Interleukin-21 Is Required for the Development of Type 1 Diabetes in NOD Mice

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OBJECTIVE—Interleukin (IL)-21 is a type 1 cytokine that has been implicated in the pathogenesis of type 1 diabetes in mice. The unique biology of the nonobese diabetic (NOD) mouse strain. The aim of this study was to investigate a causal role for IL-21 in type 1 diabetes.

RESEARCH DESIGN AND METHODS—We generated IL-21R−/- deficient NOD and C57Bl/6 mice expressing IL-21 in pancreatic β-cells, allowing the determination of the role of insufficient and excessive IL-21 signaling in type 1 diabetes.

RESULTS—Deficiency in IL-21R expression renders NOD mice resistant to insulitis, production of insulin autoantibodies, and onset of type 1 diabetes. The lymphoid compartment in IL-21R−/− NOD is normal and does not contain an increased regulatory T-cell fraction or diminished effector cytokine responses. However, we observed a clear defect in autoreactive effector T-cells in IL-21−/− NOD by transfer experiments. Conversely, overexpression of IL-21 in pancreatic β-cells induced inflammatory cytokine and chemokines, including IL-17A, IL-17F, IFN-γ, monocyte chemoattractant protein (MCP)-1, MCP-2, and interferon-inducible protein-10 in the pancreas. The ensuing leukocytic infiltration in the islets resulted in destruction of β-cells and spontaneous type 1 diabetes in the normally diabetes-resistant C57Bl/6 and NOD × C57Bl/6 backgrounds.

CONCLUSIONS—This work provides demonstration of the essential prodiabeticogenic activities of IL-21 on diverse genetic backgrounds (NOD and C57Bl/6) and indicates that IL-21 blockade could be a promising strategy for interventions in human type 1 diabetes. Diabetes 58:1144-1155, 2009

The nonobese diabetic (NOD) mouse model is the most well-characterized animal model of human type 1 diabetes and has provided important insights into the etiology and pathogenesis of this increasingly prevalent autoimmune disease (1). Rigorous genetic analysis of the NOD background has revealed the existence of multiple defined chromosomal regions known as insulin-dependent diabetes (idd) loci that confer susceptibility to or protection from the development of type 1 diabetes (2). Of the ~15 regions identified, idd3 is of particular importance, because congenic NOD lines containing alleles from protected strains at this locus are significantly less susceptible to diabetes. To date, idd3 is the most potent disease modifying the non–major histocompatibility complex (MHC) locus (3). Therefore, some of the genes within the idd3 interval must play a crucial role in regulating immune destruction of pancreatic β-cells.

Among the several candidate genes within the idd3 locus, interleukin (IL)-21 is of particular interest, because dysregulated IL-21 production and signaling has been found in the NOD mouse (4). IL-21 belongs to the type 1 cytokine family, which includes potent immune modulators such as IL-2, IL-4, IL-7, and IL-15, whose high-affinity receptor complexes all use the common γc receptor subunit (5,6). The specificity of IL-21 signaling is achieved through its specific interaction with the IL-21 receptor subunit, which forms a heterodimer with the γc subunit (7). This receptor complex delivers IL-21 signals to a variety of immune cells including CD4+ and CD8+ T-cells, B-cells, NK cells, NKT cells, and dendritic cells (8–13), all of which can play some role in the pathogenesis of type 1 diabetes in the NOD mouse (14–20). Therefore, the aim of our present study was to better understand the role of IL-21 in type 1 diabetes. We demonstrate that loss of IL-21 signaling, via knockout of the IL-21 receptor, completely abrogates diabetes development on the NOD background. In addition, we demonstrate that overexpression of IL-21 in pancreatic β-cells induces a high incidence of spontaneous type 1 diabetes on the normally diabetes-resistant C57Bl/6 genetic background. Together, these findings clearly underline the potent prodiabeticogenic activity of IL-21.

RESEARCH DESIGN AND METHODS

Mice. All mice were housed in microisolation cages under specific pathogen-free conditions at the Harvard School of Public Health and the La Jolla Institute for Allergy and Immunology. All animal studies were performed according to institutional and National Institutes of Health guidelines for animal use and care. Blood glucose levels were monitored weekly using OneTouch Ultra (LifeScan) or Ascensia Contour glucometers (Bayer). Diabetics in NOD mice was defined as two consecutive blood glucose values ≥250 mg/dl. IL-21 receptor knockout mice were generated by homologous recombination as previously described (11). NOD.LtJ mice were purchased from The Jackson Laboratories, and the IL-21 receptor-null allele was backcrossed to the NOD background for 10 generations. The IL-21 transgenic (IL-21Tg) construct was generated by cloning the full-length mature IL-21 cDNA into a transcription expression vector between the 5' human insulin promoter and 3' hepatitis virus B terminator sequence (21). The purified plasmid was linearized using the SalI and HindIII restriction sites, injected into C57Bl/6 fertilized embryos and implanted into pseudopregnant females. Founder lines were identified by Southern blot and maintained as heterozygotes for experimentation.
Tissue isolation, fixation, and immunohistochemical staining. Pancreata were harvested from IL-21R+/- and IL-21R--/- NOD mice, immersed in OCT Compound (Tissue-Tek, Sakura) and quick-frozen on dry ice. The 6-μm sections were cut at three nonoverlapping levels (200 μm apart) and fixed in acetone for 10 min at room temperature. Sections were incubated for 1 h at room temperature with guinea pig anti-corticotropin-11 (Dako, lot 3207, 1:100; Dianova, lot 108571, 1:50), and biotin-anti-mouse CD4 (BD, lot RM4-5, 1:50). Next, goat–anti-guinea pig alkaline phosphatase (Sigma, 1:50) and Avidin–HRP (Vector, 1:2,000) were incubated for 45 min at room temperature. Alkaline phosphatase or horseradish peroxidase (HRP) activity was visualized using Vector Blue Alkaline Phosphatase Kit (blue signal) and ABC substrate (red precipitates) respectively. Slides were mounted without mounting medium and counterstain (Dako Faramount Aqueous Mounting Medium). Images were digitally captured by high-magnification 20× objective by light microscopy and categorized as no insulinitis, peri-insulitis, mild infiltration (<25%), and heavy infiltration and scarring.

Pancreata were harvested from IL-21tg and littermate controls and fixed overnight with 4% paraformaldehyde (Sigma-Aldrich) before routine paraffin embedding. After dewaxing, 6-μm sections were cut and treated with 3% H2O2 in MeOH (20 min at room temperature) to quench endogenous peroxidase activity. Antigen retrieval was performed using trypsin or proteinase K digestion. Next, slides were blocked in 1% BSA and 3% normal serum in PBS for 30 min at room temperature. Primary antibodies were incubated overnight at 4°C for the following: insulin 1:100 (#A0564, Dako), IL-21 1:1,200 (#APS94 R&D Systems), CD4 1:100 (BioLegend), CD3 1:100 (#550286, BD Biosciences), IL-17A 1:100 (#555144, BD Biosciences), and IL-6 1:1,000 (#550268, BD Biosciences). Secondary antibodies were visualized using sequential detection with HRP-conjugated secondary antibodies (Jackson Immunoresearch), tyramide signal amplification (Perkin Elmer), streptavidin–HRP (Jackson Immunoresearch), and diazobenzene (Sigma-Aldrich). Slides were counterstained with hematoxylin before mounting. Cell type-specific insulin infiltration was scored using an arbitrary scale ranging from 0 = no insulin infiltration; 1 = scattered cells surrounding islets; 2 = foci of cells surrounding islets; 3 = foci of cellular infiltrates surrounding and cells within islets; and 4 = dense foci of cellular infiltrates surrounding and within islets.

**Lymphocyte preparations, transfers, and flow cytometry.** Single-cell suspensions were prepared from splenic and peripheral lymphoid nodes by mechanical disruption, filtration through a 70-μm cell strainer (BD Biosciences), erythrocyte lysis using ACK buffer, and two washes in FACS buffer (PBS/0.6% BSA/0.01% NaN3). Pancreatic lymphocytes were prepared as previously described (22). Single-cell suspensions were obtained from non-diabetic IL-21R+/−/−/+ NOD mice or age-matched IL-21R+/+/−/+ NOD mice. The 2 x 10^6 splenocytes were transferred intravenously into 6-week-old female NOD/scid mice. Blood glucose levels were monitored twice weekly for 4 weeks and then once weekly. Insulinitis was scored as above. Diabetic animals or nondiabetic animals at 7 weeks after transfer were killed.

**FTTC, PE, PerCP, APC, caspase blue-** and caspase orange-conjugated monoclonal antibodies to CD3, CD4, CD8, CD21, CD23, B220, IgM, and IgD (all BD) were used according to the manufacturer’s instructions. Cells were analyzed using an LSRII flow cytometer (BD Biosciences).

**Standardized quantitation of active B cells** were killed using CO2/70°C, and harvested tissues were snap-frozen in liquid nitrogen. Total RNA was purified using TRIzol reagents, and 2 μg was used for cDNA synthesis. Real-time quantitative PCR was performed using a Stratagene MX3005P QPCR system using β-actin as an internal reference control.

Insulin autoantibodies. Levels of autoantibodies to murine insulin (nDIA) were determined by a radioactive assay as described (23). The limit of normal (0.010) was chosen based on historical data (23).

**Statistical analysis.** For diabetes incidence, significance was calculated using the log-rank test or one-way ANOVA followed by a Bonferroni post test. For all other parameters, significance was calculated by Student’s t test, indicated as follows in the figures: *p < 0.05, **p < 0.01, and ***p < 0.005.

**RESULTS**

Genetic and cellular studies have suggested that IL-21 could be important for the pathogenesis of type 1 diabetes in the NOD mouse model (3,4). To begin, we defined the expression patterns of IL-21 and IL-21R mRNA in the pancreas and pancreatic draining lymph nodes in pre-diabetic and diabetic NOD mice. IL-21 mRNA levels, essentially unaltered in pancreas draining lymph nodes, showed an upward trend in the pancreas as diabetes developed in the NOD (Fig. 1A, P = 0.057, pre-diabetic vs. diabetic NOD). Levels of IL-21R mRNA remained unchanged in both pancreas and associated draining lymph nodes as diabetes develops (Fig. 1B). IL-21R is clearly detectable on the surface of pancreatic CD4+ and CD8+ T-cells (Fig. 1C), including diabetogenic npr-V7 (tetramer+ CD8+ T-cells) (data not shown), at levels comparable to splenic CD4+ and CD8+ T-cells from NOD mice (data not shown) and other strains (24). These data indicate that increased pancreatic IL-21 production correlated with diabetes onset in NOD mice and that T-cells infiltrating the pancreas express IL-21 receptor (IL-21R).

To assess the importance of signaling through the IL-21R during spontaneous type 1 diabetes development, we generated a colony of IL-21R−/− NOD mice and compared disease parameters with littermate control animals. The IL-21R−/− NOD littersmates developed type 1 diabetes beginning at week 11, with a median onset at 19 weeks and >90% penetrance of disease (Fig. 2A), comparable to our NOD colony (2,3). In contrast, IL-21R−/− NOD animals were completely protected from type 1 diabetes development up to 60 weeks of age (Fig. 2A). Heterozygotes displayed an intermediate phenotype with delayed onset (median onset: 29 weeks) and reduced penetrance of disease (~50%). The effect of IL-21R deficiency on mononuclear cell infiltration in the pancreas was determined by immunohistochemistry on pancreatic tissue sections. The severity of insulinitis in IL-21R−/− NOD littersmates increased with age. At 13–18 weeks, IL-21R−/− NOD islets were highly infiltrated or destroyed, before diabetes onset (Fig. 2B). The observed infiltrate was composed predominantly of CD4+ T-cells that preferentially resided in the islet zones where β-cell destruction had occurred (Fig. 2C). CD8+ T-cells were found scattered throughout the islet (Fig. 2C). In contrast, we observed minimal mononuclear cell infiltration in islets of IL-21R−/− NOD mice up to 40 weeks of age. In keeping with the lack of insulinitis, autoimmunity to islet antigens was reduced in IL-21R−/− NOD. Quantitation of serum insulin autoantibodies revealed seropositivity in 10/27 IL-21R−/− NOD mice (8–12 weeks old), in contrast to only 1/20 IL-21R−/− NOD mice (Fig. 2D). Thus, loss of IL-21 signaling protects NOD mice from diabetes, islet inflammation, and the generation of islet autoantibodies.

We next analyzed the composition of the lymphoid compartment of various IL-21R−/− NOD genotypes. We found roughly equal splenocyte numbers in IL-21R−/− NOD, IL-21R−/− NOD, and IL-21R−/− NOD at both early (7–9 weeks) and late pre-diabetic stages (12–15 weeks) (data not shown). The proportion of CD4+ and CD8+ T-cells within the lymphocyte population in spleen and the pancreas draining lymph node was not significantly influenced by IL-21R deficiency (Fig. 3A and B; supplementary Fig. 2, found in an online appendix at http://care.diabetesjournals.org/cgi/content/full/db08-0882/DC1). Moreover, the fraction of B-cells and NK cells at 12–15 weeks of age was similar between all genotypes (data not shown). Enumeration of pancreatic CD4+ and CD8+ T-cells corroborated the insulitis index scores (Fig. 2B) as CD4+ and CD8+ T-cell numbers increased from early to late pre-diabetic stage in IL-21R−/− NOD pancreata (Fig. 3A and B, lower panels). We hypothesized that an increased regulatory compartment could explain the observed diabetes resistance of IL-21R−/− NOD mice. Whereas no significant differences in CD4+FoxP3+ Tregs were observed in the spleen of late-stage pre-diabetic mice (Fig. 3C), the Treg
fraction in the pancreatic lymph nodes of IL-21R−/− NOD mice was reduced (~50%) compared with controls. This may represent a relative reduction of Tregs in IL-21R−/− NOD mice or an increase in IL-21R−/+ NOD related to disease onset (25). Regardless, we conclude that the peripheral lymphoid compartment in IL-21R−/− NOD is essentially normal, with the unexpected exception of reduced Treg numbers in the pancreatic lymph nodes, which suggests that diabetes resistance is not due to an increased regulatory compartment.

Given the absence of obvious cellular defects, we reasoned that modulation of Th effector responses from pathogenic (Th1, Th17) to protective (Th2) may account for diabetes protection in IL-21R−/− NOD mice. Lymphocytes from spleens and pancreatic lymph nodes of 8- to 9-week-old IL-21R−/+ NOD and IL-21R−/− NOD mice were restimulated in vitro with phorbol 12-myristate 13-acetate/ionomycin for 3 h for intracellular cytokine detection by flow cytometry. We found a slight increase in the proportion of CD4+ T-cells that produce IL-17 or interferon (IFN)-γ in the splenic and pancreatic lymph node cells of IL-21R−/− NOD mice (Fig. 4A). We next used enzyme-linked immunosorbent spot assays to confirm these data. Splenocytes from IL-21R−/− NOD and IL-21R−/− NOD mice were stimulated for 72 h with anti-CD3/anti-CD28 under nonpolarizing conditions. We observed significantly

FIG. 1. Expression of IL-21 and IL-21R in pancreas and pancreatic lymph nodes of NOD mice. A: IL-21 mRNA analyzed in pancreas and pancreatic lymph node at indicated ages by quantitative PCR (n = 6 per group). B: IL-21R mRNA analyzed in pancreas and pancreatic lymph node at indicated ages by quantitative PCR (n = 6 per group). C: IL-21R expression on CD4+ (left panel) and CD8+ T-cells (right panel) from pancreas of prediabetic NOD mice was determined by flow cytometry using the three-step staining protocol described before (24). Specific and control staining are represented by the solid line and tinted area, respectively. AU, arbitrary units.
increased numbers of IL-17- and IL-4-producing cells in IL-21R<sup>−/−</sup> NOD splenocytes compared with controls. We also found a trend toward increased IFN-γ- and IL-10-producing cells that failed to reach statistical significance. Thus, whereas there are increases in IL-4 production, concomitant increases in IL-17 and possibly IFN-γ make it unlikely that skewing toward protective Th2 response explains the diabetes resistance in IL-21R<sup>−/−</sup> NOD mice.

To decipher whether an IL-21R<sup>−/−</sup> NOD environment was sufficient to restore the diabetogenic potential of IL-21R<sup>−/−</sup> NOD lymphocytes, we performed parallel transfers of IL-21R<sup>−/−</sup> NOD and IL-21R<sup>−/−</sup> NOD splenocytes into lymphopenic NOD/scid recipients. As previously published, splenocytes from recently diabetic IL-21R<sup>−/−</sup> NOD mice induced diabetes upon transfer to NOD/scid mice starting at 3 weeks post-transfer (Fig. 5A). In contrast, transfer of age-matched IL-21R<sup>−/−</sup> NOD splenocytes could not induce diabetes in NOD/scid mice (Fig. 5A). Immunohistochemistry on pancreatic sections revealed limited islet infiltration by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in NOD/scid recipients of IL-21R<sup>−/−</sup> NOD splenocytes, but abundant infiltration by transferred IL-21R<sup>−/−</sup> NOD splenocytes. Defective reconstitution of lymphoid space by IL-21R<sup>−/−</sup> NOD lymphocytes could not explain these observations, as we found equivalent numbers of lymphoid cells in spleen or pancreatic lymph nodes of NOD/scid mice receiving either IL-21R<sup>−/−</sup> NOD or IL-21R<sup>−/−</sup> NOD splenocytes (Fig. 5D). These observations indicate that IL-21R<sup>−/−</sup> NOD mice lack auto-aggressive splenocytes compared with their wild-type littermates and that lymphopenia-induced proliferation of IL-21R<sup>−/−</sup> NOD lymphocytes does not confer them with diabetogenic properties.

We showed that pancreatic levels of IL-21 increase during diabetes development in NOD (Fig. 1A) and that loss of IL-21 signaling protects NOD mice from islet infiltration and diabetes development (Fig. 2). We therefore hypothesized that elevated levels of IL-21 would
exacerbate disease pathogenesis. To test this, we generated transgenic C57Bl/6 mice in which IL-21 is under the control of the human insulin promoter, resulting in pancreatic β-cell-specific overexpression of IL-21 (Fig. 6A). Next, we measured IL-21 levels by quantitative RT-PCR (Fig. 6B) and by immunohistochemistry using a polyclonal anti-mouse IL-21 antibody (Fig. 6C). These data revealed distinct overexpression of IL-21 mRNA and protein in pancreatic islets of IL-21 transgenic animals.

Analysis of lymphoid compartments revealed splenomegaly and lymphadenopathy in IL-21Tg mice. We identified an ~2.5-fold increase in total cell numbers in spleen (Fig. 6D) and pancreatic draining lymph nodes (Fig. 6E) resultant from expansion of both the T-cell (CD3+) and B-cell (B220+) compartments (data not shown). Most B-cells in our IL21Tg mice displayed a mature phenotype, while expressing reduced levels of CD21 and CD23 (IgD+, IgM+, CD21lo, CD23lo) (Fig. 6F). Other studies have shown that IL-21 can downregulate surface CD21 and CD23 on B-cells, and expansion of IgD+IgM−CD21loCD23lo B-cells was also observed in other IL-21Tg mouse lines driven by ubiquitous promoters (10). Thus, these data suggest that bioactive IL-21, expressed specifically by pancreatic β-cells, is released systemically from the endocrine pancreas to mediate effects in peripheral lymphoid compartments.

To determine whether IL-21 overexpression resulted in diabetes onset, we monitored blood glucose levels of...
IL-21Tg mice and wild-type littermate controls were maintained from the time of weaning until ~40 weeks of age. As expected, wild-type animals (C57BL/6) remained normoglycemic over the duration of the study (Fig. 7A). In contrast, IL-21Tg animals started developing diabetes from ~8 weeks of age (Fig. 7A), with a median onset at ~22 weeks and ~80% penetrance in both sexes of the experimental population. To prove specificity of the IL-21 effect and to exclude a transgenesis artifact, we crossed IL-21TgB6 to IL-21R—/— B6 mice. IL-21Tg×IL-21R—/— animals were completely protected from diabetes onset (Fig. 7B). Taken together, these data show that β-cell-specific overexpression can induce type 1 diabetes in the diabetes-resistant C57BL/6 background.

Next, we determined β-cell mass and pancreatic islet infiltration by immunohistochemistry. Pancreatic sections stained for insulin and insulin-positive islets were quantified per visual field. Islet infiltration was scored based on the presence of peri- and intra-islet cellular infiltration. The number of islets in IL-21Tg mice was significantly reduced at all time points compared with controls (Fig. 7C). In addition, ~50% of the islets in pre-diabetic (10 weeks of age) IL-21Tg mice were infiltrated (Fig. 7D). The severity of islet inflammation increased with age and, at 16 weeks of age, ~90% of the islets revealed some level of cellular infiltration.

β-Cell-specific IL-21 overexpression precipitates diabetes in diabetes-resistant C57BL/6 mice (Fig. 7A). To test whether the presence of diabetes susceptibility alleles from NOD influences disease onset, we crossed IL-21Tg mice (C57BL/6) to NOD mice. We found that IL-21Tg F1 (B6×NOD) mice developed diabetes as early as 3 weeks of age, with a median onset at ~4 weeks and 100% penetrance of disease at 6 weeks (Fig. 7E). This represents a striking acceleration of diabetes onset in IL-21Tg on the mixed B6×NOD versus the B6 background (median onset 4 vs. 22 weeks, respectively; Fig. 7A vs. E). We determined β-cell mass and pancreatic islet infiltration by immunohistochemistry and found a reduced amount of islets and distinct infiltration of the remaining islets between 2 and 3 weeks of age in the IL-21Tg B6×NOD F1 compared with wild-type B6×NOD littermates (Fig. 7F and G). Our data show that one "dose" of NOD-derived alleles exacerbates diabetes in IL-21Tg C57BL/6 mice.

Next, we used immunohistochemistry to determine which cell subsets infiltrate the islets in IL-21Tg C57BL/6 mice. We analyzed the presence of B-cells (B220+), CD4+ cells (CD4+), NK cells (NKL-1+), macrophages (F4/80+), and dendritic cells (CD11c+) in islet infiltrates from pre-diabetic (8–10 weeks; Fig. 8A, top panel) and diabetic IL-21Tg cohorts (24 weeks; Fig. 8A, bottom panel). We observed more severe infiltration by all cell types in IL-21Tg versus littermate controls, and in diabetic versus pre-diabetic mice (Fig. 8B), corroborating our data in Fig. 7. The infiltrates in the pre-diabetic IL-21Tg cohort predominantly contained F4/80+ macrophages but also CD4+ and dendritic cells. In diabetic IL-21Tg mice, the infiltrates consisted mostly of macrophages and contained focal accumulation of CD4+ cells, B-cells, dendritic cells, and NK cells. We reasoned that the distinct pattern of infiltration could result in part from the production of cytokines and chemokines. Therefore, we performed quantitative RT-PCR on pancreatic tissue from IL-21Tg and littermate controls, which revealed significantly increased production of IFN-γ, IL-17A, and IL-17F in the pancreas of IL-21Tg mice (Fig. 8C). In addition, we found a significant increase in monocyte chemoattractant protein (MCP)-1, MCP-2, and IFN-inducible protein (IP)-10 production (Fig. 8D). Thus, pancreatic β-cell-specific overexpression of IL-21 results in the production of inflammatory cytokines and chemokines and predominant infiltration of the islets by macrophages and CD4+ T-cells.

DISCUSSION

In this study, we demonstrate a causal relationship between IL-21 production and type 1 diabetes. First, IL-21 production increases as spontaneous diabetes develops in the NOD model. Second, IL-21R-deficient NOD mice are protected from type 1 diabetes. Third, β-cell-specific overexpression of IL-21 precipitates diabetes in diabetes-resistant C57BL/6 mice.

Type 1 diabetes pathogenesis in the NOD model consists of a sequence of stages. Initially, islet antigens are released during postnatal remodeling of the pancreas and captured by migratory and resident antigen-presenting cells that prime anti-islet T-cells in the pancreatic draining lymph nodes (20,26–28). At an early stage, macrophages are