Enhanced thymic selection of FoxP3+ regulatory T cells in the NOD mouse model of autoimmune diabetes


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FoxP3+CD4+ regulatory T cells (Tregs) play a key role in the maintenance of peripheral self-tolerance, and it has been suggested that diabetes-susceptible nonobese diabetic (NOD) mice are defective in the generation and numbers of Tregs. We found thymic selection of Tregs to be under genetic control. Fetal thymic organ cultures on the NOD background required 3- to 10-fold more antigen than corresponding cultures on the B6 background for optimal induction of Tregs, but once the threshold for induction was reached the NOD background yielded close to 10-fold more Tregs. This increased selection of Tregs was also found in nontransgenic NOD mice in fetal through adult stages. This trait did not map to the MHC, idd3, or the chromosome 3 (Chr3) regions that control clonal deletion, but mainly to two regions on Chr1 and Chr11. Thus, NOD mice do not have a global defect in the generation or maintenance of Tregs; if anything, they show the opposite.

Regulatory T cells (Tregs) play a key role in the maintenance of peripheral self-tolerance (1). It was found that such cells express the forkhead/winged helix transcription factor FoxP3 (2–4), and that deficiencies in FoxP3 underlie the lymphoproliferation and multiorgan autoimmunity in scurfy mutant mice and human patients with immunopathyregulation polyendocrinopathy and enteropathy, X-linked syndrome (5). FoxP3-GFP reporter mice, as well as intracellular staining for FoxP3, revealed that 60–88% of FoxP3+ cells express high levels of CD25, depending on the tissue localization (6). A distinct gene-expression signature is also characteristic of Tregs (6–8).

Tregs in normal mice are generated in the thymus, but how immature thymocytes are selected into this alternative lineage remains a question of central interest. The repertoire of Tregs is enriched in autoreactive T lymphocytes. In some experimental systems, Treg differentiation appeared to be induced when the T cell receptor (TCR) on immature thymocytes was engaged by agonist ligands (9, 10). Alternatively, some investigators have proposed that the observed high frequency of Tregs resulting from recognition of cognate ligand may reflect a higher resistance of Treg versus conventional CD4+ T cell thymic precursors to clonal deletion (11, 12). Still others have suggested that Treg selection may be favored in particular thymus stromal niches (13). In addition, mature CD4+ T cells from peripheral lymphoid organs can be converted to FoxP3+ positivity by exposure to TGFB (14) or chronic stimulation by agonist peptide (15).

The activity or inactivity of Tregs plays an important role in controlling the development of autoimmune diabetes in the nonobese diabetic (NOD) mouse model (16): transfer of Tregs can protect the recipients from diabetes, whether they be NOD mice or TCR transgenic systems derived from NOD (17–19), and genetic deficiencies that reduce Treg numbers or activity that lead to accelerated diabetes (17, 18). It has been suggested that NOD mice have a generalized defect in their ability to generate effective numbers of Tregs, with a percentage of CD4+CD25+ T cells lower that of other autoimmunity-resistant mouse strains (17, 20, 21), and other studies report age-dependent declines in Treg-suppressive activity in NOD mice (22–24). Here, we have analyzed genetic variation in the thymic generation of Tregs, focusing on NOD and the diabetes-resistant MHC-matched B6.H2b7 as well as a panel of other inbred strains. To our surprise, the NOD background proved superior at generating Tregs in the thymus, whether in response to agonist ligands or in unmanipulated conditions.

Results

Generation of Tregs in Fetal Thymus Organ Cultures (FTOCs). As mentioned above, there is a contradiction between results from certain experimental systems that suggested direct induction of Treg differentiation by TCR engagement with agonist ligands (9, 10) versus other systems where preferential survival of Tregs could account for an apparent increase in self-reactive Treg thymocytes upon agonist exposure, without an actual induction of differentiation (11, 12, 25). To address this contradiction, we investigated the generation of Tregs in FTOCs, using embryonic day 15 (E15) thymic lobes from timed matings of BDC2.5/NOD mice. The BDC2.5 mouse line was derived from a CD4+ T cell clone, restricted by the NOD MHC class II Aβ7 molecule, and specific for an unknown β cell antigen (26).

Neither FoxP3 RNA nor the corresponding protein could be detected at FTOC initiation (data not shown). Cultures were incubated for 7 days, and the appearance of Tregs was monitored by staining for CD25 and FoxP3 among CD4 single-positive (CD4SP) thymocytes. In the NOD FTOCs ~4% of the CD4SPs were Tregs, a value comparable with that of normal CD4SP thymocytes (Fig. 1A Left). Thus, the thymus is fully competent to produce this subset of Tregs in an autonomous fashion. Fewer Tregs were generated in lobes from BDC25/NOD embryos, indicating that the BDC2.5 TCR is less compatible with selection into the Treg pathway than is the diverse repertoire of NOD mice (Fig. 1A Center), as commonly observed with TCR transgenic mice. On the other hand, a robust Treg population (10–20% of CD4SPs) was induced by addition of 10 ng/ml BDC2.5 mimotope peptide agonist (Fig. 1A Right). In peptide titrations, the proportion of Tregs among CD4SPs increased from <1% (no...
peptide) to >50% (100 ng/ml peptide; Fig. 1B Left). This increase reflected a true induction of Tregs, as their total number also increased >20-fold at the optimal peptide dose (30 ng) (Fig. 1B Right). The peptide titration also reproduced the differential sensitivity of conventional and regulatory CD4SP precursors to agonist-induced deletion: Tregs were induced at a dose of peptide that resulted in the loss of conventional CD4SPs and of CD4, CD8 double-positive (DP) thymocytes (Fig. 1C). Thus, true induction of Treg differentiation occurs in the BDC2.5 system, in a range of agonist stimulation where certain degree of negative selection also occurred.

We then asked whether the agonist-induced Tregs in BDC2.5/NOD FTOC had an origin or phenotypic characteristics different from those of Tregs “naturally” selected in NOD FTOCs. Comparing the kinetics of Treg generation between NOD and peptide-stimulated BDC/NOD FTOCs revealed no differences between the two groups. The first evidence of FoxP3 expression was simultaneously detected on day 4 (Fig. 2A). There has been some debate as to the stage in thymocyte differentiation where Tregs first appear. They could potentially differentiate from immature DP precursors, as conventional SPs do, or later from committed CD4SPs that have migrated into the medulla (6, 13). The kinetic analysis revealed that DP FoxP3+ thymocytes were induced in the BDC2.5/NOD FTOCs, in highest frequency at day 5 of the culture, and decline as a fraction of the total FoxP3 population from ~35% at day 5 to 18% by day 7, with a corresponding increase in CD4SPs FoxP3+ thymocytes (Fig. 2B Left and Right). NOD FTOCs showed a parallel evolution, albeit with fewer DPs within the FoxP3+ populations (Fig. 2B Right). These data are consistent with a scenario wherein self-reactive cells enter the Treg lineage at the DP thymocytes stage, followed by a transition to CD4SP compartment, although some conversion of committed CD4SPs may also occur.

Close inspection of the data in Fig. 2A revealed that the FoxP3+ Tregs induced in the BDC2.5/NOD cultures had a different distribution of CD25 expression, with a predominance of CD25+ cells. Therefore, we compared the distribution of some of the hallmark molecules of Tregs on naturally selected and agonist-induced Treg thymocytes. Differences were detected for CD45RA, OX40 (CD134), and CD103 (Fig. 2C). GITR and CTLA4 expression showed no marked divergence in the two groups.

**Genetic Differences in Treg Generation.** Given the generally accepted notion that NOD mice have some kind of defect in Tregs, we asked whether the genetic variation between the NOD strain and the diabetes-resistance C57BL/6 (B6) reference strain might
influence the propensity for generating Tregs in FTOCs. Parallel transgenic lines were used, the same BDC2.5 transgenes being crossed onto the NOD or congenic B6.H-2d (Bg7) backgrounds (27), which share the selecting MHC class II A\(^{e}\) molecule but differ in all non-MHC genes. FTOCs were performed with embryos from BDC2.5/NOD (open symbols) mice and BDC2.5/Bg7 (filled symbols) mice and supplemented with different concentrations of BDC-specific peptide. (A) (Left and Middle) Percentage and absolute number of Foxp3\(^{+}\) Tregs per CD4SP. Mean and SD of four independent experiments with each three to six pooled FTOC lobes per concentration and group are shown. (Right) Number at peak shows absolute number of Foxp3\(^{+}\) cells at the peptide concentration with a maximum of Treg induction (3 ng for BDC/Bg7 and 30 ng for BDC/NOD). Presented are four individual experiments. (B) Deletion based on percentage of DP cells. (C) Nontransgenic FTOC experiments from NOD mice and Bg7 mice. (Left) Numbers show percentage of Foxp3\(^{+}\) Tregs per CD4SP of representative dot plots. (Right) Percentage and absolute number of Foxp3\(^{+}\) Tregs per CD4SP and mean and SD of three independent experiments with three to five FTOC per group and experiment are shown.

Fig. 3. Strain differences between NOD and Bg7 in Treg induction. FTOCs were cultured for 7 days. FTOC cultures were performed in parallel with embryos from BDC2.5/NOD (open symbols) mice and BDC2.5/Bg7 (filled symbols) mice and supplemented with different concentrations of BDC-specific peptide. (A) (Left and Middle) Percentage and absolute number of Foxp3\(^{+}\) Tregs per CD4SP. Mean and SD of four independent experiments with each three to six pooled FTOC lobes per concentration and group are shown. (Right) Number at peak shows absolute number of Foxp3\(^{+}\) cells at the peptide concentration with a maximum of Treg induction (3 ng for BDC/Bg7 and 30 ng for BDC/NOD). Presented are four individual experiments. (B) Deletion based on percentage of DP cells. (C) Nontransgenic FTOC experiments from NOD mice and Bg7 mice. (Left) Numbers show percentage of Foxp3\(^{+}\) Tregs per CD4SP of representative dot plots. (Right) Percentage and absolute number of Foxp3\(^{+}\) Tregs per CD4SP and mean and SD of three independent experiments with three to five FTOCs per group and experiment are shown.

Fig. 4. Comparison of ex vivo T cells from NOD and Bg7 mice. Thymus and spleen cells were isolated and stained for CD4, CD8, CD25, Foxp3, and TCR-\(\beta\) chain. Percentage of Foxp3\(^{+}\) Tregs per CD4SP was analyzed. (A) Thymi from NOD mice (open symbols), NOD.Ea16 mice (gray symbols, dashed line), and Bg7 mice (black symbols) were isolated at different days after birth, and percentage of Foxp3\(^{+}\) per CD4SP T cells was analyzed. Mean and SD of 3–11 mice per group and time point are shown. (B) Broad screen of Tregs among inbred mouse strains. Thymus and spleen from 10 inbred mouse strains were analyzed. Mean percentage of CD4\(^{+}\)Foxp3\(^{+}\) Tregs and SD are shown. Three mice per strain were analyzed. Mean values from spleen and thymus were plotted, and NOD and B6 mice are highlighted.

from several lymphoid organs for FoxP3 and CD25. A detailed time-course analysis of Treg appearance in the thymus was performed, which showed their proportion among CD4SPs to increase over time in both strains, consistent with a previous report (13); this proportion was consistently higher for NOD mice at all time points tested, in neonatal, infant, or adult mice (Fig. 4A). This increase was not an artifact of NOD’s autoimmune attack to the pancreas, because it was also observed in NOD.Ea16 mice, genetically identical to NOD animals, but protected from insulitis by an MHC class II Ea transgene (29).

NOD and B6 are just two mouse strains, and differences observed above could be caused by an extreme representation in either strain. To this end, we analyzed thymic and splenic in 10 mouse strains from the Mouse Phenome Project (30) (all in 5- to 6-week-old mice, in triplicate). A wide distribution of proportions was observed for Foxp3\(^{+}\) cells among thymic CD4\(^{+}\)CD8\(^{-}\)SPs (from 2.7 to 5.3%; Fig. 4B). In keeping with the results above, NOD and B6 mice were on the high and low ends of that distribution, respectively, but still within the normal range (consistent with a prior study (31), and with a broader 33-strain analysis performed earlier with a CD25\(^{hi}\) phenotype as an indicator of Tregs (SI Fig. 6)). The proportion of Tregs in spleen
Where Does the Efficient Treg Generation of NOD Mice Map? Given the demonstration that Tregs were generated more efficiently in the thymus of NOD than Bg7 mice, we sought to identify the origin of this difference.

The first issue to resolve was whether the difference was intrinsic to the differentiating thymocytes or to the thymic stroma. To this end, criss-cross reaggregation experiments were performed, where E15 DN T cell precursors were transferred into lobes depleted of their own lymphoid precursors by deoxyguanosine pretreatment. After culture, thymocytes derived from donor precursors were identified via the CD45 allotype marker (Fig. 5A). Higher proportions and numbers of Tregs were generated from NOD precursors, irrespective of the strain origin of the host lobe. Thus, T cell-intrinsic differences accounted for the strain differences in Treg selection.

The Id3 locus has been linked with higher IL-2 production and Treg number in the periphery (32). We wondered whether the id3 locus might also be responsible for the differences in Treg generation between NOD and B6 mice. FTOCs from NOD/id3b/b congenic mice demonstrated that the id3 locus was not responsible for this difference (Fig. 5B).

We next asked which genetic loci contribute to this difference, anticipating that this information might also lead to new insights into the genetic control of Treg differentiation. To address this question, we intercrossed NOD and Bg7 mice to yield (NODxBg7) F1 and F2 embryos and measured Foxp3 and CD25 expression on the CD4SP thymocytes. Compared with their parental NOD and Bg7 counterparts, F1 embryos demonstrated an intermediate percentage of Tregs; F2 embryos, which carried random combinations of alleles from the parental backgrounds, showed a broad range of Foxp3+ cells (Fig. 5C).

A genomewide scan was performed on the DNA from 137 of these (NODxBg7) F2 embryos, genotyping 106 SNPs that distinguish the NOD and B6 genomes (average spacing of 25 Mb). Marker-association analysis was performed with R/qtl software, with permutation analysis to estimate thresholds for genomewide significance. Peaks of significant association were detected on chromosome 1 (Chr1) (100−180 Mb) and Chr11 (between 100 Mb and the telomere; Fig. 5D and E). At both loci, the NOD-derived allele was associated with a higher Treg proportion, essentially dominant for the Chr1 quantitative trait loci (QTL), and additive for the QTL on Chr11 (SI Fig. 7). Together, these two QTLs account for 28.9% of the variance, which represents a very substantial proportion in such analyses. Thus, the differential selection of Tregs in NOD and B6 thymi is under traceable Mendelian control and maps away from loci that condition the basic efficacy of clonal deletion and deviation, or the maintenance of Tregs in the periphery.

Discussion

The results presented herein demonstrate that the thymic selection of Tregs is under genetic control and also lead to an unexpected perspective on Tregs in the autoimmunity-prone NOD mouse, with more effective Treg selection in the thymus than in the reference diabetes-resistant strain, B6. These data imply that defective selection of Tregs is not a major component of diabetes susceptibility in the NOD mouse.

Our initial studies focused on Treg differentiation in FTOCs to assure that all differences were purely thymus-derived and to permit greater experimental flexibility. The results confirmed that the thymus is autonomously capable of generating Tregs, and the good match between relative numbers in FTOCs and neonatal mice indicates that FTOCs can serve as a valid experimental model in which to study the selection of Tregs. Previous reports using TCR transgenic mice coexpressing cognate ligand in the thymus suggested that high-affinity engagement of a self-ligand by the transgenic TCR induced a signal that could commit thymocytes to the Treg lineage (9, 10). Alternately, we and others have argued that the higher frequency of Tregs in the thymus of other TCR/Ag double-transgenic mice could be entirely explained by selective survival of Tregs (11, 12). Here, we found evidence of both scenarios in FTOCs from BDC2.5...
TCR transgenic mice. FoxP3+ cells were certainly more resistant to an agonistic stimulus that induced clonal deletion in immature thymocytes, particularly at high antigen concentrations, but, at lower concentrations, true induction of FoxP3+ Tregs was clearly evident as well.

It had not been clear whether Tregs induced experimentally by agonist ligand are phenotypically and functionally the same as the “naturally” selected Tregs generated in the thymus of a normal mouse. Comparing normally selected Tregs from nontransgenic mice with agonist-induced transgenic Tregs in FTOCs showed their kinetic of generation to be quite similar, although the agonist-induced FoxP3+ cells were enriched in DP thymocytes (up to 40% of all FoxP3+ cells). This difference is consistent with the notion that channeling of thymocytes into the Treg lineage may occur at different stages of differentiation depending on the thymus location in which the TCR self-agonist is encountered (33). In the FTOC system, immature DPs encounter the agonist peptide in the cortex of the developing cultures, whereas some of the Tregs selected in a normal thymus may depend more on presentation of self-antigens in the medulla. Several markers typical of Tregs proved differentially expressed in Tregs selected by the two modes: agonist-induced Tregs displayed less CD25 and OX40 and more CD103. One might propose that Tregs are a heterogeneous population, harboring TCRs with a spectrum of affinities/avidities for self-antigens, and with a range of phenotypic characteristics that reflect this spectrum. The CD103+ CD25hiOX40hi phenotype might denote Tregs elicited by a high-avidity ligand, whereas the CD103- CD25hiOX40hi phenotype might be that of Tregs with lower avidity for self-ligand. Thus, the phenotypic and functional differences observed in peripheral Treg populations (7) could reflect differences in their encounter of high-avidity ligand during thymic selection, potentially continued in the periphery.

When the generation of Tregs on the NOD and B6 backgrounds were compared, clear differences in sensitivity were detected. As reported (28, 34), the NOD background confers a relative resistance to clonal deletion and also to clonal deletion toward the CD8αα and Treg lineages (35). BDC2.5/NOD FTOCs needed 3- to 10-fold more antigen than BDC2.5/Bg7 embryos for optimal induction of Tregs. But, once the threshold for induction was reached, the NOD background was much more effective, with close to 10-fold higher yields of Tregs at comparable levels of clonal deletion. This greater propensity was also manifest in the absence of agonist stimulation in FTOCs and thymi of nontransgenic NOD mice at a variety of age groups, whereas some of the Tregs selected in a normal thymus may depend more on presentation of self-antigens in the medulla. Several markers typical of Tregs proved differentially expressed in Tregs selected by the two modes: agonist-induced Tregs displayed less CD25 and OX40 and more CD103.

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Fetal thymus lobes were dissected from E15.5 embryos. Organs were cultured on sponge-supported filter membranes (Gel Foam surgical sponge; Amersham Pharmacia, Piscataway, NJ; Superf 450 mohrblad, 0.45-μm pore size, Pall Gelman Laboratory, East Hills, NY) at an interphase between 5% CO2-humidified air and RPMI medium 1640 (10% FCS/50 μM 2-mercaptoethanol/2 mM L-glutamine/10 mM Hepes/nonessential amino acids/sodium pyruvate/penicillin/streptomycin). For negative selection experiments, FTOCs were supplemented with a BDC-specific peptide mimotope at indicated concentrations (BDcmi, peptide 1040–63; ref. 44). Medium/peptide was changed after 3 days of culture.

Repopulation Thymus Organ Culture. For repopulation experiments, thymus lobes were removed from E15.5 embryos and cultured on sponge-supported filters for 5 days in complete culture medium in the presence of 1.35 mM 2-deoxyguanosine. After culture, lobes were washed overnight by immersion in 50 ml of culture medium (without 2-deoxyguanosine) at 37°C, before repopulation with DN thymocytes from E15.5 embryos. Repopulation was obtained in Terazaki plates, incubating thymus lobes (one lobe per well) in hanging drops with 2–8 × 10^5 sorted DN cells for 12 h. Repopulated lobes were cultured on filters for 7 days, and then cells were harvested for FACS analysis.

Genetic Analysis. DNA was isolated from (B6.129g7 × NOD) F2 embryos and genotyped for SNP markers distinguishing B6 and NOD alleles by using 106 sets of TaqMan primers and probes (Assays by Design; Applied Biosystems, Foster City, CA) developed from the GNF database (http://snp.gnf.org) or NOD BAC end sequences (www.sanger.ac.uk/Projects/M.musculus-NOD). SNP markers covered all 19 autosomes and IdId loci with an average spacing of ~25 Mb. The full list of primer/probe sets along with specific genomic locations (Mb positions based on the latest version of the National Center for Biotechnology Information genome assembly) can be accessed in SI Table 2. Genotyping reactions were carried out in 384-well plates by using 5-μl reaction volumes containing 8 ng of dehydrated genomic DNA and 2.5 μl of ABI Master Mix (2× concentrated), 900 nM of each primer, and 200 nM probes of each 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxy fluorescein or 6-carboxy fluorescein-labeled probe. Standard TaqMan thermocycling conditions were followed, with endpoint fluorescence reads on an ABI 7900HT instrument and genotypes calls with SDS software (Applied Biosystems). Genetic analyses were performed with R/qtl (45). Experiment empirical P values were established by permutation tests (10,000 permutations; R/qtl). QTLs were confirmed by using nonparametric interval mapping algorithms (R/qtl). 2D interval mapping for epistasis was performed by using R/qtl.

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