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T-bet-Deficient NOD Mice Are Protected from Diabetes Due to Defects in Both T Cell and Innate Immune System Function

Jonathan H. Esenstein,* Michael R. Lee,* Laurie H. Glimcher,† and Jeffrey A. Bluestone2*†

The transcription factor T-bet (Tbx21) is critical for Th1 polarization of CD4+ T cells. Genetic deletion of Tbx21 can cause either exacerbation or attenuation of different autoimmune diseases in animal models. In the nonobese diabetic (NOD) mouse, genetic deletion of the Ifng or the Il12b (IL-12p40) genes, which are both critical Th1 cytokines, does not reduce the incidence of autoimmune diabetes. These results suggest that autoimmune diabetes in the NOD may not be a Th1-driven disease. However, we report that Tbx21 deficiency in the NOD mouse completely blocks insulitis and diabetes due to defects both in the initiation of the anti-islet immune response and in the function of CD4+ effector T cells. We find defective priming of naive islet-reactive T cells by the innate immune system in Tbx21+/− animals. By contrast to naive cells, activated islet-reactive T cells do not require Tbx21 in recipient animals for efficient adoptive transfer of diabetes. However, when these BDC2.5 TCR-transgenic T cells are transferred, the Th2-like disease in Tbx21−/− mice is due to intrinsic defects in both T cells and cells of the innate immune system paired with the relative preservation of regulatory T cell function.

Germline deletion of the Th1-lineage transcription factor T-bet (Tbx21) can alternatively exacerbate or attenuate autoimmune and inflammatory diseases in animal models. Tbx21 null animals have attenuated clinical symptoms of autoimmune experimental encephalomyelitis (1) and CD4+ Tbx21 null cells do not cause colitis when transferred into SCID or Rag-deficient mice (2). Tbx21 also plays a role in CD8+ T cell-driven disease. For example, OVA-specific Tbx21-deficient CD8+ OT-I cells have intrinsic defects in tissue homing and cytotoxicity in a mouse model of myocarditis (3). Importantly, Tbx21 null animals with a transgene expressing a lymphocytic choriomeningitis virus protein in pancreatic islets are partially protected from diabetes when infected with lymphocytic choriomeningitis virus due to defects in the generation of antiviral CD8+ T cells (4). In collagen Ab-induced arthritis in mice, Tbx21 expression in dendritic cells was necessary to drive the disease in the absence of an adaptive immune response (5). Tbx21 deficiency in B cells reduces autoantibody titers and renal immune complex deposition in Fas-deficient mice (6).

By contrast to these examples of attenuated disease, several disease models show exacerbated disease in the absence of Tbx21. Tbx21−/− animals immunized with heart myosin develop exacerbated autoimmune myocarditis compared with controls due to increased IL-17 production by effector T cells (7). Tbx21 null animals are more susceptible to Th2-mediated diseases such as airway inflammation similar to human asthma (8) and bleomycin-induced skin sclerosis (9). Some strains of Tbx21-deficient mice that also lack an adaptive immune system due to Rag2 gene deficiency develop spontaneous colitis due to dysregulated cytokine production in the gut mucosa (10). These examples show that Tbx21 is important for the function of lymphocytes and nonlymphocytes in disease models and that the effects of Tbx21 deficiency on a particular disease model are difficult to predict.

This complexity in different disease models is explained in part by the many different functions of Tbx21 that have been described in lymphocytes and dendritic cells. CD8+ T cells that lack Tbx21 can produce IFN-γ in vitro (11), likely due to the expression of the Tbx21 paralog Eomesodermin (12). However, the functions of Tbx21 and Eomesodermin overlap only partially, since Tbx21 null CD8+ T cells showed reduced IFN-γ production in mice infected with Toxoplasma gondii (13), Tbx21 in dendritic cells promotes IFN-γ production and is necessary for effective in vivo priming of Ag-specific T cells by dendritic cells (14). Deficiency of Tbx21 in B cells skews class switching away from IgG2a (6). Conversely, up-regulation of Tbx21 in cultured B cells is associated with decreased class switching to IgE and IgG1 (15). NK cells require Tbx21 for control of tumor metastasis in mice inoculated with a melanoma cell line (16). NK cells that lack Tbx21 have intrinsic cytotoxicity defects and survive poorly compared with Tbx21+/+ NK cells in vivo and in vitro (16). In sum, Tbx21 controls a wide range of Th1-related cellular phenotypes in many cell types of both the adaptive and the innate immune system.

There is evidence that polymorphisms in Th1-related genes contribute to risk of type 1 diabetes mellitus (T1DM)3 in humans. A polymorphism of Tbx21 that increases transcription from the IFN-γ promoter has been implicated as a risk gene in human T1DM in a Japanese study population (17). However, the region of

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1 Abbreviations used in this paper: T1DM, type 1 diabetes mellitus; NOD, nonobese diabetic; Treg, regulatory T cell; SNP, single nucleotide polymorphism.
human chromosome 17 that contains Tbx21 has not been implicated as a risk region for T1DM in a recent genome-wide association study (18). Separately, a polymorphism of the IL-12p40 gene has been linked to the risk of T1DM in humans (19).

Germline deletion of the Ifng gene or the IFN-γ receptor gene has been reported to delay only slightly the onset of diabetes in the nonobese diabetic (NOD) mouse (20–23). Since Tbx21 drives IFN-γ production in both CD4+ T cells (11) and dendritic cells (14), we sought to test whether Tbx21 was required for spontaneous autoimmune diabetes by backcrossing the Tbx21 null mouse to the NOD. Our results show that loss of Tbx21 completely blocks diabetes in NOD mice. The NOD.Tbx21+/− mice are protected from insulitis and show defects in both effector T cell function and in innate immune cell function. Regulatory T cells (Tregs) in NOD.Tbx21+/− animals function normally in vitro and in vivo assays, suggesting that the balance of effector vs regulatory function of T cells is tipped toward regulation in these animals. Our results suggest that spontaneous diabetes in the NOD mouse requires a Tbx21-dependent Th1 response and that Tbx21 impacts disease pathogenesis in multiple cell types. A role for the Th1 T cell subset in diabetes in the NOD mouse was previously uncertain (22). Thus, our results highlight the importance of the Th1 effector function for diabetes in the NOD mouse.

Materials and Methods
Mice
Mice with the Tbx21(−/−) allele were backcrossed to NOD/MtTac mice purchased from Taconic Farms. NOD.BDC2.5 TCR-transgenic, NOD.Cg-H9253/H11001 (SNPs) across the genome of the NOD.Tbx21+/− mouse revealed that all chromosomes were NOD derived except for the telomeric end of chromosome 11, which contains Tbx21 (supplemental Tables I and II). Diabetes incidence was followed by periodic checks for elevated urine glucose levels using Diastix strips (Bayer) and confirmed with blood glucose measurements of >250 mg/dl on at least two separate days using an Accu-Chek glucose meter (Roche). All animal experiments were approved by the University of California, San Francisco Animal Care and Use Committee.

Histopathology
Pancreata were harvested and immediately fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E. Scoring of insulitis was conducted with an AutoMACS (Miltenyi Biotec). Typical purities were collected for adoptive transfer.

Cell culture and adoptive transfer
For activation assays, CD4+ T cells from NOD and NOD.Tbx21−/− and +/− mice were purified from bulk lymph node cells with a RoboSep (Stem-Cell Technologies) and the StemCell Mouse CD4+ T cell enrichment kit or with an AutoMACS (Miltenyi Biotech). Typical purities were >90% CD4+. For adoptive transfer experiments, CD4+CD25+ cells were removed by incubating the cells for 20 min in supernatant from the 7D4 hybridoma (IgM anti-CD25) followed by incubation with rabbit complement for 30 min at 37°C. For in vitro activation experiments, cells were resuspended in DMEM supplemented with 10% FBS, glutamine, HEPES buffer, 2-ME, nonessential amino acids, and antibiotics. The cells were activated in plates precoated with 1 µg/ml anti-CD3 (clone 145-2C11) and 1 µg/ml anti-CD28 (clone PV1). For Th1 skewing, the medium was supplemented with 100 U/ml recombinant human IL-2, 20 ng/ml recombinant mouse IL-12, and 20 µg/ml anti-IL-4 (clone 11B.11). For adoptive transfer experiments with naïve T cells, CD4+ cells were isolated as described above and injected into recipient mice via the tail vein. For adoptive transfer experiments with activated BDC2.5- transgenic cells, the cells were isolated as described above and activated by mixing together the mimotope peptide p31 (25) with T-depleted, irradiated splenocytes from nondiabetic NOD mice. Activation was done in supplemented DMEM as described above without skewing cytokines. Cells were collected 4 days after activation and adoptively transferred into mice via tail vein injection.

RNA isolation and real-time PCR
RNA from primary mouse T cells was extracted using the RNeasy kit (Qiagen) using the manufacturer’s recommended protocol. Reverse transcription was done with a SuperScriptIII reverse transcriptase kit (Invitrogen), using random DNA hexamers as primers according to the manufacturer’s instructions. Real-time PCR was performed on a 7500 Fast Realtime PCR System (Applied Biosystems) using TaqMan primer probes Mm99999054_x1 (Ccr3) and Mm00801778_m1 (Ifng) with a TaqMan primer-probe set for the eukaryotic 18S RNA as an internal control. cDNA was diluted 10,000-fold for assay of 18S rRNA levels. Both the Ccr3 and Ifng primer-probes were validated before use by titration against the 18S rRNA primer-probe using lymphocyte cDNA and the amplification efficiency of the individual primer-probes was calculated. Calculation of relative expression was done using the ΔΔCt method according to the recommendations from Applied Biosystems.

Flow cytometry
Intracellular staining for FOXP3 was conducted with the eBioscience FOXP3 staining kit according to the manufacturer’s instructions. For CCRX3 surface staining, spleens were harvested, disrupted, and the splenocytes were incubated for 10 min in supernatant from the 2.4G2 hybridoma to block nonspecific binding of IgG to splenocytes. Primary stain was done for 30 min with purified goat anti-mouse CCRX3, SC-9902 (Santa Cruz Biotechnology) at a 1/5 dilution. For control stains, this primary Ab was omitted. Cells were washed and incubated with donkey anti-goat biotin (F(ab')2 (Jackson ImmunoResearch Laboratories) at 1/50 dilution for 30 min. Cells were washed again and streptavidin-PE (BD Biosciences) was added at a 1/350 dilution for 30 min. BDC TCR surface staining was done with a purified anti-BDC cloneotype Ab produced from the original hybridoma (26). The secondary Ab was 1/2000 diluted rat anti-mouse IgG2b-horseradish peroxidase (BD Biotechnologies), which was detected with 1/5000 diluted streptavidin-PE (BD Biotechnologies). Cells were washed between all incubation steps. All cytometry data were collected on a BD Biosciences FACSCalibur or LSRII machine.

CFSE labeling
CD4+CD25+ T cells were resuspended at 20 × 10^6 cells/ml in PBS. CFSE at a stock concentration of 10 mM (Invitrogen/Molecular Probes) was diluted 1/2000 in PBS and this dilution was mixed with an equal volume of cells. After 5 min, the reaction was quenched with FBS and the cells were collected for adoptive transfer.

Treg isolation and expansion
Tregs were sorted from bulk lymph node cells of NOD and NOD.Tbx21−/− animals. Suppression assays in 96-well plates were set up as previously described (27). BDC2.5 TCR-transgenic Tregs were FACs purified and expanded in vitro as previously described before adoptive transfer of 3 × 10^6 cells into NOD.CD28−/− mice (27).

Mouse genome SNP scan
A scan of SNPs across the genome of the NOD.Tbx21−/− mice was done by the University of Texas Southwestern Medical Center Microarray Core Facility using a Mouse Medium Density Linkage Panel (Illumina).

Results
Tbx21−/− mice are protected from diabetes
The Tbx21−/− mouse was backcrossed to NOD for 10 generations. A scan of SNPs throughout the genome of the NOD.Tbx21−/− mouse revealed that all chromosomes were NOD derived except for part of chromosome 11, which contains the null allele of Tbx21 (supplemental Tables I and II). To determine diabetes incidence in these animals, female offspring of NOD.Tbx21−/− parents were followed for diabetes incidence for 30 wk. Both NOD.Tbx21−/− and NOD.Tbx21−/− mice were completely protected from disease (Fig. 1A). The NOD.Tbx21−/− littermates became diabetic with normal kinetics for our colony, with 56% of female mice diabetic by 30 wk of age. Histological analysis of pancreata showed that 12-wk-old NOD.Tbx21−/− females are protected
from insulitis (Fig. 1B). Heterozygous mice show partial protection from insulitis, indicating that gene dosage of Tbx21 affects progression to insulitis.

**Priming of diabetogenic T cells is defective in Tbx21−/− mice**

Since defects in APC function have been described in Tbx21−/− mice (14), we tested initial priming of diabetogenic T cells in Tbx21−/− mice (14), we tested initial priming of diabetogenic T cells in Tbx21−/− mice (14), we tested initial priming of diabetogenic T cells in Tbx21−/− mice (14), we tested initial priming of diabetogenic T cells in Tbx21−/− mice (14), we tested initial priming of diabetogenic T cells in Tbx21−/− mice. Naive CD4+CD25− BDC2.5 TCR-transgenic cells, which have a TCR specific for an islet Ag in the context of I-A^b^ (28), were labeled with CFSE and adoptively transferred into NOD or NOD.Tbx21−/− recipients. After 3 days, the pancreatic lymph nodes of the recipient mice were harvested and analyzed by flow cytometry. The percentage of Tbx21+/+ BDC2.5 TCR-transgenic T cells that went into cycle in the pancreatic lymph nodes of the recipient animals was reduced by about half in the Tbx21−/− recipients (Fig. 2A). Deficiency of Tbx21 in the T cells did not affect proliferation when the recipients were NOD.Tbx21−/−. Interestingly, Tbx21-deficient cells proliferated better in NOD.Tbx21−/− hosts compared with NOD. Tbx21+/+ cells in NOD.Tbx21−/− hosts (p < 0.01), suggesting that NOD.Tbx21+/+ T cells may have a greater requirement for Tbx21 in APCs than NOD.Tbx21−/− T cells. In sum, the expression of Tbx21 in the recipient animals was critical for optimal priming of diabetogenic T cells.

**FIGURE 1.** Tbx21 deficiency protects NOD mice from insulitis and diabetes. A. Female NOD.Tbx21−/− mice and their +/+ and +/− littermates were followed for diabetes for 30 wk. B. Mice from the above cross were sacrificed for histopathological analysis of the pancreas at 12 wk of age. Grade 0, No insulitis; grade 1, perivascular/periductal mononuclear cell infiltration outside of islet perimeter; grade 2, mononuclear cell penetration of up to 25% of islet; grade 3, mononuclear cell penetration of up to 75% of islet; grade 4, islet destruction with <20% of islet mass remaining. Results are from at least 100 islets from four animals per genotype.

**FIGURE 2.** Tbx21 is necessary in both T cells and APCs in an adoptive transfer model of diabetes. A. CD4+CD25− BDC2.5 TCR-transgenic T cells from Tbx21−/− and Tbx21+/+ mice were labeled with CFSE. The cells were collected, recounted, and 1 × 10^6 or 5 × 10^3 freshly isolated CD4+CD25− BDC2.5-transgenic T cells from Tbx21+/+ mice were adoptively transferred into NOD.Rag2−/− or NOD.Rag2−/−, Tbx21−/− mice. The mice were followed for diabetes incidence. Results are from two or three independent experiments. C. One × 10^6 or 5 × 10^3 freshly isolated CD4+CD25− BDC2.5-transgenic T cells from Tbx21−/− mice were adoptively transferred into NOD.Rag2−/−, Tbx21−/− or NOD.Rag2−/−, Tbx21−/− mice. The mice were followed for diabetes incidence. Results are from three independent experiments.
To test for a functional role of Tbx21 in APCs that prime autoreactive diabetogenic T cells, low numbers of naïve CD4<sup>+</sup>CD25<sup>+</sup> Tbx21<sup>−/+</sup> BDC2.5 TCR-transgenic cells were transferred into Rag2<sup>−/−</sup> animals. At a dose of 5 × 10<sup>3</sup> BDC2.5-transgenic cells per mouse, all of the Tbx21<sup>−/+</sup> recipients but only 3 of 12 Tbx21<sup>−/−</sup> recipients had become diabetic by 90 days after adoptive transfer. At a high dose of 1 × 10<sup>6</sup> cells/animal, all recipients became diabetic between 11 and 21 days after adoptive transfer, regardless of the Tbx21 genotype of the recipient animal (Fig. 2B).

These results suggest that there is a defect in T cell priming in Tbx21<sup>−/−</sup> mice after adoptive transfer of a low number of autoreactive T cells. However, this defect can be overcome with the transfer of higher cell numbers. A statistically significant difference (p = 0.03) was found between the percentage of CD4<sup>+</sup> cells in the pancreatic lymph nodes of Tbx21<sup>−/+</sup> and Tbx21<sup>−/−</sup> recipient mice 11 days after adoptive transfer. In the Tbx21<sup>−/+</sup> recipients, 20% (range, 14.8–23%; n = 3) of cells were CD4<sup>+</sup>, compared with 11.1% (range, 10.5–12.1%; n = 3) of cells in the Tbx21<sup>−/−</sup> recipients. Together, these results suggest that Tbx21 is necessary in the innate immune system for efficient priming and proliferation of diabetogenic T cells.

**Effector T cells require Tbx21 for efficient disease transfer**

Different numbers of naïve CD4<sup>+</sup>CD25<sup>−</sup> Tbx21<sup>−/−</sup> BDC2.5-transgenic T cells were transferred into Rag2<sup>−/−</sup> recipients to test for the capacity of the Tbx21-deficient cells to cause diabetes. When 1 × 10<sup>6</sup> Tbx21<sup>−/−</sup> cells were transferred, all Rag2<sup>−/−</sup>, Tbx21<sup>−/+</sup> and Rag2<sup>−/−</sup>.Tbx21<sup>−/−</sup> animals became diabetic with rapid kinetics (Fig. 2C). However, when only 5 × 10<sup>3</sup> cells were transferred, the disease incidence was decreased to 8% (Tbx21<sup>−/−</sup> cells into Rag2<sup>−/−</sup>.Tbx21<sup>−/−</sup> hosts, n = 12) and 20% (Tbx21<sup>−/−</sup> cells into Rag2<sup>−/−</sup>.Tbx21<sup>−/−</sup> hosts, n = 1) by 50 days after adoptive transfer. By comparison, when 5 × 10<sup>4</sup> naïve CD4<sup>+</sup>CD25<sup>−</sup> Tbx21<sup>−/−</sup> BDC2.5-transgenic T cells were transferred into Rag<sup>−/−</sup>.Tbx21<sup>−/−</sup> animals, 100% of mice became diabetic within 40 days of adoptive transfer (Fig. 2B). These results show a clear defect in the ability of Tbx21-deficient T cells to cause diabetes when the number of diabetogenic cells is limited in this system.

In the experiments described above, differences in initial priming and homeostatic expansion could account for the observed defects in Tbx21-deficient T cells. We sought to test for a cell-intrinsic defect in the Tbx21<sup>−/−</sup> T cells unrelated to these processes. Lymph nodes were harvested from BDC2.5 TCR-transgenic NOD or NOD.Tbx21<sup>−/−</sup> mice and CD4<sup>+</sup> cells were purified by magnetic bead selection. These cells were then cultured with T-depleted irradiated splenocytes from NOD mice in the presence of the peptide mimotope Acp31. After 4 days, no difference in T cell proliferation (Fig. 3A) or CD25 expression (data not shown) was detected between the −/− and +/+ transgenic cells.

Adoptive transfer of 1 × 10<sup>6</sup> of these activated cells into NOD or NOD.Tbx21<sup>−/−</sup> animals revealed that activated Tbx21<sup>−/−</sup> T cells induce diabetes at lower rates and with slower kinetics than activated Tbx21<sup>−/+</sup> cells (Fig. 3B). These results show that Tbx21 is required in the activated transgenic T cells but not in the recipient animals for robust diabetes transfer. These results could be explained by a defect in tissue trafficking in the activated Tbx21<sup>−/+</sup> cells or by some other intrinsic defect in effector function. To distinguish between these possibilities, we harvested pancreas from prediabetic animals from the above experiment at day 5 after transfer for histological scoring of islet infiltration. This analysis showed that the animals transferred with activated Tbx21<sup>−/−</sup> cells had infiltration in fewer than 50% of their islets (Fig. 3C). By contrast, animals transferred with activated Tbx21<sup>−/+</sup> BDC2.5 T cells showed complete destruction of >75% of their islets, with few islets free of mononuclear infiltration.

In a study of autoimmune myocarditis induced by activated Tbx21<sup>−/−</sup> transgenic T cells, it was found that these cells become sequestered in the heart draining lymph nodes after adoptive transfer, likely due to a defect in CXCR3 expression (3). However, TCR-specific staining for activated BDC2.5 TCR<sup>+</sup> T cells revealed no significant differences in the percentage of transgenic T cells among the total CD4<sup>+</sup> population of the pancreatic lymph nodes of recipients of activated BDC2.5 T cells 5 days after adoptive transfer (Fig. 4A). To test whether differences in CXCR3
expression could play a role in the efficacy of BDC2.5 T cell transfers. Cxcr3 mRNA was measured by quantitative PCR in the activated BDC2.5 TCR+ T cells used for the adoptive transfer experiments described above. Ifng expression was decreased 100-fold in Tbx21-deficient T cells. In contrast, Cxcr3 mRNA expression in Tbx21−/− cultures was decreased to 25% of the levels in Tbx21+/+ cultures (Fig. 4B). Thus, the activated Tbx21−/− T cells appeared to enter the pancreas in lower numbers than Tbx21+/+ T cells, although they were not sequestered in the pancreatic lymph nodes despite lower levels of CXCR3 expression. Tbx21−/− CD4+ T cells activated under Th1 skewing conditions showed an inter-

mediate phenotype, with Ifng mRNA and protein expression decreased by half and Cxcr3 mRNA expression levels between 80 and 96% of Tbx21+/+ (supplemental Fig. 1, A and B). These results are consistent with previous reports showing that a single copy of Tbx21 is not sufficient to drive normal immune cell effector function (8, 29). The decrease in Cxcr3 expression in Tbx21−/− cells in the experiments described above was modest compared with the observed 30-fold decrease in Cxcr3 mRNA previously reported in CD4+ cells activated in a Th0 culture (30) and even larger decreases reported in other settings (3). In fact, surface staining revealed only a modestly lower level of CXCR3 protein compared with Tbx21+/+ controls (Fig. 4C). Although this difference was statistically significant in CD4+ splenocytes, there was no difference in CXCR3 protein expression between Tbx21+/+ and −/− CD8+ splenocytes (Fig. 4D).

**Tregs contribute to protection of Tbx21-deficient mice from diabetes**

CD4+ FOXP3+ Treg function is critical for controlling the balance between immune effector function and regulation in diabetes in the NOD mouse (31). There was no difference in the percentage of
T-bet DEFICIENCY PROTECTS NOD MICE FROM DIABETES

In this article, we show that the Tbx21 transcription factor, which is a critical regulator of Th1 responses, is necessary for diabetes in the NOD mouse. We have found defects in both the innate and adaptive immune systems in the NOD.Tbx21−/− mouse. Cells of the innate immune system in Tbx21−/− mice are defective in driving activation and cycling of adoptively transferred naive BDC2.5 TCR-transgenic T cells. This cycling defect was paralleled by a defect in diabetes transfer by naive islet-reactive cells into Rag2−/−/Tbx21−/− mice. The observed failure of small numbers of anti-islet Ag-specific transgenic T cells to cause disease could be overcome with high numbers of cells. This result suggests that efficient in vivo proliferation, which is absent in Tbx21−/− recipients, is critical to generate large enough numbers of islet-reactive cells to cause disease. The defects in initial T cell priming and proliferation helps to explain why the NOD.Tbx21−/− animals fail to develop insulinitis, which is normally completely penetrant in the NOD mouse (33). Further work with a conditional allele of Tbx21 will more clearly elucidate the role of this transcription factor in cells of the innate immune system.

In addition to defects in the innate immune system, there are also profound defects in diabetogenic CD4+ T cells that lack Tbx21. Activated NOD.BDC2.5 TCR-transgenic T cells that lack Tbx21 fail to enter the pancreas and cause islet destruction similar to Tbx21−/− cells. The Tbx21−/− cells were not sequestered in the target organ draining lymph nodes, a result reported in another adoptive transfer model using transgenic Tbx21−/− cells (3). Expression of Cxcr3 mRNA is decreased in Tbx21−/− BDC2.5 TCR-transgenic T cells activated in vitro compared with Tbx21+ cells. However, this decrease is much more modest (25% of wild type) than that described by others in Tbx21−/− cells (3, 30). By contrast, IFN-γ expression is completely dependent upon Tbx21 expression. This modest difference in CXCR3 expression implies that the trafficking defects in Tbx21−/− T cells may be due to other factors besides decreased CXCR3 expression. We speculate that Tbx21 is important in the innate immune system in the early initiating stages of autoimmune diabetes and that the T cell defects
The absence of diabetes in the NOD Tbx21+/− mouse is unexpected. Although there is some evidence that Th1 cytokines such as IL-12 help to promote diabetes in the NOD (34, 35), both the IL-12 knockout (36) and the IFN-γ knockout (20) NOD mice show robust progression to diabetes. These results have led to uncertainty about the importance of IFN-γ-producing Th1 T cells in the pathogenesis of diabetes in the NOD. Importantly, a recent report argues that IFN-γ can protect against diabetes by decreasing a pathogenic anti-islet Th17 response (37). These results raise the prospect that diabetes in the NOD is a Th17-driven disease. This hypothesis is unlikely for two reasons. First, Stat4−/− mice on the NOD background are mostly but not completely protected from diabetes (38, 39). Stat4 is critical for IL-12 receptor signaling (40) and its functions in driving gene expression during Th1 differentiation only partially overlap with those of Tbx21 (41). Second, we have found that the phenotype of the NOD Tbx21−/− mouse is even stronger than that of the NOD Stat4−/−, since none of the Tbx21−/− NOD mice develop diabetes or exhibit insulitis. This profound block in diabetes and insulitis shows that the effects of Tbx21 in diabetes extended beyond driving IFN-γ production. Indeed, many other reports have shown that Tbx21 is necessary not only for the production of IFN-γ or IL-12, but also for a wide range of phenotypes including lymphocyte trafficking (30), cytotoxic activity of T cells (42), and priming of T cells by dendritic cells (14).

The 129S6/SvEvTac-derived genetic region surrounding the null Tbx21 allele in the NOD Tbx21−/− mouse extends for up to 15 Mb, raising the possibility that 129-derived genes other than Tbx21 could contribute to the protection of the NOD Tbx21−/− mouse from diabetes. Importantly, chromosome 11 contains Idd4 (43–45), a region initially derived from B10.H-2k mice that partially protects NOD mice from developing diabetes. In these studies, mice heterozygous for the B10-derived portion of chromosome 11 had rates of diabetes of about half of the homozygous controls (44). Several other non-NOD regions spread along the length of mouse chromosome 11 have been associated with protection from diabetes, but none of the NOD Idd4-congenic mice in the published literature show a complete absence of diabetes as in the Tbx21−/− and Tbx21−/− mice (46–48). The region of chromosome 11 defined as Idd4 by The Jackson Laboratory is completely NOD derived in the NOD Tbx21−/− mouse (supplemental Table I). Thus, although, we cannot exclude the possibility that some part of the observed phenotype of the NOD Tbx21−/− mouse is due to 129-derived genes linked to Tbx21, the genetic ablation of Tbx21 is very likely to be critical for a large part of the observed phenotype of the mouse.

Tregs play a role in controlling diabetes in the NOD Tbx21−/− mouse. Deficiency of CD28 in the NOD mouse has been shown to decrease the number of Tregs and increase the kinetics and penetrance of diabetes (49). Strikingly, the NOD Tbx21+/− CD28−/− mouse shows robust progression to diabetes, implying that Tbx21+/− Tregs are necessary to prevent disease in this animal. To directly test whether Tbx21+/− Tregs are capable of preventing diabetes in vivo, we adaptively transferred in vitro-expanded BDC2.5 Tregs that were either Tbx21+/+ or Tbx21−/− into NOD CD28−/− mice. The Tbx21+/− Tregs are competent to prevent or delay disease in these animals compared with untreated controls. However, two of five CD28−/− mice in the group treated with Tbx21+/− Tregs became diabetic during the course of the experiment, compared with zero of five in the Tbx21+/+ treated group. These results suggest that although Tbx21+/− Tregs are functional, they may be less effective than Tbx21+/+ Tregs at similar doses. However, the survival curve of the mice treated with Tbx21+/− Tregs was not statistically different from those treated with Tbx21+/+ Tregs. Furthermore, NOD Rag2−/− Tbx21−/− BDC2.5 TCR-transgenic mice, which lack endogenous Tregs, show rapid progression to diabetes between 4 and 8 wk of age. Taken together, these data demonstrate that Tregs contribute to the control of diabetes in Tbx21−/− animals and that Tbx21−/− effector cells are still competent to cause diabetes if Tregs are removed. Thus, the balance between regulation and autoimmunity in the NOD is shifted toward tolerance by the deficiency in Tbx21, but the system can still be pushed into autoimmunity. Additional studies using tissue-specific genetic deletion of Tbx21 will be needed to define precisely which Tbx21-expressing cell types are the most important for spontaneous diabetes in the NOD.

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L.H.G. is on the Board of Directors of and holds equity in Bristol-Myers Squibb Pharmaceutical Company.

References


SUPPLEMENTAL FIGURE 1. CD4+ T cells from Tbx21+/− mice express half as much IFN-γ and Tbx21 as Tbx21+/+ cells. A, CD4+ T cells were purified and activated with plastic-bound anti-CD3 and anti-CD28 for 6 days in Th1-skewing conditions (20 ng/mL IL-12, 20 μg/mL anti-IL-4, 100 U/mL IL-2). The cells were re-stimulated for 3 hours with PMA and ionomycin in the presence of monensin before fixation and staining for intracellular IFN-γ. B, RNA was extracted from the cells after primary activation described in part A and Taqman realtime PCR assays were performed. The 18S ribosomal RNA was used as an endogenous control. Each data point in a given gene’s column represents an independent experiment.

SUPPLEMENTAL TABLE 1. Genetic markers on the distal portion of chromosome 11 in the NOD. Tbx21−/− mouse. A combination of SNPs, microsatellite markers, and coding sequence polymorphisms were used to determine the border of the genomic region derived from the 129/S6SvEvTac strain, which was used to derived the TC1 ES cells used to make the Tbx21−/− mouse (11). N signifies a NOD-derived allele and S signifies a 129/S6SvEvTac-derived allele. The markers used by the Jackson Laboratory to genotype for the Idd4 locus are highlighted in gray and labeled “JAX Idd4.” (33) The Tbx21 locus is also highlighted in gray.

SUPPLEMENTAL TABLE 2. Full set of SNP genotyping data from the NOD. Tbx21−/− mouse. Reference genotypes for the NOD/LtJ and 129S1/SvImJ are included. Note that the SNP genotyping data fails to distinguish between strains at many points. Reference genotypes occasionally do not match with the genotypes of the 129S6/SvEvTac substrain
(source of ES cells used to make $Tbx21$ null allele) or the NOD/MrkTac substrain (used for the backcross of the $Tbx21$ null allele to NOD).
Supplemental figure 1
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Supplemental table 1