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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. Esensten,* 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 BDC2.5 TCR-transgenic T cells do not require Tbx21 in recipient animals for efficient adoptive transfer of diabetes. However, when these BDC2.5 TCR-transgenic effector cells lack Tbx21, they are less effective at entering the pancreas and promoting diabetes than Tbx21+/+ cells. Tbx21−/− regulatory T cells function normally in vitro and diabetes can be restored in Tbx21−/− mice by reducing regulatory T cell numbers. Thus, the absence of diabetes in the NOD, Tbx21−/− 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. The Journal of Immunology, 2009, 183: 75–82.

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-deficient animals immunized with heart myosin develop exaggerated autoimmune myocarditis compared with controls due to increased IL-17 production by effector T cells (7), Tbx21 null animals are more susceptible than wild-type animals 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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3 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 versus 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 Tbx21fl/fl allele were backcrossed to NOD/MrkTac mice purchased from Taconic Farms. NOD.BDC2.5 TCR-transgenic, NOD.Cg-Rag2tm1Sor/Jbsl, and NOD.129S2(B6)-Cd28tm1Sor/Jbsl mice were bred and housed in a specific pathogen-free barrier facility at the University of California, San Francisco. A scan of single nucleotide polymorphisms (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 for diabetes incidence 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.

Histopathology

Pancreata were harvested and immediately fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E. Scoring of insulitis was done blinded to the genotype of the mouse according to the protocol found in Ref. 24.

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 (StemCell Technologies) and the StemCell mouse CD4+ T cell enrichment kit or with an AutoMACS (Miltenyi Biotec). 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 2.4G2 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 μg/ml recombinant mouse IL-12, and 20 μg/ml anti-IL-4 (clone 11B.11). For adoptive transfer experiments with naive 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 RNAeasy 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 rRNA 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 lymphotox cDNA and the amplification efficiency of the individual primer-probes was calculated. Calculation of relative expression was done using the ∆∆Ct method according to the recommendations from Applied Biosystems.

Flow cytometry

Intracellular staining for FOXP3 was done with the eBioscience FOXP3 staining kit according to the manufacturer’s instructions. For CCRX3 surface staining, splenocytes 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 with 30 min with purified goat anti-mouse CCRX3, SC-9002 (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 1/5000 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 clonotype Ab produced from the original hybridoma (26). The secondary Ab was 1/2000 diluted rat anti-mouse IgG2b-biotin (BD Biotechnologies), which was detected with 1/5000 diluted streptavidin-PE (BD Biosciences). 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 MediumDensity 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−/− mice were followed for diabetes incidence for 30 wk. Both NOD.Tbx21−/− males and NOD.Tbx21−/− females 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

4 The online version of this article contains supplemental material.
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.

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−/− hosts. Naive CD4+CD25− BDC2.5 TCR transgenic cells, which have a TCR specific for an islet Ag in the context of I-Aβ7 (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 × 10⁶ or 5 × 10⁵ freshly isolated CD4+CD25− BDC2.5-transgenic T cells from Tbx21+/+ mice were adoptively transferred into NOD.Tbx21+/+ recipients. After 72 h, pancreatic lymph nodes from the recipients were harvested and bulk lymph node cells were stained for CD4+ and the BDC2.5 TCR. Each data point is an individual mouse and the line is the mean of all mice in a group. **Indicates a p < 0.01 using a two-tailed unpaired t test. n.s. indicates p > 0.05. B, One × 10⁶ or 5 × 10⁵ 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 two or three independent experiments. C, One × 10⁶ or 5 × 10⁵ 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 naive CD4+CD25− BDC2.5 TCR-transgenic cells were transferred into Rag2−/− animals. At a dose of 5 × 10^3 BDC2.5-transgenic cells per mouse, all of the Tbx21+/+ recipients but only 3 of 12 Tbx21−/− recipients had become diabetic by 90 days after adoptive transfer. At a high dose of 1 × 10^6 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−/− 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+ cells in the pancreatic lymph nodes of Tbx21+/+ and Tbx21−/− recipients 11 days after adoptive transfer. In the Tbx21+/+ recipients, 20% (range, 14.8−23%; n = 3) of cells were CD4+, compared with 11.1% (range, 10.5−12.1%; n = 3) of cells in the Tbx21−/− 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 naive CD4+CD25− Tbx21−/−/BDC2.5-transgenic T cells were transferred into Rag2−/− recipients to test for the capacity of the Tbx21-deficient cells to cause diabetes. When 1 × 10^6 Tbx21−/− cells were transfened, all Rag2−/−/Tbx21+/+ and Rag2−/−/Tbx21−/− animals became diabetic with rapid kinetics (Fig. 2C). However, when only 5 × 10^3 cells were transferred, the disease incidence decreased to 8% (Tbx21−/− cells into Rag2−/−/Tbx21+/+ hosts, n = 12) and 20% (Tbx21−/− cells into Rag2−/−/Tbx21−/− hosts, n = 11) by 50 days after adoptive transfer. By comparison, when 5 × 10^3 naive CD4+CD25− Tbx21+/+ BDC2.5-transgenic T cells were transferred into Rag−/−/Tbx21+/+ 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 homoeostatic expansion could account for the observed defects in Tbx21-deficient T cells. We sought to test for a cell-intrinsic defect in the Tbx21−/− T cells unrelated to these processes. Lymph nodes were harvested from BDC2.5 TCR-transgenic NOD or NOD/Tbx21−/− mice and CD4+ 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 × 10^6 of these activated cells into NOD or NOD/Tbx21−/− animals revealed that activated Tbx21−/− T cells induce diabetes at lower rates and with slower kinetics than activated Tbx21+/+ 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−/− cells or by some other intrinsic defect in effector function. To distinguish between these possibilities, we harvested pancreata 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−/− cells had infiltration in fewer than 50% of their islets (Fig. 3C). By contrast, animals transferred with activated Tbx21+/+ 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−/− 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+ T cells revealed no significant differences in the percentage of transgenic T cells among the total CD4+ 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 intermediate 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 expression on freshly isolated splenocytes than in Tbx21$^{-/-}$ splenocytes; and dark line is Tbx21$^{+/+}$ splenocytes. Data are representative of three experiments and analyzed with a one-sample t test. Error bars represent the SEM. n.s., not statistically significant.

**FIGURE 4.** Tbx21-deficient BDC2.5 TCR-transgenic T cells are defective in IFN-$\gamma$ production and Cxcr3 expression. A, Pancreatic lymph nodes were harvested from mice mentioned in Fig. 3C at day 5 after adoptive transfer of activated BDC2.5 TCR-transgenic T cells. Lymph node cells were stained for the BDC2.5 TCR. Percentages indicated are out of total CD4$^+$ cells. B, CD4$^+$CD25$^+$ BDC2.5 TCR-transgenic T cells from Tbx21$^{-/-}$ and Tbx21$^{+/+}$ mice were activated in vitro using irradiated T-depleted NOD splenocytes and the Acp31 mimeotope peptide. Total cellular RNA was collected 4 days after activation and assayed by real-time PCR for levels of Ifng and Cxcr3 mRNA transcript. Expression of each gene in Tbx21$^{+/+}$ samples was indexed to 1. Data were pooled from three independent experiments and analyzed with a one-sample t test. C, Freshly isolated CD4$^+$ splenocytes were stained using a polyclonal Ab for cell surface expression of CXCR3 protein and measured by flow cytometry. Gray tracing is no primary Ab; dotted line is Tbx21$^{+/+}$ splenocytes; and dark line is Tbx21$^{-/-}$ splenocytes. Data are representative of three experiments. D, Quantitative analysis of CXCR3$^+$ cells in freshly isolated splenocytes in the total CD4$^+$ population (top panel) and the CD8$^+$ population (bottom panel). Data are pooled from three independent experiments and analyzed with a two-tailed unpaired t test. Error bars represent the SEM. n.s., not statistically significant.

**FIGURE 5.** Tbx21-deficient mice have normal levels of functional Tregs. A, Splenocytes and pancreatic lymph node cells from NOD and NOD.Tbx21$^{-/-}$ mice were stained for CD4 and FOXP3. Each data point represents an individual mouse. Groups were compared with an unpaired two-tailed t test. n.s. is not statistically significant. B, CD4$^+$CD25$^+$CD62L$^+$ Treg were purified by FACS and titrated in a suppression assay with CD4$^+$CD25$^+$ responder cells. Responders were activated with soluble anti-CD3 and irradiated syngeneic splenocytes. A 1:1 ratio of Tregs to responders has 5 $\times$ 10$^6$ of each cell type. Data are representative of three independent experiments. Error bars are SEM. PLN, Pancreatic lymph node.
NOD. Tbx21−/− mice were crossed onto a NOD.CD28−/− background. The NOD.CD28−/− mouse exhibits rapid, synchronized diabetes onset at ~12 wk of age due to a defect in Treg survival and function (31). In contrast to the NOD.Tbx21−/− mouse, the NOD.Tbx21−/−.CD28−/− animals showed diabetes onset starting from 9 wk of age. Overall incidence of diabetes in this genotype increased until ~20 wk of age before reaching a plateau at 72% (Fig. 6A). These data suggest that Tbx21−/− Tregs are necessary in vivo to prevent diabetes. To more rigorously test this hypothesis, NOD.CD28−/− mice were adoptively transferred with in vitro-expanded NOD.BDC2.5 Tregs that were either Tbx21+/+ or Tbx21−/−. The recipient mice were then followed for spontaneous diabetes incidence (Fig. 6B). Protection of CD28−/− mice from diabetes with Tbx21−/− Tregs was statistically significant (log rank test, p < 0.05). There was no statistical difference between the groups treated with Tbx21+/+ and Tbx21−/− Tregs (log rank test, p = 0.16). Lastly, we bred NOD.Tbx21−/− mice that expressed the BDC2.5 transgene and were also Rag2 deficient. These animals showed rapid diabetes onset at or soon after weaning (Fig. 6C), similar to published data on this genotype in the context of Tbx21 sufficiency (32). By contrast, Rag2−/− animals were protected from early onset of diabetes (log rank test, p < 0.01). These results point to a critical role for Tregs in controlling diabetes in Tbx21-deficient animals, since expression of endogenously rearranged TCRα chains is necessary for the generation of BDC2.5 Tregs.

Discussion
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 insulitis, 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...
enter or delay disease in these animals compared with untreated mice. 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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Disclosures
L.H.G. is on the Board of Directors of and holds equity in Bristol-Myers Squibb Pharmaceutical Company.

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T-bet DEFICIENCY PROTECTS NOD MICE FROM DIABETES


