatory cytokines, chemokines, and their receptors in the pancreas (supplementary Table 1). Although neutralization of IL-16 protects from type 1 diabetes, we also noted that some anti-IL-16 treated mice developed type 1 diabetes at the same age as control-treated mice. It is likely that several other chemoattractant factors also target the immune system against islets in the absence of IL-16, which is consistent with the pleiotropic and redundant biology of chemoattractant cytokines.

A diminished inflammatory response against islets and prevention of type 1 diabetes seems to result in part from the decreased expression of several inflammatory mediators. Nonetheless, neutralization of IL-16 led to an increase in CCL4 and IL-10 expression in the PLN, which is relevant because CCL4 and IL-10 expression are associated with protection against type 1 diabetes. Of note, whereas activated T-cells were found to secrete elevated levels of CCL4 in the PLN of anti-IL-16 treated mice, CCL4 is also expressed by a variety of immune cells that could represent additional cellular sources of CCL4 in anti-IL-16 treated mice. Because we showed that neutralization of CCL4 reverses anti-IL-16 mediated protection against type 1 diabetes, our results agree with several reports that CCL4 protects against type 1 diabetes in mice and is not associated with disease onset in humans (22,27,45,46). Although

an increase in the frequency of $FoxP3^+$ regulatory T-cells was not observed in anti-IL-16 treated mice, we cannot exclude the possibility that Tr1 regulatory T-cells and their secreted IL-10 product may be protective in these mice, as comparison of the IL-10 MFI indicated that $CD4^+$ T-cells do indeed produce greater amounts of IL-10 in the PLN of anti-IL-16 treated mice (supplementary Fig. 4).

Because CD4 and CCR5 can act synergistically to selectively recruit Th1 cells to sites of inflammation (4,47), CCL4 expression may be elevated in anti-IL-16 treated mice to compensate for deficient IL-16 activity. However, if IL-16 and CCL4 function cooperatively to promote islet inflammation, then neutralization of CCL4 would not be expected to abrogate anti-IL-16 mediated protection. Thus, our results support the notion that IL-16 may regulate or antagonize CCL4 function in the spleen and islets during the onset of type 1 diabetes. These findings are consistent with the observations that reciprocal cross-desensitization occurs between CD4 and CCR5 upon the binding of IL-16 and CCL4, respectively (15), and that CCL4 treatment can prevent type 1 diabetes (27). How CCL4 functions in anti-IL-16-induced protection from type 1 diabetes is currently not well understood, but this protection may result from the lack of IL-16-induced desensitization of CCR5 according to a model shown in Fig. 8 (4,15). This model proposes that CCL4 signaling through CCR5 mediates protection from type 1 diabetes, an idea supported by two recent reports that transient blockade of CCR5 with an anti-CCR5 mAb during 11–13 weeks of age or CCR5 deficiency significantly accelerates rather than prevents type 1 diabetes in NOD mice (48,49). Further experimentation is required to ascertain the functional role of CCL4 in anti-IL-16 mediated protection against type 1 diabetes.

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