IL-21Tg mice and wild-type littermate controls were killed immediately after weaning until approximately 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 developed diabetes from 8 weeks of age (Fig. 7A), with a median onset at 22 weeks and a 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 inflammation was scored based on the presence of peri- and intras-ile 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 (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 (LGL1+), 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, while 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 tissues 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 cell that prime anti-islet T-cells in the pancreatic draining lymph nodes (20,26-28). At an early stage, macrophages are
recruited to the islets (29) and are a necessary cellular component of diabetes pathogenesis (30). Next, chemotactic factors, produced by β-cells in response to inflammatory stimuli, attract mononuclear cells to the islets, particularly CD4+ and CD8+ effector T-cells. The transition from nondestructive islet inflammation to a β-cell-destructive state is a key event that precipitates type 1 diabetes (18,31). Because IL-21 is broadly expressed throughout the immune system and other nonhematopoietic lineages (63–35), there are multiple time points and sites of action for IL-21 during the pathogenesis of type 1 diabetes.

We show here that IL-21 levels are increased in the pancreas as NOD mice develop diabetes and that CD4+ and CD8+ T-cells infiltrating the pancreas can respond to local IL-21 as they express IL-21R. Our data are consistent with recent studies by the labs of Leonard and Sarvetnick (36,37) showing that IL-21R+/− NOD mice are protected from insulitis and type 1 diabetes. Similar to Spolski et al. (37), we find unaltered numbers of T-cells, B-cells, and NK cells in IL-21R−/− NOD lymphoid organs (Fig. 3 and data not shown). In contrast to ours and other studies, Datta and Sarvetnick detected higher lymphocyte numbers in IL-21R+/− NOD mice, interpreting this as a normalization of IL-21-induced, type 1 diabetes–promoting lymphopenia (4,36). We see no differences in the expansion of IL-21R+/+ NOD and IL-21R−/− NOD splenocytes when transferred to lymphopenic NOD/scid recipients (Fig. 5D), yet IL-21R−/− NOD splenocytes still fail to induce diabetes. We therefore think it unlikely that IL-21 catalyzes diabetes development by regulating homeostatic proliferation.

Given that T-cell numbers and responses are intact in IL-21R−/− mice (8,11), we hypothesized that altered cytokine production may partially account for the protection from type 1 diabetes. Our analyses show that production of various effector cytokines was not impaired in IL-21R−/− NOD mice (Fig. 4A and B). One of these cytokines, IL-17, has recently been shown to modulate some aspects of the type 1 diabetes pathogenesis in NOD (38), and recent studies identify IL-21 as an amplifying factor for Th17 responses (39,40). Spolski et al. (37) identify defective polarization toward the Th17 lineage in IL-21R−/− NOD lymphocytes and reason that defective IL-17 production may explain diabetes resistance in IL-21R−/− NOD mice. We (data not shown) and others (39) find similarly defective in vitro Th17 polarization using IL-21R−/− T-cells. Moreover, our data show increased IL-17 production in the pancreas of β-cell–specific IL-21 overexpressing mice (Fig. 8D). However, we show increased numbers of IL-17-producing cells in IL-21R−/− NOD mice when cells are restimulated directly ex vivo, which is likely to be more reflective of the in vivo context. Thus, we conclude that reduced IL-17 production in IL-21R−/− NOD mice is unlikely to be the mechanism for the protection from type 1 diabetes.

The reduced frequency of insulin autoantibodies and insulins in IL-21R−/− NOD mice (Fig. 2B and D) shows...
that the anti-islet response is impaired at multiple levels. Reduced autoantibody levels may reflect impairments in CD4+ T helper cell function or antibody production in the absence of IL-21R (9,41,42). Anti-islet IL-21R−/− T-cells may be primed ineffectively or possess inherent defects in migration to islet tissue. Since IL-21R−/− NOD mice have normal or fewer numbers of regulatory T-cells (Fig. 3C), and the function of these cells is not altered (37), it is unlikely that increased regulatory function explains the reductions in autoimmunity. Transfer experiments using diabetogenic T-cell receptor–transgenic T-cells may elucidate the existence of defects in priming or trafficking and are the subject of ongoing studies.

Although IL-21R deficiency protects diabetes-prone NOD mice from type 1 diabetes, β-cell–specific overexpression of IL-21 causes severe diabetes in otherwise diabetes-resistant C57BI/6 mice. Few other models of cytokine overexpression in pancreatic islets cause diabetes of similar severity (43,44). The phenotype of IL-21Tg mice most closely resembles that of IFN-γTg mice in terms of onset and severity of disease. The IFN-γTg model is both T-cell and macrophage dependent (21,45,46). Similar to the IFN-γTg model, the high numbers of macrophages in the islet infiltrates of IL-21Tg mice suggest an important role for macrophages, since macrophage-derived inflammatory cytokines and reactive oxygen species are directly
toxic to β-cells (47). In vitro stimulation of macrophages with IL-21 enhances their T-cell priming capacity (48); thus, phagocytosis of damaged islets and presentation of β-cell antigens to CD4$^+$ T-cells may cause enhanced killing of islets in the IL-21Tg model. In IL-21Tg pancreatic tissue, we showed upregulation of chemokine transcripts such as MCP-1, MCP-2, and IP-10, which recruit inflammatory cells such as macrophages and CXCR3$^+$ T-cells (Fig. 8E) (49). Previous studies identified β-cells as an important source of chemokines during diabetes pathogenesis, but our experiments have failed to identify IL-21R expression on β-cells (supplementary Fig. 1). Regardless, we believe that
IL-21-dependent inflammatory chemokine production could be an important element of the pathogenesis of type 1 diabetes and partially explain the protection afforded by IL-21R deficiency in the NOD model (33,50,51).

In conclusion, we demonstrate a critical role of IL-21 for diabetes pathogenesis in animal models. The disease-promoting activities of IL-21 involve the recruitment of CD4+ cells and macrophages to inflamed islets and may
reflect events that occur in response to IL-21 production by infiltrating cells. In addition, the partial protection from diabetes in IL-21-/- NOD mice shows the sensitivity of the diabeticogenic response to alterations in IL-21 signaling and, by inference, IL-21 levels. Thus, both of our experimental models suggest that the use of IL-21 blocking agents, antibodies, or IL-21R-Fc fusion proteins has potential therapeutic value for the prevention or treatment of human type 1 diabetes.

ACKNOWLEDGMENTS

A.P.R.S. is a CJ Martin Fellow of the National Health and Medical Research Council of Australia. T.V.B. is a fellow of the Belgian American Educational Fundation and funded by The Brehm Center for Type 1 Diabetes Research and Analysis. No potential conflicts of interest relevant to this article were reported.

We thank Kirsten Sigrist, Jennifer Donovan, Diana Pascual, Therese Juntti, Jeanette Liao, and the staff at the Harvard School of Public Health Animal Testing Facility for assistance with animal care.

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DIABETES, VOL. 58, MAY 2009

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