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 (T1D). This inflammatory process is mediated by several mediators that are potential therapeutic targets to arrest development of T1D. In this study, we investigate the role of one of these mediators, interleukin-16 (IL-16), in the pathogenesis of T1D in nonobese diabetic (NOD) mice.

**Research design and methods**—At various stages of progression of T1D, 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 T1D.

**Results**—The increased expression of IL-16 in islets correlated with the development of invasive insulitis. IL-16 immunoreactivity was found in islet infiltrating T cells, B cells, NK cells, and dendritic cells (DC), 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 T1D 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 insulitis during the onset of T1D. IL-16 and CCL4 appear to function as counter-regulatory proteins during disease development. Neutralization of IL-16 may represent a novel therapy for the prevention of T1D.
novel therapeutic target for the prevention of T1D.

**RESEARCH DESIGN AND METHODS**

**Mice.** 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 (SuperArray Biosciences). A detailed protocol is presented in the Supplemental Material and Methods.

**In vivo treatment of mice.** NOD mice were injected intraperitoneally (i.p.) 3x 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 3-7, 9-16 or 17-22 weeks of age. Control mice received isotype-matched mouse IgG2a(κ) (UPC 10, 200 µg, Sigma) (17). To determine if CCL4 mediates anti-IL-16 induced protection from T1D, 0.2 mg of anti-IL-16 (14.1) and 0.1 mg of anti-mouse CCL4 mAb (R&D Systems) were co-administered 3x weekly from 9-16 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 mM on two consecutive readings indicated the onset of T1D.

**Histopathology.** Pancreata were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 7 µm intervals. The incidence and severity of insulitis were examined after hematoxylin-eosin staining. At least 10 non-adjacent sections were analyzed per pancreas, and average percentages of insulitis were determined from ≥ 100 islets per treatment group.

**Adoptive T cell transfer.** NOD.Scid mice (5-7 week-old, n=8/group) were injected i.p. with spleen T cells (4 x 10⁶/mouse) isolated at 16 weeks of age from NOD mice treated 3x weekly from 9-16 weeks of age with 200 µg of anti-IL-16 (14.1) or isotype control IgG2a(κ).

**Statistical analysis.** Significant differences were determined using the Student’s t test for unpaired samples, the Mantel-Haenszel log rank test, and the Mann Whitney non-parametric test where indicated. Statistical comparisons were performed using GraphPad Prism (www.graphpad.com; San Diego, CA). A probability value of $P < 0.05$ was considered significant.

An expanded RESEARCH DESIGN AND METHODS section is available in the Online Appendix at http://diabetes.diabetesjournals.org.

**RESULTS**

**IL-16 expression during development of insulitis.** In our NOD female mice, non-destructive peri-insulitis develops from 6-10 weeks of age and is followed by an invasive and destructive insulitis in 15-25 week-old mice (Fig. 1A). Onset of diabetes is observed as early as 13 wk of age progressing to a cumulative disease incidence of 80-90% by 30 wk 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 maximum by 15 weeks of age (Fig. 1B). IL-16 mRNA expression decreased between 15-25 weeks of age even though insulitis 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 insulitis, 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 3-19 weeks of age. Intra-pancreatic pro-IL-16 (80 kDa) expression increased during this timeframe and peaked near 16 weeks of age (Fig. 1B). Similarly, mature IL-16 (18 kDa) was detected between 8-16 weeks of age. Since 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 insulitis 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 between 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 between 3-15 weeks of age, with the largest increase detected between 9-15 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 pro-inflammatory responses against islets.

**In situ localization of IL-16 in islets.** To examine which cells produce IL-16 in an insulitic lesion, immunohistochemistry and confocal imaging was used. As IL-16 is synthesized as a precursor protein (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 insulitic lesions, including Thy1+ T cells, B220+ B cells, CD4+ and CD8+ T cells, p46+ NK cells, and CD11c+ DC (Fig. 2 and Supplemental Fig. 1). For all experiments, the appropriate isotype controls were used to rule out non-specific staining (Supplemental Fig. 2A). 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. Co-localization 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. Since IL-16 expression was not detected in insulin-producing islet beta 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 (Supplemental Fig. 2B). Thus, in an insulitic 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 insulitis and T1D.** Having determined that intra-islet expression of mature IL-16 increases with the severity of insulitis, we
next examined whether blockade of endogenous IL-16 can prevent T1D. NOD mice were treated with a neutralizing anti-IL-16 mAb or isotype-matched control antibody from 3-16 weeks of age when intra-pancreatic levels of IL-16 are increased. This treatment reduced the overall severity of insulitis, and prevented the onset of destructive insulitis 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 insulitis (insulitis 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 possessed 52% normal healthy islets (insulitis score = 0), while only 20% of islets were healthy in the control-treated mice. In addition, the incidence of T1D 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 insulitis and T1D.

Since intra-pancreatic IL-16 expression peaks between 9-16 weeks of age, we reasoned that neutralization of IL-16 may prevent T1D 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 insulitis. At 33 weeks of age, a similar incidence of T1D was initially observed for NOD mice treated between 3-14 weeks of age with either 100 or 200 µg of anti-IL-16 mAb per injection (8/18 = 44%; Figure 3B) and 3/8 = 37.5%; data not shown), respectively. Thus a dose range of 100-200 µg 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 to control mice treated from 9-16 weeks of age (Group 3; H&E images not shown), anti-IL-16 treatment prevented the development of destructive insulitis (Group 4) with protection being equally effective to that in mice treated with anti-IL-16 from 3-16 weeks of age (Group 2). Importantly, the incidence of T1D 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 T1D (*P*=0.451) when administered between 3-7 weeks of age (Fig. 3D). In addition, anti-IL-16 treatment from 17-22 weeks of age (after disease onset when severe insulitis predominates) was ineffective (*P*=0.194) at protecting against T1D (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 non-invasive to destructive insulitis. Neutralization of IL-16 during this transition elicits maximum protection against T1D.

**Anti-IL-16 treatment decreases T cell activation in the PLN and diminishes T cell recruitment to islets.** Since IL-16 recruits CD4<sup>+</sup> T cells to sites of inflammation and mature IL-16 levels increase more than 3-fold in the islets and pancreas of 3-15 week-old NOD mice, we analyzed whether anti-IL-16 treatment from 9-16 weeks of age protects from T1D by blocking the recruitment to and activation of CD4<sup>+</sup> T cells in the pancreas. Consistent with our result that anti-IL-16 therapy from 9-16 weeks of age prevents invasive insulitis and T1D, CD69 expression on CD4<sup>+</sup> T cells (*P*=0.010) and CD8<sup>+</sup> T cells (*P*=0.012) from the 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 insulitis. Moreover, a lower percentage of CD3<sup>+</sup> T cells and higher
percentage of B220+ B cells was detected in islets of anti-IL-16 treated versus control treated mice (Supplemental Fig. 3A), decreasing the CD3+,:B220+ ratio of islet-infiltrating cells from 2.2 to 1.1. Although B220+CD11c-neg immunosuppressive DC-like cells can prevent T1D (18), we did not detect an increased frequency of this cellular subset in the pancreas of anti-IL-16 treated mice (Supplemental 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. As 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 (Supplemental Table 1). Comparative analyses of changes in gene expression of 96 different cytokines and chemokines and their receptors performed using GEArrayTM 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 more than 2-fold in anti-IL-16 versus control treated mice. The activity of MIF, CCL22, CXCL10, CXCL12, ligands for CCR4 (CCL22), CCR5 (CCL5), CXCR3 (CXCL10) has been associated with the development of T1D (2,3,20-24). Only CCL4 and IL-10 mRNA expression was up-regulated in the PLN of anti-IL-16 versus control treated mice, which supports our result that T cell production of CCL4 is enhanced in the PLN of mice protected from T1D. Note that while a detectable increase in frequency of CD4+ IL-10+ T cells in the PLN of anti-IL-16 treated mice, 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 (Supplemental Figure 4). 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 diabetogenic potential of spleen T cells mediates anti-IL-16 induced protection against T1D. Transfer of T1D was significantly prevented (P=0.011) in NOD.Scid recipients of spleen T cells from anti-IL-16 treated NOD mice (16 week-old) (Fig. 5). Whereas only 25% (2/8) of recipients of spleen T cells from anti-IL-16 treated mice developed T1D by 12 weeks after transfer, 100% (8/8) of NOD.Scid recipients of spleen T cells from control treated mice developed T1D by 8 weeks post-transfer. Thus, the diabetogenic potential of splenic T cells is diminished appreciably in anti-IL-16 treated mice at 16 weeks of age. As 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 control 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.** As Th2-type cytokine responses can prevent T1D (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 T1D 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 TCR-stimulated spleen cells. At 48 h post-stimulation, 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 T1D 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 16 weeks of age. At 14 weeks of age, both groups of NOD mice received CD4+CD25neg T cells from NOD.BDC2.5.Thy1.1 mice. At 7 days post-transfer, 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 recipients (Supplemental Fig. 5). Minimal proliferation 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 T1D.** Since CCL4 protects from T1D (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 T1D occurs in a CCL4-dependent manner. The effect of co-administration of neutralizing anti-CCL4 and anti-IL-16 antibodies to female NOD mice from 9-16 weeks of age on the incidence of T1D was evaluated. This combination treatment abrogated (P=0.05) the ability of anti-IL-16 to protect against T1D (Fig. 7). Thus, anti-IL-16 therapy augments CCL4 production by T cells, and anti-IL-16 mediated protection against T1D is CCL4-dependent.

**DISCUSSION**

In this study, we demonstrate that progression to the onset of T1D 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 T1D. 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 insulitis 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 DCs, 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 insulitis and T1D. Consequently, we have demonstrated for the first time that neutralization of endogenous IL-16 activity may have
therapeutic value for the treatment of T1D due to its ability to arrest the transition from peri-insulitis to an invasive/destructive insulitis 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 T1D (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).

While our study is the first to characterize the function of IL-16 during the development of T1D, earlier reports have described how administration of a non-depleting anti-CD4 mAb also prevents T1D (33,34). Similar to anti-IL-16 immunotherapy, this non-depleting anti-CD4 mAb prevents T1D when administered after the development of invasive insulitis (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 non-depleting 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 T1D 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 T1D by 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 T1D to NOD.Scid mice. Thus, by enhancing susceptibility of 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 (Supplemental Table 1). Although neutralization of IL-16 protects from T1D, we also noted that some anti-IL-16 treated mice developed T1D 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 T1D 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 as CCL4 and IL-10 expression are associated with protection against T1D. Of note, while 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. Since we showed that neutralization of CCL4 reverses anti-IL-16 mediated protection against T1D, our results agree with several reports that CCL4 protects against T1D in mice and is not associated with disease onset in humans (22,27,45,46). While 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 mean fluorescent intensity indicated that CD4+ T cells do indeed produce greater amounts of IL-10 in the PLN of anti-IL-16 treated mice (Supplemental Figure 4).

Since 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 T1D. 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 T1D (27). How CCL4 functions in anti-IL-16 induced protection from T1D is presently not well understood, but this protection may result from the lack of IL-16 induced desensitization of CCR5 according to a model shown in Figure 8 (4,15). This model proposes that CCL4 signaling through CCR5 mediates protection from T1D, 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 T1D in NOD mice (48,49). Further experimentation is required to ascertain the functional role of CCL4 in anti-IL-16 mediated protection against T1D.

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FIG. 1. Quantitation of IL-16 mRNA and chemotactic activity in the pancreas and islets. A, 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 GAPDH and normalized to 3 weeks of age. One of two representative experiments is shown. B, Immunoprecipitation of IL-16 isoforms in pancreata from NOD and NOD.Scid (NS) mice. Lysate aliquots were immunoprecipitated as described in Materials and Methods. All blots were initially developed by electrochemiluminescence. Visualization of mature IL-16 was enhanced by stripping, re-blotting, and detecting with the more sensitive ECL-Plus reagent (lower panel). Stripping and re-blotting with non-specific isotype-matched biotinylated control failed to detect IL-16. Results are from one of three representative and reproducible experiments. C, 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 x 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 µg/ml; white bars). *, 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.

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; panels C-E, I-J, M-N), anti-insulin (blue; panels F, L, O) and various cell surface markers in green (Thy1.2 in panels A, D, E; B220 in panels G, J, K; CD4 in panels M-N). Magnified images of areas indicated by the white boxes in merged images (panels D, J, and M) are presented in panels E, K, and N. Colocalized signals of IL-16, activated caspase-3, and Thy1.2 (panel E) or B220 (panel K) or CD4 (panel N) are denoted by arrows. Adjacent serial sections stained for IL-16 and insulin are shown in panels F, L, and O. Scale bars represent 50 µm for all images except for the magnified images (panels E, K, N) where the scale bar represents 10 µm.

FIG. 3. Neutralization of IL-16 prevents destructive insulitis and T1D 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 3x weekly from 3-16 weeks of age with 100 µg of isotype control (1) or anti-IL-16 (2), or from 9-16 weeks of age with 200 µg isotype control (3) (note: H&E images are not shown) or anti-IL-16 (4). At 33 weeks, the severity of insulitis was similar for mice treated with isotype control antibody from 9-16 (3) or 3-16 weeks of age (1). Bar graphs represent the percent of islets in each insulitis scoring category for each denoted treatment group. A representative islet of the most prevalent insulitis 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 3x weekly with anti-IL-16 from either 3-16 (B) (P=0.005) or 9-16 weeks of age (C) (P=0.015), but not from 3-7 (D) (P=0.45) or 17-22 weeks of age (E) (P=0.19), are protected against T1D compared to control treated mice.

FIG. 4. Anti-IL-16 protective therapy inhibits T cell activation in the pancreas and islet infiltration by T cells. Independent groups of NOD mice were treated with anti-IL-16 (■) or control antibody (○) 3x per week from 9-16 weeks of age, at which time PLN-derived T cells
pooled from 7 groups of 3 mice (A) were isolated 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 (*, \(P < 0.03\)).

FIG. 5. Diabetogenic potential of spleen T cells is decreased in anti-IL-16 treated NOD mice. NOD mice were treated i.p. with anti-IL-16 or isotype control antibody 3x per week from 9-16 weeks of age, and their spleen T cells were pooled at 16 weeks of age and transferred i.p. to NOD.Scid recipients (4 x 10^6 cells/mouse, 8 mice/group). BGL were monitored until 11 weeks post-transfer, and two consecutive BGL > 11.1 mM was indicative of diabetes onset (*, \(P=0.011\)). Results are representative of three independent and reproducible experiments.

FIG. 6. Anti-IL-16 treatment enhances Th1-type cytokine secretion and apoptosis of spleen T cells. (A) Spleen T cells or (B) PLN-derived T cells (10^6/ml) purified from mice treated 3x/week from 9-16 weeks of age with anti-IL-16 or control antibody were activated for 48 h in vitro with plate-bound anti-CD3\(\varepsilon\). Cytokine and chemokine accumulation in supernatants from triplicate cultures were determined by ELISA. Mean values ± 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 x 10^6/ml) from anti-IL-16 or isotype control treated mice were stimulated with plate-bound anti-CD3\(\varepsilon\), and at the indicated time points, the percentage of CD4^+ T cells undergoing apoptosis was determined by TUNEL staining. The results of triplicate cultures are expressed as the mean ± SEM (***, \(P = 0.005\)). Results are representative of two independent and reproducible experiments.

FIG. 7. Anti-IL-16 induced protection from T1D in NOD mice is CCL4-dependent. NOD mice were treated with 200 \(\mu\)g of the 14.1 anti-IL-16 mAb (n=12), 200 \(\mu\)g of anti-IL-16 plus 100 \(\mu\)g of anti-CCL4 (n=11), or 200 \(\mu\)g of isotype control IgG2a(\(\kappa\)) plus 100 \(\mu\)g control rat IgG (n=10) 3x/week from 9-16 weeks of age. Mice were monitored for the development of T1D until 33 weeks of age, and two consecutive BGL of \(\geq 11.1\) mM was indicative of onset of T1D (*, \(P \leq 0.05\) relative to anti-IL-16 treated mice).

FIG. 8. IL-16 and CCL4 may function as counter-regulatory cytokines during the development of T1D. 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^{\text{Lck}} independent signal desensitizes CCR5 and blocks the activity of CCL4 and other CCR5 ligands. A second non-p56^{\text{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 T1D.
Figure 1

IL-16 BLOCKADE PROTECTS FROM TYPE 1 DIABETES
Figure 2

IL-16 BLOCKADE PROTECTS FROM TYPE 1 DIABETES
**Figure 3**

| Treatment                  | Isotype control 3-16 wk (1) | Anti-IL-16 3-16 wk (2) | Anti-IL-16 9-14 wk (4) |
|----------------------------|-----------------------------|------------------------|------------------------|
| H&E                        | ![H&E image]                | ![H&E image]          | ![H&E image]          |
| Insulin                    | ![Insulin image]            | ![Insulin image]       | ![Insulin image]       |

**Graphs:**

- **B:** Comparison of TID incidence over weeks of age between Isotype (n=18) and Anti-IL-16 (n=18).
- **C:** Additional data on TID incidence over weeks of age.
- **D:** Further comparison with Isotype (n=7) and Anti-IL-16 (n=7).
- **E:** Additional data with Isotype (n=11) and Anti-IL-16 (n=9).
Figure 4

A

Frequency of CD4^+ CD8^+ T cells (%)

B

Frequency of CD4^+ CD8^+ T cells (%)

No. of infiltrating cells per mouse (10^6)

CD4+    CD8+    B220+

176X    1.6X    *

Figure 5

% Incidence of T1D

Wk Post-transfer

Control (n=8)    Anti-IL-16 (n=8)
Figure 6

A

Cytokine levels (pg/mL)

0 100 200 300 400 500

Control

Anti-IL-16

IL-2

IL-4

CCL4

IFN-γ

B

C

% Apoptotic CD4+ T cells

0 20 40 60

0 h, unstimulated

48 h, unstimulated

48 h, stimulated

Control

Anti-IL-16

Figure 7

Cumulative T1D Incidence (%)

0 20 40 60 80

0 3 6 9 12 15 18 21 24 27 30 33

Wk of Age

Anti-IL-16

Anti-IL-16 + Anti-CCL4

Rat IgG2a (k) + Rat IgG

(8/10)

(8/11)

(4/12)
Figure 8

Development of Insulitis and T1D

- APC
- Ag-MHC II
- CD4
- p56<sup>lck</sup>

IL-16
- CCL4
- CCR5

Modulation of CD4-TCR interaction, T cell activation suppressed

1. CCL4 induced chemotaxis blocked
2. IL-16 induced chemotaxis

Prevention of T1D

- APC
- Ag-MHC II
- CD4
- p56<sup>lck</sup>

Anti-IL-16: IL-16 complex
- CCL4
- CCR5

Enhanced T cell activation and AICD, production of CCL4

CCL4 induced chemotaxis

Desensitization