

Singular role for T-BET⁺CXCR3⁺ regulatory T cells in protection from autoimmune diabetes

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Foxp3⁺ regulatory T (Treg) cells are crucial for restraining inflammation in a variety of autoimmune diseases, including type 1 diabetes (T1D). However, the transcriptional and functional phenotypes of Treg cells within the pancreatic lesion remain poorly understood. Here we characterized pancreas-infiltrating Treg cells in the NOD mouse model of T1D and uncovered a substantial enrichment of the Treg subpopulation expressing the chemokine receptor CXCR3. Accumulation of CXCR3⁺ Treg cells within pancreatic islets was dependent on the transcription factor T-BET, and genetic ablation of T-BET increased the onset and penetrance of disease, abrogating the sex bias normally seen in the NOD model. Both male and female mice lacking T-BET⁺ Treg cells showed a more aggressive insulinitic infiltrate, reflected most prominently by elevated production of type 1 cytokines. Our results suggest the possibility of fine therapeutic targeting of Treg cells, in a tissue- and cell-subset-specific fashion, as a more focused immunotherapy for T1D.

immunoregulation | immunogenomics | Treg subsets

Regulatory T cells (Tregs) characterized by the transcription factor FoxP3 are critical for maintaining immunologic homeostasis, enforcing tolerance to self, and preventing runaway immune responses (1). They regulate the activation and differentiation of conventional CD4⁺ T cells (Tconvs) and of many other cell lineages of the innate and adaptive immune systems through a variety of effector mechanisms (2). There is increasing realization that Tregs have an extraimmunologic role in several tissues, controlling the noxious side effects of inflammation and promoting effective tissue repair. Examples of such “tissue-Tregs” include populations in the visceral adipose tissue (VAT) and in injured muscle (3). Treg cells reside in secondary lymphoid organs [spleen and lymph nodes (LN)] but also radiate to barrier sites where they mediate harmonious coexistence with symbiotic microbes (4).

In line with these wide-ranging roles, Treg cells can adopt distinct subphenotypes that differ by their preferential expression of chemokine receptors, effector molecules, and cofactors that collaborate with FoxP3 to drive these functional nuances (5, 6). These subphenotypes often are driven by transcription factors central to the differentiation and effector functions of the cells they regulate. For instance, BCL6 is induced in Tregs that regulate the interplay between Tconv and B cells in the germinal center (7), and IRF4 and STAT3 are required for optimal control of Th2 and Th17 responses, respectively (8, 9). One of the best characterized of these subphenotypes is the CXCR3⁺ Treg subset driven by the transcription factor T-BET (encoded by *Tbx21*), which has a particular role in controlling type 1 inflammation (10, 11). Treg cells that cannot up-regulate T-BET fail to persist and control autoimmunity when transferred into FoxP3-deficient Scurfy mice (10), or to attenuate IFN- γ -driven pathology in mice infected with *Toxoplasma gondii* (11). Similar Treg subsets expressing CXCR3 and T-BET have been described in humans (12).

Type-1 diabetes (T1D) is an organ-specific autoimmune disease keyed to a breakdown in T lymphocyte tolerance to islet-cell antigens. After an occult phase of pancreatic infiltration that reduces the number and function of insulin-producing β -cells, transition to a metabolically overt phase of diabetes occurs when islets

become sufficiently damaged and/or when local inflammation impairs β -cell function. The genetics of T1D in humans and the nonobese diabetic (NOD) mouse model point primarily to a dysfunction of CD4⁺ T cells, because class II genes of the MHC and several other loci that modify T-cell activation and regulation are linked to T1D susceptibility (13). Although a constellation of immunocytes is present in the islet infiltrate, both CD4⁺ and CD8⁺ T cells are dominant and are required for pathogenesis (13). IFN- γ -producing Th1 CD4⁺ T cells comprise the majority of effector T cells (13), but evidence for an essential function of IFN- γ has been inconclusive: A knockout mutation in *Tbx21* confers significant protection from T1D (14), but deficits in *Ifng*, *Ifngr1*, or *Ifngr2* have little or no effect (15–17). Early evidence of a requirement for *Ifngr1* in disease (18) was likely artifactual, resulting from a closely linked protective allele (16).

Consistent with the importance of Treg cells in immunologic tolerance, there is much evidence that they control the progression of T1D in mice and humans. T1D is one of the autoimmune manifestations in FOXP3-deficient patients, occurring within the first months of life (13). In NOD mice, Treg depletion by various means leads to accelerated diabetes, whereas augmentation of the Treg compartment by transfer or therapeutic manipulations mitigates disease progression (reviewed in ref. 19). Although Treg cells may limit the priming of islet-reactive Tconv cells in the pancreatic lymph nodes (PLNs) or their subsequent migration into the islets (20–22), their primary role is to prevent the runaway destruction of the target organ by locally restraining inflammatory responses of both innate and adaptive immunocytes (21, 23–25).

Here, to understand the mode of action of insulinitic Tregs in T1D better, we have revisited earlier work (26) and assessed the transcriptome and homeostasis of pancreatic Tregs in NOD mice. Pancreas-infiltrating Tregs appeared quite distinct from other tissue-Treg populations, with a preponderance of the

Significance

We analyzed the transcriptome, phenotype, and function of Foxp3⁺ regulatory T cells (Tregs) infiltrating the pancreatic lesion of NOD mice and found a unique and nonredundant role for T-BET-dependent, CXCR3⁺ Tregs in the control of autoimmune diabetes. In particular, pancreatic Tregs were enriched for the T-BET-dependent CXCR3⁺ population. Genetic deficiency of T-BET in Treg cells dramatically accelerated diabetes and eliminated the sex bias common to NOD mice. These findings have implications for the therapeutic targeting of Treg cells in type 1 diabetes and other Treg-associated disorders.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo/> (accession no. GSE87677).

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CXCR3⁺ subset that appears to play a unique and nonredundant role in limiting the progression of T1D.

Results

Distinct Transcriptional Profile in Pancreas-Infiltrating Tregs. To characterize the phenotype of pancreatic Treg cells, we analyzed the transcriptional profile of Treg cells purified from infiltrated whole pancreata of 10-wk-old prediabetic NOD.*Foxp3^{ires-gfp}* reporter mice (these preparations include cells from peri-insulitic and insulitic lesions) and compared that profile with the profile of splenic Treg cells from the same mice (microarray, in triplicate). Pancreatic Treg cells had a transcriptome quite distinct from that of their splenic counterparts, with 184 differentially expressed transcripts [at a fold-change (FC) ≥ 2 and P value < 0.05] (Fig. 1A and Table S1). (A few differential transcripts were typical of exocrine pancreatic cells and were discounted as residual contamination, despite double-sorting the cells.) Differential transcripts encoded molecules with known involvement in Treg inhibitory activity (e.g., *Il10*, *Fgl2*, and *Lag3*) or specific chemokine receptors (*Cxcr3* and *Ccr5*) and several transcription factors that typically denote cell activation (*Nr4a2*, *Fos*, and *Jun*).

Some of these mRNAs (e.g., *Il10* and *Lag3*) were shared with other tissue-Tregs, but the direct comparison of various tissue-Treg populations in Fig. 1B showed that Tregs from the infiltrated pancreas were distinct from those found in the VAT, injured muscle, or colon. This segregation was confirmed by principal component analysis (Fig. 1C). The cluster of transcripts more specifically represented in insulitic Tregs (Fig. 1B) was especially

enriched for genes involved in cell growth and proliferation, suggesting rapid turnover, in line with previous reports (24, 26). The diversity of tissue-Tregs is derived, in part, from the activity of transcription factors that are specific to the corresponding tissue, such as PPAR- γ in the VAT (27–30). These factors were not overexpressed in insulitic Tregs (Fig. 1D), further underscoring the distinction of pancreatic Tregs from other tissue-Tregs.

To parse the pancreatic Treg transcriptome further for alterations in Treg-specific pathways, we overlaid various Treg signatures onto the comparison of pancreatic vs. splenic Treg gene-expression profiles. In agreement with the enrichment of cell-division transcripts, pancreatic Tregs demonstrated a significant up-regulation of a previously determined Treg activation signature (Fig. 1E, Left) (31). The transcription factors BLIMP-1 and IRF4 are required for Treg effector functions and their maintenance at tissue sites (8, 32). Indeed, BLIMP-1- and IRF4-dependent Treg signatures were also biased in pancreatic Tregs (Fig. 1E, Center), possibly reflecting increased effector Treg differentiation in response to local inflammation. However, the strongest bias was observed when overlaying a signature specific to CXCR3⁺ Treg cells (Fig. 1E, Right and Fig. S1). This signature, which was derived by comparing splenic CXCR3⁺ and CXCR3[−] Tregs from C57BL/6 (B6) mice, included transcripts typical of the CXCR3⁺ Treg subset (*Tbx21*, *Cxcr3*) (10, 11) and others that have not been described (e.g., *Lilrb4*, *Fgl2*, *Ccr5*, and *Gzmb*). CXCR3 induction is dependent on T-BET in Tregs and other leukocyte subsets (10, 11, 33), and both transcripts were overrepresented in insulitic Tregs (Fig. 1F). However, we observed an intriguing disjunction

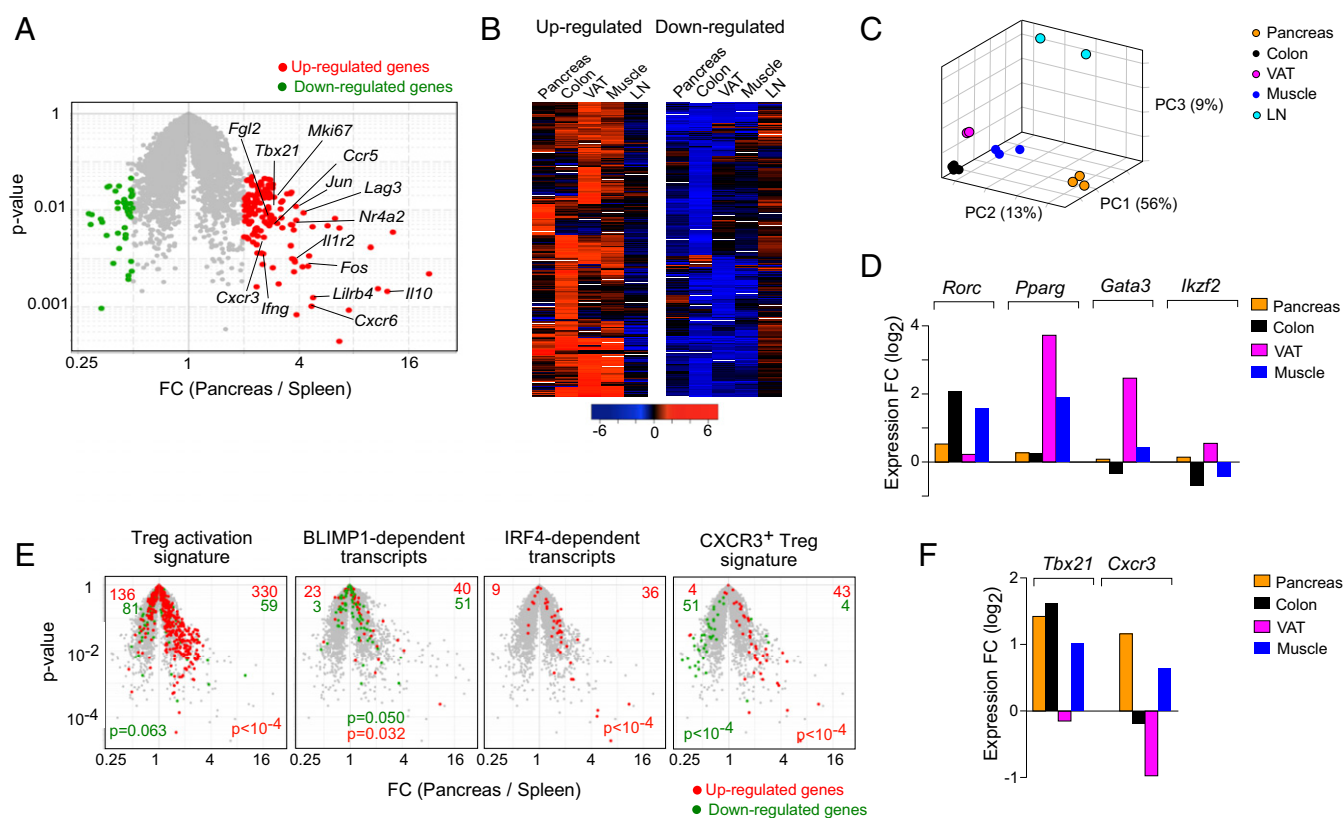


Fig. 1. Pancreatic Treg cells possess a unique transcriptome. The transcriptional profiles of Tregs from the pancreata and spleens of 10-wk-old prediabetic NOD *Foxp3^{ires-gfp}* females were analyzed in biological triplicate by microarray. (A) Volcano plot of pancreatic vs. splenic Treg cells. Red and green indicate transcripts up- and down-regulated, respectively, by pancreatic Treg cells with FC ≥ 2 and $P < 0.05$ (Student's t test). (B) Heatmap of transcripts differentially expressed in various tissue-Treg populations. Log₂ of FC values (tissue-Tregs vs. matching splenic or LN Tregs) are shown. VAT, visceral adipose tissue; Muscle, cardiotoxin-treated muscle, 4 d after injury; LN, subcutaneous lymph nodes. (C) Principal components analysis comparing pancreatic Tregs with other tissue-Tregs (computed from FC relative to matched splenic/LN Treg controls on genes with FC ≥ 2). (D) Transcript levels of key transcription factors in tissue-Treg populations. (E) Pancreatic vs. splenic Tregs (as in A) overlaid with various Treg signatures. Red and green indicate genes up- and down-regulated, respectively, in each signature (χ^2 test for P value). (F) Levels of transcripts encoding T-BET and CXCR3 in tissue-Treg populations. $n = 3$ for pancreatic and splenic Tregs; $n = 2-4$ for other tissue-Tregs.

CXCR3⁺ Treg Cells Are Required to Control T1D. To evaluate the function of T-BET⁺CXCR3⁺ Treg cells in T1D, we monitored disease progression in *Foxp3^{Cre}Tbx21^{fl/fl}* NOD mice, in comparison with their T-BET-proficient littermates that harbored one or none of the *Tbx21^{fl/fl}* alleles together with the *Foxp3^{Cre}* transgene. NOD.*Foxp3^{Cre}Tbx21^{fl/fl}* females (five-generation backcross) developed T1D at a higher incidence and with a markedly earlier onset than their control littermates (Fig. 3A) (diabetes is essentially never observed in our NOD colony before 10 wk of age). T1D penetrance in control littermates was lower than usual in NOD mice, likely because of the incomplete backcross, emphasizing the effect of deficiency. The difference in diabetes progression was even greater in males, which exhibited an incidence of overt disease unprecedented in our NOD colony, with proportions and timing of onset similar to those of their female siblings (Fig. 3B). It was as if the pronounced sex bias in disease penetrance of T1D in NOD mice was abrogated by the absence of CXCR3⁺ Treg cells. Accordingly, insulinitis was already profound in prediabetic *Foxp3^{Cre}Tbx21^{fl/fl}* mice of both sexes at 6 wk of age, when insulitis is usually incipient, as it was here in control littermates (Fig. 3C and D).

We then asked whether this profound effect of eliminating CXCR3⁺ Tregs would also be seen in a model of accelerated diabetes, BDC2.5/B6^{g7} mice. These mice express in most of their CD4⁺ T cells the islet-reactive BDC2.5 TCR, introgressed from the NOD onto the B6.H2^{g7/g7} genetic background, which provides disease-accelerating alleles (36). Most, but not all, BDC2.5/B6^{g7} mice developed early-onset diabetes between 4 and 6 wk of age, without sex bias. Disease penetrance in BDC2.5/B6^{g7}.*Foxp3^{Cre}Tbx21^{fl/fl}* mice devoid of CXCR3⁺ Tregs was significantly increased (Fig. 3E). Curiously, however, mice that did not develop early diabetes remained disease-free thereafter, as do standard BDC2.5/B6^{g7} mice (36). Hence T-BET-dependent CXCR3⁺ Treg cells are indispensable for Treg-cell-mediated control of T1D in mice.

T-BET Promotes the Regulation of Type 1 Inflammation by Treg Cells Without Affecting Their Accumulation in the Insulitic Lesion. In theory, deficiency in T-BET and the CXCR3⁺ Tregs that it controls might modulate T1D progression either by influencing CXCR3-

dependent Treg traffic to and retention in the pancreas or by affecting Treg-suppressive function within the islets. To determine if heightened T1D in *Foxp3^{Cre}Tbx21^{fl/fl}* mice arose from a defect in Treg recruitment into the islets, we measured Treg frequencies in the pancreata of prediabetic *Foxp3^{Cre}Tbx21^{fl/fl}* mice at 9 wk of age. Pancreatic Treg frequencies were essentially unchanged in *Foxp3^{Cre}Tbx21^{fl/fl}* vs. *Foxp3^{Cre}Tbx21^{fl/+}* mice (11.1 vs. 14.4% for females; 14.5 vs. 17.3% for males) (Fig. 4A). The comparable frequencies of pancreatic Tregs in mice of the two genotypes could arise from enhanced proliferation and/or survival of Treg cells that lacked T-BET, thereby compensating for impaired CXCR3-mediated migration into the islets. However, proliferation of pancreatic Treg cells was equivalent in *Foxp3^{Cre}Tbx21^{fl/fl}* mice and control littermates (Fig. 4B).

Thus, disease exacerbation in mice lacking T-BET-dependent CXCR3⁺ Tregs likely resulted from defects in Treg function rather than from their recruitment to or homeostasis in the islets. Indeed, impaired Treg activity was apparent, in that many immunocyte populations in the pancreatic infiltrates of prediabetic *Foxp3^{Cre}Tbx21^{fl/fl}* mice had a more inflammatory tone [elevated frequencies and proliferation of CD4⁺ and CD8⁺ T cells; increased IFN- γ production by CD4⁺, CD8⁺, and $\gamma\delta$ T cells and by innate lymphoid cells (ILCs); and heightened activation of antigen-presenting cells (APCs) (Fig. 4C)] than seen in control littermates. Not all effector functions of immunocytes were equally affected, however, because the frequencies of Th17 cells and IFN- γ -producing NK cells remained constant; the latter observation is interesting in light of the documented first response of NK cells to Treg ablation (25, 37). Thus, T-BET-dependent CXCR3⁺ Tregs played a crucial role in controlling autoimmune inflammation in the pancreas in a manner that could not be adequately compensated by numerically identical CXCR3⁺ Treg cells.

Discussion

Several lines of evidence have established the concept that FoxP3⁺ Treg cells have a key role in limiting autoinflammation in T1D. Here we examined the transcriptome and phenotype of Treg cells in the insulitic lesion and found them to be distinct from other

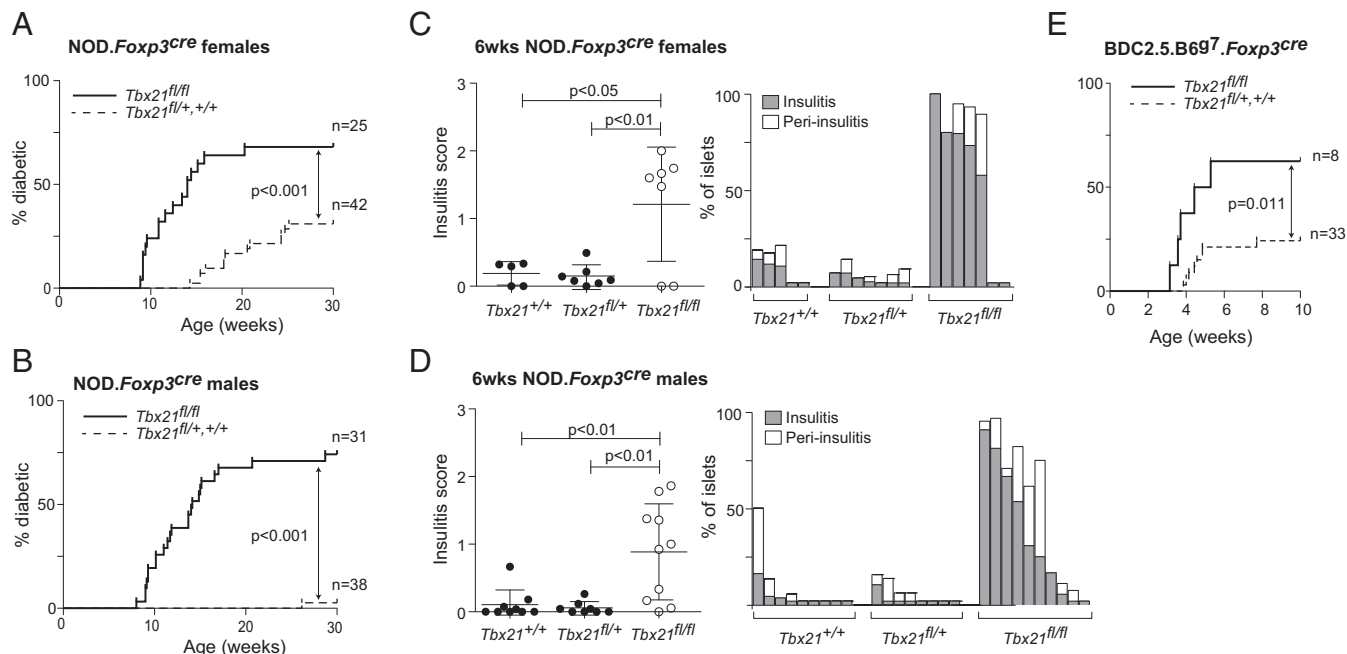


Fig. 3. Genetic deficiency of T-BET in Treg cells results in exacerbated diabetes and insulitis. (A and B) Diabetes incidence in *Foxp3^{Cre} × Tbx21^{fl/fl}* mice on the NOD background (backcrossed for five generations) of the indicated genotypes and sexes. Log-rank test. (C and D) Insulitis scores in 6-wk-old *Foxp3^{Cre} × Tbx21^{fl/fl}* mice on the NOD background (backcrossed for eight generations) of the indicated genotypes and sexes. (Left) Summary data: mean \pm SD; Kruskal-Wallis test with Dunn's multiple corrections. (Right) Proportion of infiltrated islets for each mouse. (E) Diabetes incidence in *Foxp3^{Cre} × Tbx21^{fl/fl}* BDC2.5/B6^{g7} mice of the indicated genotypes (males and females pooled) (log-rank test).

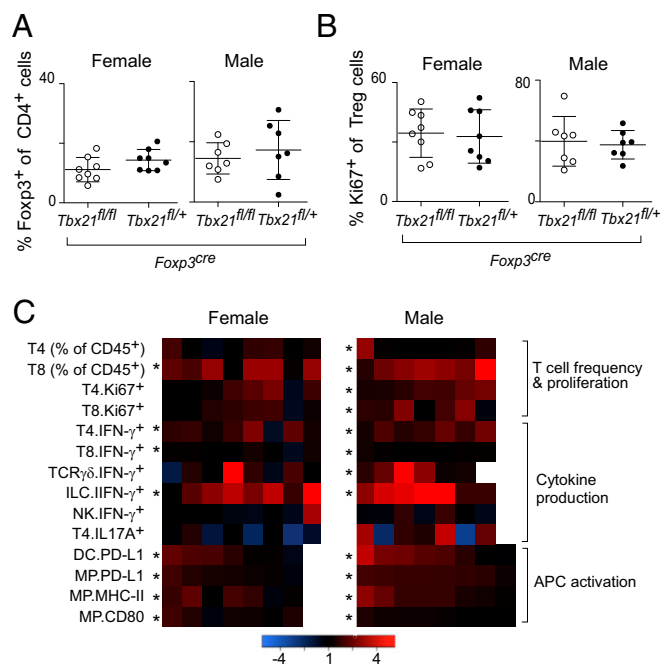


Fig. 4. NOD mice lacking T-BET⁺CXCR3⁺ Treg cells manifest heightened insulinitic inflammation. Prediabetic 9- to 10-wk-old prediabetic mice were used. (A and B) Frequencies of total Foxp3⁺ Treg cells (A) and Ki67⁺ Tregs (B) in NOD mice of the indicated genotypes and sexes. Data pooled from at least four independent experiments are shown as mean \pm SD; Mann-Whitney *u* test. (C) Heatmap of various inflammatory parameters of the insulinitic infiltrate in Foxp3^{Cre}Tbx21^{fl/fl} mice, normalized to those of Foxp3^{Cre}Tbx21^{fl/fl} littermates. T4, CD4⁺ T cells; T8, CD8⁺ T cells; Ki67⁺ and IFN- γ ⁺: frequency of cells expressing the indicated proteins within each leukocyte subset; MP, CD11b⁺F4/80⁺ macrophages. The mean fluorescence intensity of the PD-L1, CD80, and MHC-II staining was compared. Each column represents one mouse. *n* = 7 or 8 pairs of littermates per comparison. **P* < 0.05; Wilcoxon signed rank test.

tissue-Treg populations. Pancreatic Tregs were especially enriched for the T-BET-dependent, CXCR3⁺ subset. Importantly, these CXCR3⁺ Tregs seemed uniquely suited to restrain autoimmune attack of the pancreas, with a nonredundant role that could not be assumed by other Treg cells. These findings are pertinent to the pathogenesis pathways in T1D and have implications for therapeutic targeting of Tregs in human diabetes.

It is likely that IFN- γ , secreted locally by several types of immunocytes (T, NK, type 1 ILCs), is critical to the accrual of CXCR3⁺ Treg cells in the pancreas. This cytokine might induce the production of the CXCR3 ligands CXCL9/10 (38), thereby attracting CXCR3⁺ Tregs to or retaining them in the islets. Alternatively, IFN- γ might up-regulate T-BET and CXCR3 in Treg cells (10, 11) already present in the islets. This dual involvement of T-BET in both the aggressive Th1 cells and in their essential CXCR3⁺ Treg foil might help reconcile the confusing observations on the importance of IFN- γ , its receptor, and T-BET itself to disease progression in NOD mice (14, 15, 17, 18).

A striking finding from this study was the abrogation of the sex bias in T1D incidence when T-BET⁺ Treg cells were absent, with T1D occurring with quasi-superimposable incidences in males and females. Several explanations for the sex bias have been proposed, including hormonal effects and differences in the intestinal microbiota, but actual microbes have not been consistent between laboratories (39, 40). Whatever the root, we might speculate that the relevant pathways involve CXCR3⁺ Treg cells, modifying their frequency or activity in a sex-biased manner. In this context, surveys at the Jackson Laboratory of the rates of diabetes incidence in different KO strains crossed onto the NOD background revealed that the normal sex bias disappeared in mice carrying mutations

targeting the IL-12-IFN- γ signaling pathway (*Ifng*, *Ifngr1*, and *Stat4*) (40). Increased secretion of IFN- γ in the PLNs of males relative to females has been reported and proposed to account for protection from T1D (40), and one might extend this speculation by proposing that this overabundance of IFN- γ enhances CXCR3⁺ Treg function. However, we did not observe significant differences in CXCR3⁺ Treg frequencies between males and females.

What accounts for the nonreplaceable role of CXCR3⁺ Tregs in T1D? Tbx21^{fl/fl} Treg cells have suppressive activity equivalent to that of WT controls in in vitro suppression assays (14, 41). In several in vivo contexts, such as the T-cell transfer model of colitis or the experimental autoimmune encephalomyelitis model of multiple sclerosis, CXCR3⁺ Tregs appear functionally identical to bulk Treg cells and are dispensable for protection from disease (42, 43). On the other hand, specific functional defects in controlling Th1 inflammation have been reported in the absence of CXCR3⁺ Tregs, including protection from autoinflammatory disease in Treg-deficient Scurfy mice and from allograft rejection (10, 41). Our finding that mice lacking T-BET-dependent CXCR3⁺ Treg cells developed heightened insulinitic inflammation without substantial changes in Treg frequency indicates that pancreatic autoimmunity is one of the contexts in which the activity of T-BET⁺ Tregs is essential. T-BET likely modulates a specific facet of Treg activity relevant to the control of local Th1 responses and/or innate lymphocytes rather than promote a nonspecific activation of Treg cells, because the generation of Blimp-1⁺ effector Treg cells does not require T-BET (32).

The transcripts differentially expressed in pancreatic vs. splenic Treg cells, which closely overlapped the CXCR3⁺/CXCR3⁻ Treg differential (Fig. S1), likely include transcripts that are functionally relevant to the control of Th1 responses. No transcript is an immediately obvious candidate, although some (e.g., *Fgl2* and *Lag3*) encode molecules previously described as being important in the control of Th1 responses (44, 45). Another candidate is *Il1r2* which specifies the decoy receptor for IL-1. Expression of IL-1R2 by CXCR3⁺ Treg cells would enable them to sequester IL-1, much as the high level of IL-2 receptor subunit alpha (IL-2RA) on Tregs allows them to outcompete other cells for IL-2 (1, 25). Because IL-1 signaling in Tconv cells renders them refractory to Treg-mediated suppression, sequestering IL-1 would enhance the inhibition of Th1 effector differentiation (46). Is Treg-intrinsic expression of CXCR3 relevant to the control of pancreatic autoimmunity by T-BET⁺ Treg cells? Pancreatic Treg cells express several chemokine receptors besides CXCR3, including CCR2, CCR8, and CXCR6, that might be expected to compensate for the absence of CXCR3 in promoting Treg localization to the islets. However, CXCR3 may have a role in mediating other facets of pancreatic Treg cell function. A precedent exists in a model of vaccinia virus infection, in which CXCR3 was not required for the recruitment of CD8⁺ T cells to infected skin but was crucial for their locating and killing of virus-infected cells in situ (47). CXCR3 might perform an analogous function in pancreatic Treg cells, enabling the proper positioning of Treg cells in relation to APCs and IFN- γ -producing cells for optimal suppressive function. Chemokine receptor signaling also can trigger cellular responses independent of chemotaxis [e.g., CXCR1 induction of IL-10 production in macrophages (48)], and one might speculate that CXCR3 similarly potentiates the suppressive function of Tregs in the islets. These hypotheses would account for the earlier finding that fully *Cxcr3*-deficient NOD mice developed accelerated T1D (49), the loss of CXCR3⁺ Tregs being the dominant effect.

Whether T-BET⁺CXCR3⁺ Tregs also have a central role in human T1D is conjectural at this point, but CXCR3⁺ Treg cells specific for Th1-inducing microbes have been identified in healthy humans (12), and one might speculate that microbial influences on these cells could condition the propensity to progress from prediabetes to overt disease. Our results suggest that the therapeutic transfer of CXCR3⁺ Tregs might be more efficacious in treating T1D than the transfer of bulk Tregs expanded nonspecifically. Alternatively, one can imagine that targeting Treg-trophic factors via CXCR3 might be an effective means of enhancing this subset precisely for a targeted impact on T1D.

Materials and Methods

Mice. NOD/ShiLtJ (NOD), NOD.*Foxp3ires-gfp*, NOD.*Foxp3Cre*, B6.*Foxp3YFP-Cre*, and BDC2.5/B6^{g7} mice were maintained in the specific pathogen-free colony at Harvard Medical School (Institutional Animal Care and Use Committee-approved protocol 02954). *Tbx21*^{fl/fl} mice (50) were obtained from S. Reiner, Columbia University, New York, and were backcrossed to NOD mice for five or eight generations (genotyping for H-2^{g7/g7} early in the backcross) before they were intercrossed.

Diabetes and Insulinitis Assessments. NOD and BDC2.5/B6^{g7} mice were monitored weekly for diabetes, from 8–30 or from 3–10 wk of age, respectively, as described (51). Pancreata were processed and stained for flow cytometry as described (25).

Gene-Expression Profiling and Analysis. Treg cells (30,000) were double-sorted into TRIzol (Invitrogen). Subsequent sample processing and hybridization

onto Affymetrix MoGene 1.0 ST microarray chips were performed as described (52). Normalized data were analyzed with the Multiplot Studio module from GenePattern. Other Treg transcriptomes were used as a reference: VAT, LN, injured muscle, and colon (30), Treg activation (31), BLIMP-1-dependent (32), and IRF4-dependent (8) transcripts. The CXCR3⁺ Treg signature was derived by comparing the transcriptome of splenic CXCR3⁺ Tregs with that of their CXCR3[−] counterparts from B6 mice, performed in triplicate, and defining transcripts with FC ≥ 2 and $P < 0.05$.

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- Josefowicz SZ, Lu LF, Rudensky AY (2012) Regulatory T cells: Mechanisms of differentiation and function. *Annu Rev Immunol* 30:531–564.
- Vignali DA, Collison LW, Workman CJ (2008) How regulatory T cells work. *Nat Rev Immunol* 8(7):523–532.
- Panduro M, Benoist C, Mathis D (2016) Tissue Tregs. *Annu Rev Immunol* 34:609–633.
- Sun M, He C, Cong Y, Liu Z (2015) Regulatory immune cells in regulation of intestinal inflammatory response to microbiota. *Mucosal Immunol* 8(5):969–978.
- Feuerer M, Hill JA, Mathis D, Benoist C (2009) Foxp3⁺ regulatory T cells: Differentiation, specification, subphenotypes. *Nat Immunol* 10(7):689–695.
- Campbell DJ, Koch MA (2011) Phenotypic and functional specialization of FOXP3⁺ regulatory T cells. *Nat Rev Immunol* 11(2):119–130.
- Sage PT, Sharpe AH (2016) T follicular regulatory cells. *Immunol Rev* 271(1):246–259.
- Zheng Y, et al. (2009) Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 458(7236):351–356.
- Chaudhry A, et al. (2009) CD4⁺ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* 326(5955):986–991.
- Koch MA, et al. (2009) The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 10(6):595–602.
- Hall AO, et al. (2012) The cytokines interleukin 27 and interferon- γ promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity* 37(3):511–523.
- Duhen T, Duhen R, Lanzavecchia A, Sallusto F, Campbell DJ (2012) Functionally distinct subsets of human FOXP3⁺ Treg cells that phenotypically mirror effector Th cells. *Blood* 119(19):4430–4440.
- Bluestone JA, Herold K, Eisenbarth G (2010) Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature* 464(7293):1293–1300.
- Esensten JH, Lee MR, Glimcher LH, Bluestone JA (2009) T-bet-deficient NOD mice are protected from diabetes due to defects in both T cell and innate immune system function. *J Immunol* 183(1):75–82.
- Hultgren B, Huang X, Dybdal N, Stewart TA (1996) Genetic absence of γ -interferon delays but does not prevent diabetes in NOD mice. *Diabetes* 45(6):812–817.
- Kanagawa O, Xu G, Tevaarwerk A, Vaupel BA (2000) Protection of nonobese diabetic mice from diabetes by gene(s) closely linked to IFN- γ receptor loci. *J Immunol* 164(7):3919–3923.
- Serreze DV, et al. (2000) Interferon- γ receptor signaling is dispensable in the development of autoimmune type 1 diabetes in NOD mice. *Diabetes* 49(12):2007–2011.
- Wang B, et al. (1997) Interferon- γ impacts at multiple points during the progression of autoimmune diabetes. *Proc Natl Acad Sci USA* 94(25):13844–13849.
- ElEssawy B, Li XC (2015) Type 1 diabetes and T regulatory cells. *Pharmacol Res* 98:22–30.
- Bour-Jordan H, et al. (2004) Costimulation controls diabetes by altering the balance of pathogenic and regulatory T cells. *J Clin Invest* 114(7):979–987.
- Tritt M, Sgouroudis E, d'Hennezel E, Albanese A, Piccirillo CA (2008) Functional waning of naturally occurring CD4⁺ regulatory T-cells contributes to the onset of autoimmune diabetes. *Diabetes* 57(1):113–123.
- Sarween N, et al. (2004) CD4⁺CD25⁺ cells controlling a pathogenic CD4 response inhibit cytokine differentiation, CXCR-3 expression, and tissue invasion. *J Immunol* 173(5):2942–2951.
- Chen Z, Herman AE, Matos M, Mathis D, Benoist C (2005) Where CD4⁺CD25⁺ T reg cells impinge on autoimmune diabetes. *J Exp Med* 202(10):1387–1397.
- Tang Q, et al. (2008) Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* 28(5):687–697.
- Sitirin J, Ring A, Garcia KC, Benoist C, Mathis D (2013) Regulatory T cells control NK cells in an insulinitis lesion by depriving them of IL-2. *J Exp Med* 210(6):1153–1165.
- Herman AE, Freeman GJ, Mathis D, Benoist C (2004) CD4⁺CD25⁺ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J Exp Med* 199(11):1479–1489.
- Cipolletta D, et al. (2012) PPAR- γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* 486(7404):549–553.
- Schiering C, et al. (2014) The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* 513(7519):564–568.
- Wohlfert EA, et al. (2011) GATA3 controls Foxp3⁺ regulatory T cell fate during inflammation in mice. *J Clin Invest* 121(11):4503–4515.
- Sefik E, et al. (2015) MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of ROR γ ⁺ regulatory T cells. *Science* 349(6251):993–997.
- Hill JA, et al. (2007) Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27(5):786–800.
- Cretney E, et al. (2011) The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* 12(4):304–311.
- Lazarevic V, Glimcher LH, Lord GM (2013) T-bet: A bridge between innate and adaptive immunity. *Nat Rev Immunol* 13(11):777–789.
- McClymont SA, et al. (2011) Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *J Immunol* 186(7):3918–3926.
- Kornete M, et al. (2015) Th1-Like ICOS⁺ Foxp3⁺ Treg Cells Preferentially Express CXCR3 and Home to β -Islets during Pre-Diabetes in BDC2.5 NOD Mice. *PLoS One* 10(5):e0126311.
- Gonzalez A, et al. (1997) Genetic control of diabetes progression. *Immunity* 7(6):873–883.
- Feuerer M, Shen Y, Littman DR, Benoist C, Mathis D (2009) How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. *Immunity* 31(4):654–664.
- Groom JR, Luster AD (2011) CXCR3 ligands: Redundant, collaborative and antagonistic functions. *Immunol Cell Biol* 89(2):207–215.
- Markle JG, et al. (2013) Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* 339(6123):1084–1088.
- Yurkovetskiy L, et al. (2013) Gender bias in autoimmunity is influenced by microbiota. *Immunity* 39(2):400–412.
- Xiong Y, Ahmad S, Iwami D, Brinkman CC, Bromberg JS (2016) T-bet regulates natural regulatory T cell afferent lymphatic migration and suppressive function. *J Immunol* 196(6):2526–2540.
- Yu F, Sharma S, Edwards J, Feigenbaum L, Zhu J (2015) Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. *Nat Immunol* 16(2):197–206.
- McPherson RC, Turner DG, Mair I, O'Connor RA, Anderton SM (2015) T-bet Expression by Foxp3⁺ T Regulatory Cells is Not Essential for Their Suppressive Function in CNS Autoimmune Disease or Colitis. *Front Immunol* 6:69.
- Joller N, et al. (2014) Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* 40(4):569–581.
- Bettini M, et al. (2011) Cutting edge: Accelerated autoimmune diabetes in the absence of LAG-3. *J Immunol* 187(7):3493–3498.
- Schenten D, et al. (2014) Signaling through the adaptor molecule MyD88 in CD4⁺ T cells is required to overcome suppression by regulatory T cells. *Immunity* 40(1):78–90.
- Hickman HD, et al. (2015) CXCR3 chemokine receptor enables local CD8⁺ T cell migration for the destruction of virus-infected cells. *Immunity* 42(3):524–537.
- Hadis U, et al. (2011) Intestinal tolerance requires gut homing and expansion of FoxP3⁺ regulatory T cells in the lamina propria. *Immunity* 34(2):237–246.
- Yamada Y, et al. (2012) Acceleration of diabetes development in CXC chemokine receptor 3 (CXCR3)-deficient NOD mice. *Diabetologia* 55(8):2238–2245.
- Intlekofer AM, et al. (2008) Anomalous type 17 response to viral infection by CD8⁺ T cells lacking T-bet and eomesodermin. *Science* 321(5887):408–411.
- Kriegel MA, et al. (2011) Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. *Proc Natl Acad Sci USA* 108(28):11548–11553.
- Feuerer M, et al. (2010) Genomic definition of multiple ex vivo regulatory T cell subphenotypes. *Proc Natl Acad Sci USA* 107(13):5919–5924.